Oral Abstracts

Presidential Symposium

1. Atidarsagene Autotemcel (Hematopoietic Stem Cell GeneTherapy) Preserves Cognitive and Motor Development in Metachromatic Leukodystrophy with up to 12 Years Follow-Up

Valeria Calbi[1], Francesca Fumagalli[1], Fabiola De Mattia[1], Alberto Zambon[1], Vera Gallo[1], Salvatore Recupero[1], Elena Sophia Fratini[2], Alessia Ippolito[1], Francesca Ciotti[1], Maddalena Fraschini[1], Marina Sarzana[1], Stefano Scarparo[1], Eugenio Montini[1], Sara Locatelli[1], Marcella Facchini[1], Alessandra Clerici[1], Francesco Morena[3], Sabata Martino[3], Muska Yarzi[4], Sean L. Moro[5], Andrew Shenker[6], Jean Brooks[4], Alan Richardson[4], Laura Campbell[4], Alessandro Aiuti[2]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Milan, Italy,[2]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget); Vita Salute San Raffaele University, Milan, Italy,[3]University of Perugia, Perugia, Italy,[4]Orchard Therapeutics, London, United Kingdom,[5]Orchard Therapeutics, Boston, MA,[6]Clinical Consultant, Pennington, NJ

Introduction: Metachromatic leukodystrophy (MLD) is a rare neurometabolic disorder caused by deficiency of arylsulfatase A (ARSA), leading to accumulation of sulfatides in the central and peripheral nervous systems, and subsequently progressive demyelination, neurodegeneration, loss of all motor and cognitive skills and early death. **Methods:** We present long-term results from an integrated analysis of 39 patients with early-onset MLD (19 late infantile, 20 early juvenile) treated with autologous *ex vivo* hematopoietic stem cell gene therapy (atidarsagene autotemcel, "arsa-cel") across two prospective clinical trials (n=30) and expanded access frameworks (n=9). Arsa-cel consists of autologous $CD34^+$ cells transduced *ex vivo* with a lentiviral vector encoding for the human *ARSA* gene. After busulfan conditioning, arsa-cel was administered intravenously. Clinical outcomes were compared with natural history (NHx) data from 49 untreated early-onset MLD patients. **Results:** The median length of follow-up was 6.76 years (range 0.64-12.19). All patients demonstrated stable multilineage engraftment of gene-corrected cells and restoration of ARSA activity in peripheral blood mononuclear cells to normal or supranormal levels by 3 months post-treatment and restoration in cerebrospinal fluid to normal levels by 3-6 months post-treatment, which were sustained throughout follow-up. The risk of experiencing severe motor impairment or death was significantly reduced in the pre-symptomatic late-infantile (PSLI, $p<0.001$), pre-symptomatic early-juvenile (PSEJ, $p=0.042$), and early-symptomatic early-juvenile (ESEJ, able to walk independently and without cognitive impairment prior to treatment, $p<0.001$) treated subgroups versus MLD subtype-matched NHx patients. Arsa-cel treatment resulted in substantial improvements in motor and cognitive outcomes as compared with NHx, particularly among patients treated before symptom onset. Over 95% (25/26) of treated PSLI and PSEJ patients retained the ability to walk at last follow-up, and most treated patients showed clinically meaningful preservation of cognitive abilities and speech. In contrast, most NHx patients experienced rapid motor and cognitive decline and loss of speech, progressing to a severely debilitated state or death. Three treated patients died, all considered unrelated to arsa-cel. There were no serious adverse events related to arsa-cel, no malignancies, and no evidence of abnormal clonal expansion or replication-competent lentivirus. The most common adverse events (AEs) were consistent with the known safety profile of busulfan or symptoms of MLD. The only arsa-cel-related AEs were 6 events of transient and low titer anti-ARSA antibodies with no impact on pharmacodynamic or clinical outcomes. **Conclusions:** With up to 12 years follow-up in the earliest treated patient and a cumulative 251 patient-years of follow-up, arsa-cel shows a favorable benefit-risk profile with clinically meaningful, sustained efficacy, preventing severe motor and cognitive impairment, preserving speech, and slowing disease progression in early-onset MLD patients.

2. *In Vivo* Generation of Both CAR T Cells and CAR NK Cells Using a CD7 Targeted Lentiviral Vector

Karla F. Mullen, Daniel Grubaugh, Kayla R. Welsh, Jeffery Foss, Julia L. A. Nguyen, Nishi Gheewala, Leticia Kuri-Cervantes, James I. Andorko, Bruce C. Schnepp, Timothy D. Culp, Philip R. Johnson

Interius BioTherapeutics, Philadelphia, PA

CAR-T cell therapy has shown remarkable clinical efficacy against various cancers, including hematologic malignancies. CAR-NK cells have shown promising results in cancer therapy, especially B-cell lymphoma, with a lower risk of GVHD, serious cytokine release syndrome (CRS) and immune effector-cell associated neurotoxic syndrome (ICANS) compared to allogenic CAR-T cells[1]. INT2104 utilizes a lentiviral vector platform that incorporates CD7-targeting to specifically transduce T cells and NK cells upon intravenous (i.v.) administration. Here we describe the ability of a single i.v. administration of INT2104 to transduce both T and NK cells *in vivo* generating functional CAR20+ cells resulting in the depletion of CD20+ B cells. *In vitro* assessments of INT2104 confirmed transduction of both CD7+ NK cells and T cells, including CD4+ and CD8+ subsets. A humanized CD34-engrafted NSG-Tg (Hu-IL15) mouse model, which constitutively expresses human interleukin-15 to enhance NK-cell development in these mice, was utilized to demonstrate effective NK cell transduction following INT2104 administration. Mice receiving a single bolus INT2104 dose of 1×10^7 transducing units by tail vein injection were compared to control mice receiving no vector. Weekly analysis of blood confirmed CD20+ B cell depletion compared to untreated control mice. At select timepoints throughout the study, the distribution and persistence of transduced CAR+ NK cells and T cells was assessed examining select tissues from euthanized animals. Beginning on Day 7 post INT2104 administration, CAR T and NK cells were detected in bone marrow, spleen, and liver. After demonstrating targeted gene delivery to T and NK cells using humanized mouse models, CAR20 vector was administered i.v. to cynomolgus macaques. Depletion of peripheral blood B cells in vector-treated NHPs was evident as early as Day 4 post administration. The complete absence of circulating B cells was maintained in one animal and analysis of secondary lymphoid organs in this animal confirmed

systemic B cell depletion at necropsy 1-year post treatment. Separately, to demonstrate that both CAR+ T cells and NK cells were generated in NHPs following i.v. administration of a CAR20 vector, two NHPs were treated with CAR20 vector and sacrificed 4 days post treatment. Immunohistochemical analyses of spleen and liver were conducted with an anti-CAR antibody and markers for T cells (CD3), NK cells (NKG2A), as well as hepatocytes (HNF4α). CAR+ cells were detected in spleen with CAR+ signal colocalizing to both CD3+ T cells (Figure 1a) and NKG2A+ NK cells (Figure 1b). No CAR+ hepatocytes were detected among the 8685 hepatocytes present in the representative liver sections analyzed to date. These data highlight the selectivity in targeting and transducing CD7+ T cells and NK cells, with no off-target transduction observed in hepatocytes. A FIH study for INT2104 is scheduled to begin in 4Q2024.

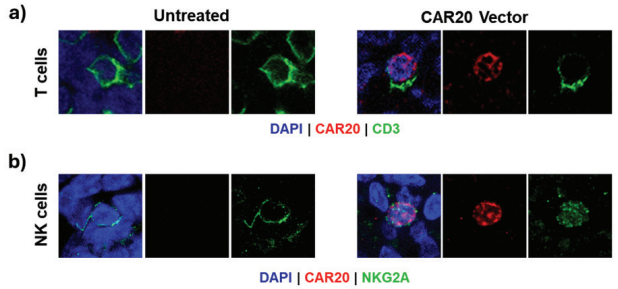


Figure 1: CD7 targeted CAR20 vector treatment in NHPs results in CAR+ T cells and NK cells. Representative CD3+ T cells (a) and NKG2a+ NK cells (b) colocalize with CAR+ signal in NHP spleen.

[1]Liu, E., et al. "Use of CAR-Transduced Natural Killer Cells in CD19-Positive Lymphoid Tumors." The New England Journal of Medicine. 382(6) (2020): 545-553.

3. A Recombinant Human Bocavirus Vector Delivers Therapeutic Levels of CTFR to Cystic Fibrosis Primary Human Airway Cells and Transduces Airway Epithelium *In Vivo*

Jeff Moffit, Salvatore Iovino, Graham Casey, Leila Malkoc, Gillian Payne, Josh Merritt, Robert M. Kotin, Marc Abrams, Joel Schneider

Carbon Biosciences, Waltham, MA

Gene therapies for severe pulmonary diseases, including cystic fibrosis (CF) could achieve meaningful clinical outcomes if the physical and cellular barriers of airway epithelial cell transduction could be overcome. Current viral-based gene delivery approaches are also limited by genome capacity and immunogenicity. Human Bocavirus Type 1 (HBoV1) is a nonpathogenic parvovirus of genus bocaparvovirus that readily infects human airway epithelial cells repeatedly in nature and offers 20% extra genomic capacity beyond adeno-associated virus 2 (AAV2). CBN-1000 is a hybrid viral vector featuring an HBoV1 capsid engineered with conventional AAV2-vector genome elements. The hybrid vector displays exquisite lung tropism while simultaneously leveraging much of the safety, regulatory, and CMC profile of AAV vectors. In the present study we evaluated the in vivo biodistribution and functional rescue by CBN-1000 in relevant species as well as in patient-derived human airway epithelial cells from two genotypes of cystic fibrosis. Primary human airway basal cells cultured at an air-liquid interface and differentiated into mature human airway epithelial cells were transduced with CBN-1000 carrying CFTR, the gene mutated in CF. Western blot demonstrated restoration of mature CFTR protein equal to that of non-CF cultures, and electrophysiology confirmed rescue of CFTR-mediated ion current. For biodistribution studies, an 124I-labeled HBoV1 capsid was administered intratracheally resulting in similar biodistribution and clearance in both WT and CF ferrets, despite a moderate-to-severe lung disease phenotype in the transgenic animals. Furthermore, CBN-1000 was well tolerated in both NHP and ferrets at clinically relevant dose levels after pulmonary delivery, and demonstrated airway-specific gene expression. These data demonstrate that CBN-1000, a new and selective viral vector for lung delivery, can deliver large transgenes including full-length CFTR which is out-of-reach for AAV vectors. CBN-1000 addresses several of the most daunting challenges of current pulmonary gene delivery and exemplifies the potential of capsids derived from non-AAV, autonomous parvoviruses to expand the scope of gene therapy for diverse applications.

Clinical Trials Spotlight

4. Gene Therapy for Adult and Pediatric Patients with Severe Pyruvate Kinase Deficiency: Results from a Global Study of RP-L301

Ami Shah[1,2,3], Julián Sevilla[4,5], Jóse Luis López Lorenzo[6,7], Susana Navarro[5,7,8], Lucía Llanos[6,7], Belgoña Pérez de Camino Gaisse[6,7], Sol Sanchez[6,7], Josune Zubicaray[4], Bert Glader[2,3], May Chien[2,3], Oscar Quintana Bustamante[5,7,8], Marta Gonzalez Vicent[4,5], June Iriondo[4,5], Michael Rothe[9], Philipp John-Neek[9], Antonella Bastone[9], Axel Schambach[9], Miriam Zeini[10], Grace Choi[10], Eileen Nicoletti[10], Gayatri Rao[10], Maria-Grazia Roncarolo[1,2,3], Juan A. Bueren[5,7,8], Jonathan Schwartz[10], Jose-Carlos Segovia[5,7,8]

[1]Ctr Definitive and Curative Med, Stanford Univ, Stanford, CA,[2]Dept Pediatrics, Div of Hematol Oncol Stem Cell Transplant Regenerative Med, Stanford Univ Sch Med, Stanford, CA,[3]Lucile Packard Children's Hosp, Palo Alto, CA,[4]Hematol y Hemoterapia, Fundación Invest Bioméd, Hosp Infantil Univ Niño Jesús (HIUNJ), Madrid, Spain,[5]Ctr Invest Bioméd en Red de Enfermedades Raras (CIBERER), Madrid, Spain,[6]Hosp Univ Fundación Jiménez Díaz, Inst Invest Sanitaria Fundación Jiménez Díaz (IIS-FJD), Madrid, Spain,[7]Unidad Mixta Terapias Avanzadas, Inst Invest Sanitaria Fundación Jiménez Díaz (IIS-FJD), Madrid, Spain,[8]Unidad Innovación Bioméd, Ctr Invest Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain,[9]Hannover Med Sch, Hannover, Germany,[10]Rocket Pharmaceuticals, Cranbury, NJ

Pyruvate kinase deficiency (PKD) is a rare hemolytic anemia caused by *PKLR* gene mutations. Manifestations include anemia, splenomegaly and iron overload, which may be life-threatening. Current treatments are limited to chronic blood transfusions, iron chelation and splenectomy which are associated with significant side effects, or PK activators. A Phase 1 trial (NCT04105166) is evaluating lentiviral (LV)-mediated hematopoietic stem and progenitor cell (HSPC)-targeted

gene therapy (RP-L301) in splenectomized patients with severe PKD (Hb < 8g/dL and/or transfusion-dependent anemia). Following apheresis, HSPCs were transduced with LV vector and cryopreserved, in part for analysis of the drug product. Myeloablative therapeutic drug monitoring-guided busulfan was administered prior to RP-L301 IV infusion. Follow-up was 2 years to assess safety (including insertion site analysis [ISA]) and efficacy (genetic correction, transfusion requirements, anemia and hemolysis markers), followed by a long-term follow-up study. As of November 2023, two adult and two pediatric patients have received RP-L301. All achieved neutrophil engraftment by day 15. Genetic correction (PB VCN up to 2.0) was observed in all patients. The adults demonstrated sustained Hb normalization and transfusion independence up to the latest follow-up visit (36 months). Concurrent improvement in anemia and hemolysis markers (bilirubin, haptoglobin, reticulocytes, and erythropoietin) were observed. Both adult patients reported improved quality of life (increased FACT-An and SF-36 scores, with marked improvement in SF-36 energy/fatigue, physical functioning, and general health domains). The pediatric cohort (with ≤9 months of follow up), had Hb improvement relative to pre-treatment baseline. No patients required transfusions following engraftment. No serious adverse events are attributed to RP-L301. PB ISA analyses up to 36 months follow-up indicate highly polyclonal patterns; longitudinal results delineating clonal diversity will be presented. RP-L301 is a potential treatment for patients with severe PKD, including those who did not derive benefit from available therapies (including splenectomy and mitapivat). Robust and sustained efficacy up to 36 months post-treatment is demonstrated by normalized Hb (adult cohort) and clinically significant Hb increases (pediatric cohort) with both cohorts demonstrating improved hemolysis parameters, with no transfusion requirements after engraftment.

5. Personalized Neoantigen DNA Vaccine (GNOS-PV02) and Pembrolizumab Develop Antitumor Poly-Functional Neoantigen-Specific CD4+ and CD8+ Effector T Cells in Advanced Hepatocellular Carcinoma Therapy

Renzo Perales[1], Jian Yan[2], Mark Yarchoan[3], Edward Gane[4], Thomas Marron[5], Neil Cooch[1], Daniel Shu[6], Elana Fertig[7], Luciane Kagohara[8], Gabor Bartha[9], Josette Northcott[9], John Lyle[9], Sarah Rochestie[1], Joann Peters[1], Jason Connor[10], Elizabeth Jaffee[11], Alfredo Perales-Puchalt[1], David B. Weiner[12], Ildiko Csiki[1], Niranjan Y. Sardesai[1]

[1]Geneos Therapeutics, Philadelphia, PA,[2]Research & Development, Geneos Therapeutics, Philadelphia, PA,[3]Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD,[4]New Zealand Liver Transplant Unit, University of Auckland, Auckland, New Zealand,[5]Early Phase Trials Unit, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY,[6]Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD,[7]Department of Applied Mathematics and Statistics, Johns Hopkins University Whiting School of Engineering, Baltimore, MD,[8]Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD,[9]Personalis, Inc., Fremont, CA,[10]ConfluenceStat, LLC, Orlando, FL,[11]The Johns Hopkins University School of Medicine, Baltimore, MD,[12]The Wistar Institute, Philadelphia, PA

A personalized therapeutic cancer vaccine (PTCV) tailored against neoantigens identified in an individual's tumor may enhance responses to PD-1 inhibitors through the induction of tumor-specific immunity. Here, we present results from a single-arm Phase Ib/2a trial evaluating a DNA plasmid (GNOS-PV02) encoding up to 40 neoantigens co-administered with plasmid-encoded IL-12 (pIL12) in combination with pembrolizumab (PEMBRO) in patients (pts) with advanced HCC. Pts were eligible for study therapy upon progression or intolerance with a 1L tyrosine kinase inhibitor (TKI). The PTCV [GNOS-PV02 (1mg) and pIL12 (0.34mg)] was administered intradermally via in vivo electroporation Q3w x 4 doses, and Q9w thereafter. PEMBRO was administered at 200mg IV Q3w. The primary endpoints were safety and immunogenicity. The data cut date was August 18, 2023. Among the 36 enrolled pts who received at least one dose of treatment, there were no DLTs or treatment-related grade ≥3 events. ORR (mITT) per RECIST 1.1 was 30.6% (11/36; 9 confirmed and 2 unconfirmed) with 8.3% (3/36) of pts achieving a CR. A complete molecular response (100% ctDNA clearance) was detected in 7 pts including the 3 radiographic CRs, and 4 additional pts who continue to show durable tumor control (3PR, 1 SD). Immunological analyses confirmed the induction of neoantigen-specific T cell responses by IFNγ-ELISpot in 19/22 (86.4%) evaluable pts, and pts with a larger ELISpot response showed a trend towards longer OS. PTCV immunization resulted in a significant increase of positive epitopes in both clinically responding and non-responding patients. The expression of T cell- biomarkers - CD8A, CD8B, CCL5, CXCR6, LCK, and TIGIT was significantly increased in the responding patients. Multi-parametric cellular profiling and single-cell analysis revealed active, proliferative, and cytolytic vaccine-specific CD4+ and CD8+ effector T cells in the blood of immunized pts, with a higher

CD8-to-CD4 ratio. In 14/14 (100%) of pts with paired pre- and on-treatment blood and tumor biopsies, we identified by TCRβ bulk sequencing expanded T cell clones in the peripheral blood that also trafficked into the tumor. Our data shows that the personalized DNA-based vaccine (GNOS-PV02) in combination with pembrolizumab generates a significant poly-functional neoantigen-specific antitumor CD8+ and CD4+ T cell responses, including T cell clonal expansion and infiltration to the tumor. These features could explain the mechanism of action behind the clinical activity observed in advanced HCC patients.

6. Allogeneic NKT Cells Expressing a CD19-Specific CAR in Patients with Relapsed or Refractory B cell Malignancies: An Interim Analysis

Leonid S. Metelitsa, Amy N. Courtney, Carlos A. Ramos
Baylor College of Medicine, Houston, TX

T cells expressing CD19-specific chimeric antigen receptors (CARs) mediate high complete response rates in patients with B cell malignancies. However, autologous cell products are costly and time-consuming to produce and demonstrate variability in potency and toxicity. Monomorphic CD1d-restricted Vα24-invariant natural killer T cells (NKTs) are not alloreactive like HLA-restricted T cells and therefore can be made from allogeneic donors without risking graft-versus-host disease. We generated a bank of healthy donor NKTs that co-express a CD19-specific CAR, IL-15, and shRNA targeting beta-2-microglobulin and CD74 to downregulate HLA class I and II expression, respectively. Five lots of allogeneic CD19-CAR-NKTs have been produced for this bank with each lot averaging 14.8 ± 2.5 days in culture, yielding 1.18 - 2.27x10(10) cells per lot and an average NKT purity of 99.0% ± 1.02%. We are assessing the safety, persistence, and efficacy of this allogeneic product in ANCHOR (NCT00840853), a first-in-human phase 1 dose-escalation trial for patients with relapsed/refractory B cell non-Hodgkin's lymphoma (NHL) and relapsed acute B lymphoblastic leukemia (ALL). No dose-limiting or grade 2+ toxicities related to CAR-NKTs have been observed in patients treated on three dose levels (DLs): 1x107 (DL1), 3x107(DL2), and 1x108 (DL3) CAR-NKT cells/m2. So far, seven NHL patients have been infused on three DLs, and two ALL patients have been infused on DL1. Three NHL patients achieved an initial partial response four-to-six weeks after infusion that in two cases evolved into a complete response (CR)(patients NHL-2 and NHL-4) by three months post-infusion. Additionally, one ALL patient (ALL-1) achieved a CR with incomplete hematologic recovery. We detected expansion of CAR-NKTs in the peripheral blood of patients NHL-5 (DL2) and NHL-7 (DL3) that peaked one week after infusion, and CAR-NKTs were also found in biopsied tumor tissue from patient NHL-3 at one week and NHL-2 at five weeks post-infusion. In patient NHL-2, who achieved a CR at three months post-infusion, we detected a population of recipient NKTs that underwent a 2000-fold expansion event, with numbers peaking at six weeks post-infusion and remaining elevated through 12 weeks. Our data indicate that allogeneic CAR-NKTs are well tolerated and can mediate objective responses in relapsed/refractory NHL/ALL patients, suggesting that NKTs represent a promising platform for "off-the-shelf" cancer immunotherapy.

7. Reprogramming of the Tumor Microenvironment in Glioblastoma Multiforme by Transplantation of Genetically-Engineered Hematopoietic Stem Cells

Bernhard Rudolf Gentner[1], Matteo Barcella[2], Francesca Farina[3], Marica Eoli[4], Alessia Capotondo[2], Filippo Gagliardi[5], Silvia Snider[5], Quintino Giorgio D'Alessandris[6], Valeria Cuccarini[7], Elena Anghileri[4], Mariagrazia Garramone[2], Valentina Brambilla[8], Francesco Di Meco[9], Paolo Ferroli[9], Valeria Ferla[3], Federico Legnani[9], Stefania Mazzoleni[8], Alessandro Olivi[6], Roberto Pallini[6], Marina Grisoli[7], Gaetano Finocchiaro[10], Carlo Russo[11], Fabio Ciceri[3], Luigi Naldini[2]
[1]CHUV, Lausanne, Switzerland,[2]SR-Tiget, Milan, Italy,[3]Hematology and Bone Marrow Transplant Unit-HSR, Milan, Italy,[4]Neuro-Oncology Unit - INCB, Milan, Italy,[5]Neurosurgery Unit - HSR, Milan, Italy,[6]Neurosurgery Unit - Policlinico Gemelli, Rome, Italy,[7]Neuroradiology Unit - INCB, Milan, Italy,[8]Genenta Science, Milan, Italy,[9]Neurosurgery Unit - INCB, Milan, Italy,[10]Neuro-Oncology Unit - HSR, Milan, Italy,[11]Genenta Science, New York, NJ

Introduction: Glioblastoma multiforme (GBM) is an incurable, highly aggressive tumor affecting the central nervous system, with a reported median survival of only 12.7 months in the unfavorable subgroup of patients with unmethylated (u)MGMT promoter. Immunotherapy has largely failed, due to intratumor heterogeneity, low T cell infiltration and a highly immunosuppressive myeloid tumor microenvironment (TME). Methods: We are conducting a phase 1/2a study in newly diagnosed patients presenting with (u)MGMT GBM (NCT03866109) evaluating the safety and biological activity of Temferon, a hematopoietic stem cell (HSC)-based gene therapy that induces local release of alpha-interferon (IFNα) from tumor-infiltrating macrophages. We performed scRNAseq and scTCRseq analysis of second surgery GBM tumors from n=5 patients enrolled in the Temferon study (post gene therapy) and n=6 control patients treated according to best standard-of-care to evaluate Temferon's effect on immune cell reprogramming. Findings were validated on an extended control group encompassing >100 GBM patients from publicly available scRNAseq datasets. Results: As of June 30th, 2023, 21 uMGMT GBM patients in 7 cohorts received 4 incremental doses of Temferon (0.5-3.0x10^6/kg). With a median follow-up of 29 months, all patients show long-term engraftment of genetically engineered cells, with no dose-limiting toxicities. The interim survival rate at 2 years is 28% (5 out of 18 patients; 3 patients excluded due to FU<12 months), which is higher than what is reported in the literature (15%). One patient is alive for >3 years after diagnosis, without further therapy. Genetically engineered cells were locally detected within the tumor lesion at levels >3% in 2/5 patients, and tumors showed increased IFNα-induced gene expression compared to the pre-treatment sample. These data indicate the recruitment of Temferon progeny into the TME, where they locally release IFNα. scRNAseq allowed unbiased identification of multiple subclusters within myeloid, lymphoid, tumor and stroma cell compartments. Most of these clusters showed strong up-regulation of IFNα and inflammatory responses. Tumors from Temferon patients were characterized by an increase in M1-like and a

decrease in M2-like macrophages. Of note, this population shift was particularly evident when comparing a stable with a progressive tumor lesion biopsied contemporaneously in 1 GBM patient, reproducing findings from a GBM mouse model (PMID: 35857642). Concomitant with myeloid cell reprogramming, it is striking that Temferon patients had more CD8 effector cells that were enriched with signatures of antitumor neoantigen-reactive T cells, a novel finding that challenges the prevailing dogma that GBM is an immune wasteland. Conclusion: Temferon, a first-in-class gene therapy targeting the myeloid TME in GBM patients is safe and biologically active at the tumor site favoring anti-tumor immunity. Preliminary survival data are promising, but longer follow-up is needed.

8. Long Term Safety and Integration Site Analysis Over a Large Cohort of Patients Treated with T Cells Modified by Lentiviral or Gammaretroviral Gene Addition

Frederic D. Bushman[1], Joseph Fraietta[1,2], Amy Marshall[2], Jan Joseph Melenhorst[3], Aoife M. Roche[1], John K. Everett[1], Sonja Zolnoski[1], Walter Rogal[2], Cory Czuczman[2], The CART Therapy Team[2], Carl H. June[2,4], Elizabeth Hexner[2], Julie Jadlowsky[2]

[1]Microbiology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA,[2]Center for Cellular Immunotherapies, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA,[3]Cleveland Clinic Lerner College of Medicine, Cleveland, OH,[4]Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

CART therapy has benefitted tens of thousands of cancer patients, but recent data has raised concerns about safety following observations of secondary T cell malignancies after treatment. Long-term follow-up (LTFU) is mandated for recipients of many gene therapies, and is intended to identify delayed toxicities and clarify mechanisms of cell product persistence. Here we summarize LTFU for 780 patients, including longitudinal tracking for 182 patients via integration site sequence analysis to quantify behavior of transduced cell clones. We analyzed 780 patients treated in modified T cell trials at the University of Pennsylvania between 2001 and 2023. With 1323 patient years of follow up, 21 (2.69%) of patients had a second primary malignancy. Tumor types included myelodysplastic syndrome (2) and acute myeloid leukemia (3); diffuse large B cell lymphoma (1), anaplastic large cell lymphoma (1), skin cancer (1 basal cell, 1 squamous cell, 1 melanoma); prostate (2), lung (3), neuroendocrine (1), thymoma (1) and uncategorized neoplasms (3). Median onset time was 1.86 years (range 51 days - 8.7 years). One case of anaplastic large cell lymphoma of the stomach was observed 344 days after infusion and was considered unrelated to CAR T cells with no detectable CAR product in lesional tissue. There were no instances of T cell malignancies related to the product reported. Integration site sequence analysis allowed characterization of possible insertional mutagenesis and quantification of clone sizes. Gene addition by both lentiviral and gammaretroviral vectors was analyzed. Comparison of these data to measures of vector copy number per cell clarified the extent of expansion of gene modified T cells within the full blood cell population. One extreme example, reported previously, involved integration within the TET2 gene and sharp clonal expansion, so that at the peak the TET2 clone comprised ~27% of all blood cells, both gene modified and unmodified. This case was distinctive in that the subject was already mutant in the other TET2 allele, helping explain the strong response. While notable for the expansion and dominance, this was a non-transformed clonal population of T cells that subsequently contracted. Further analysis of the remaining subjects showed that even modestly expanded clones were rare. Other than the previously described TET2 patient, maximum clone sizes, taking account of both gene modified and unmodified cells, averaged 0.16% over all samples analyzed to date, with a maximum of 1.3 %. Modest effects of integration on cell growth could be detected—there was increased frequency of integration in specific genes after T cell infusion, and cells with integration in specific genes were associated with longer persistence and modest clonal expansion. However, in our data, no example of vector insertion was associated with transformation or clinically concerning clonal skewing. In summary, we observed no examples of pathogenic insertional mutagenesis.

9. Safety and Efficacy of AB-1002 Gene Therapy in Patients with Advanced Heart Failure: Results from an Ongoing Phase 1 Clinical Trial

Luke Roberts[1], Timothy Henry[2], Eugene Chung[2], Monica Alvisi[1], Ferzin Sethna[1], David Murray[3], Jay Traverse[4], Lothar Roessig[1], Tugba Ozkan[1], Stacy Webb[1], Monika Mittal[1], Leigh Ervin[1], Kaitlyn Walker[1], Hesham Sadek[5], Sheila Mikhail[6], Kobra Haghighi[7], Canwen Jiang[1], R. Jude Samulski[6], Evangelia G. Kranias[7], Anna Tretiakova[1], Roger Hajjar[8]

[1]Asklepios BioPharmaceutical, Inc, Durham, NC,[2]Christ Hospital, Cincinnati, OH,[3]University of Wisconsin, Madison, WI,[4]The Minneapolis Heart Institute, Minneapolis, MN,[5]UT Southwestern Medical Center, Dallas, TX,[6]Asklepios BioPharmaceutical, Inc., Durham, NC,[7]University of Cincinnati, Cincinnati, OH,[8]Massachusetts General Brigham, Cambridge, MA

Congestive heart failure (CHF) is a progressive disease in need of innovative therapies. A key characteristic of failing hearts is abnormal intracellular Ca^{2+} handling and increased protein phosphatase 1 activity (PP1). Inhibition of PP1 by a constitutively active inhibitor-1 (I-1c) has been shown to enhance cardiac function in pre-clinical models of heart failure. We developed a novel cardiotropic and liver de-targeted capsid, AAV2i8, designed to overcome poor transduction in cardiac tissue. In a Phase 1 clinical trial the AAV2i8 capsid delivered a constitutively active I-1c protein to cardiac tissue via a single intracoronary infusion of AB-1002 (AAV2i8.I-1c). Eligible patients were >18yrs with non-ischemic cardiomyopathy, New York Heart Association (NYHA) class III with Left Ventricular Ejection Fraction (LVEF) of ≥15% to ≤35%. Efficacy measures included changes from baseline to month 12 in NYHA classification, Peak VO_2, 6-minute walk test (6MWT), LVEF and Minnesota Living with Heart Failure Questionnaire (MLHFQ). Safety and tolerability were assessed by collection of AEs and SAEs, laboratory tests, vital signs and ECGs. Eleven patients have received either 3.25E13 (Cohort 1; n=6) or 1.08E14 (Cohort 2; n=5) viral genomes (vg) of AB-1002. Three patients in cohort 1 have completed at least 36 months follow up and a further three have completed 3

months follow up. Three patients in cohort 2 have completed at least 30-months observation, and one completed 24 months of observation. Patients were aged between 53 and 76yrs, with 9 males and 2 females. Mean LVEF at baseline was 30.33% (SD 3.88; cohort 1) and 23.28% (SD 5.92; cohort 2). The first 3 participants in Cohort 1, showed clinically meaningful improvements in LVEF, NYHA Class, MLHFQ, Peak VO_2 and 6MWT at 12 months. Of the 3 remaining patients in cohort 1, two have shown initial improvement in NYHA Class, 6MWT and MLHFQ score at 3 months post administration. Among participants in Cohort 2, four of the 5 patients enrolled could be evaluated. Two of 4 patients showed improvements in MLHFQ and NYHA score, and all 4 showed improvements in LVEF at 12 months, compared to baseline. Peak VO2 declined in three but improved in one at 12 months while 6MWT declined in one patient, worsened in one, and was stable in two at 12 months. In support of this encouraging early clinical efficacy signal, left ventricular cardiac tissue was obtained from 1 patient in cohort 2 who underwent left ventricular assist device (LVAD) placement 13 months post-intracoronary gene transfer. AB-1002 demonstrated high myocardial transduction efficiency at 1.19 vg per cardiomyocyte and phosphorylation levels of phospholamban similar to normal hearts. To date AB-1002 has been well tolerated. Over the total follow up time available, 4 of 6 patients experienced 25 treatment emergent adverse events (TEAEs) and 5 patients experienced 56 TEAEs in cohorts 1 and 2 respectively, most of which were mild or moderate in severity. There was 1 fatal event in cohort 2 not considered related to investigational product. We believe our Phase 1 clinical trial demonstrates that AB-1002 is well-tolerated and results in positive efficacy outcomes in patients with non-ischemic HF. Further development of AB-1002 will be conducted in a phase 2 trial of up to 150 patients, GenePHIT, which is currently ongoing.

10. Intracochlear Administration of DB-OTO Gene Therapy in Pediatric Patients with Profound Hearing Loss Due to Otoferlin Mutations: The CHORD Phase 1/2 Open-Label Trial

Lawrence Lustig[1], Manohar Bance[2], Akira Ishiyama[3], Robert Nash[4], Ruben Polo[5], Angel Ramos[6], Manuel Jesus Manrique Rodriguez[7], Jay T Rubinstein[8], Tera Quigley[9], Jason Riggs[9], Eduardo Corrales[9], Jeffery Anderson[9], Vassili Valayannopoulos[9]

[1]Department of Otolaryngology - Head & Neck Surgery, Columbia University Medical Center, New York, NY,[2]Cambridge University, Cambridge, United Kingdom,[3]UCLA, Los Angeles, CA,[4]Great Ormond Street Hospital, London, United Kingdom,[5]Hospital Universitario Ramon y Cajal, Madrid, Spain,[6]Las Palmas University, Las Palmas Gran Canaria, Spain,[7]Clinica Universidad de Navarra, Pamplona, Spain,[8]University of Washington, Seattle, WA,[9]Regeneron Pharmaceuticals, New York, NY

Introduction: Biallelic otoferlin gene (*OTOF*) mutations commonly result in auditory neuropathy characterized by severe-to-profound sensorineural hearing loss. In these patients, the auditory brainstem response (ABR) is absent or severely reduced contrasting with normal outer hair cell function. *OTOF* gene replacement may provide instatement of high-quality, physiological hearing and be a more effective and durable treatment than cochlear implants. DB-OTO is a dual adeno-associated virus (AAV1) vector designed for intracochlear delivery of a full-length copy of the human *OTOF* gene under the control of the Myo15 hair-cell specific promoter. In this first-in-human clinical trial with DB-OTO (CHORD, NCT05788536), the safety, tolerability and preliminary efficacy of DB-OTO is being evaluated in pediatric patients with profound hearing loss caused by *OTOF* mutations. **Methods**: CHORD is a global, phase 1/2 trial of DB-OTO enrolling pediatric patients with biallelic pathogenic *OTOF* variants, profound hearing loss, presence of otoacoustic emissions or cochlear microphonic if >2 years. In Part A, the initial unilateral dose escalation phase of the study, DB-OTO is being administered by intracochlear injection using a typical facial recess approach through the round window. A 10-month-old female received a single intracochlear injection of 7.2×10^{12} vector genomes of DB-OTO unilaterally with a cochlear implant placed in the contralateral ear. At baseline the patient had no detectable hearing by behavioral pure tone audiogram (PTA) nor ABR. **Results**: Through week 12 after treatment, no dose-limiting toxicities and no DB-OTO related adverse events (AEs) were reported, including absence of vestibular manifestations. Hearing recovery was detected at 4 weeks post-treatment, with average behavioral PTA thresholds improving to 55 dB in the best frequencies (2, 4 and 8 kHz) at 12 weeks posttreatment. Hearing recovery measured by ABR showed a positive wave V amplitude through week 12 (thresholds of 40-80 dB, no response at 100 dB at baseline) in the DB-OTO treated ear, while no improvement was observed in the untreated ear. According to parental reports, as indicated by the LittlEARS auditory questionnaire, an improvement in the patient's global auditory skill development was also observed at week 12. Parents reported also more natural vocalizations when the patient was not wearing the cochlear implant on the other ear, indicating improved hearing acuity on the DBOTO-treated ear. Additional data from this and other enrolled patients will be presented. **Conclusions**: In this first-in-human gene therapy trial of DB-OTO, an overall positive tolerability profile was reported, with no AEs related to DB-OTO treatment reported. Audiometric data showed improvement in hearing from baseline corroborated by early auditory skills testing and parental reporting. These encouraging results prompt further investigation in other patients with profound hearing loss due to *OTOF* mutations.

New Technologies for Gene Targeting and Gene Correction

11. Efficacy and Integration of a Non-Viral *ABCA4* Transposon in Treating Stargardt Disease: Evidence from Mice and Primate Studies

Michelle E. LeBlanc, Chuanqi Peng, Shashank Shukla, Mitchell R. Kopacz, Kelsey A. Rush, Jungyeon Hwang, Michael J. Crowley, Subhadeep Dutta, Hugues Bernard, Ria Vashishth, Jonathan T. Lu, Sandeep Nema, Joseph J. Higgins, Joseph J. Senn

SalioGen Therapeutics, Lexington, MA

Introduction: Stargardt disease (STGD1) is a genetic disorder causing progressive central blindness. Novel integrative non-viral gene therapy applications overcome the limitations of low immunogenicity and durability. In addition, other advantages specific to STGD1 include the potential to deliver the large (6.8 kb) ATP Binding Cassette Subfamily A Member 4 (*ABCA4*) gene to correct the numerous *ABCA4* mutations in patients. The dysfunctional ABCA4 transporter causes STGD1 by altering the transfer of all-trans-retinal (ATR) and the accumulation of diretinoid-pyridinium-ethanolamine (A2E) and retinal dimer conjugates. In *Abca4*$^{-/-}$ and *Rdh8*$^{-/-}$ double knock-out (dKO) mice, A2E accumulation is accelerated compared to *Abca4*$^{-/-}$ single KO mice because the deficient all-trans-retinol dehydrogenase enzyme (Rdh8) augments the ATR defects. As such, we performed subretinal injections of an optimized lipid nanoparticle (LNP) to deliver an mRNA encoding a novel bioengineered transposase derived from the mammal, *Myotis lucifugus* (*bMLT*), and a DNA expressing full-length human *ABCA4* (*fl-ABCA4*) under the control of a constitutive promoter. This transposition process, called Gene Coding technology, was also tested in non-human primates (NHPs) to confirm interspecies translation. **Methods:** We co-encapsulated *bMLT* mRNA and *fl-ABCA4* cDNA in LNPs to evaluate dosing, tolerability, and subretinal administration (1uL per eye) in three groups of *dKO* mice at postnatal day 14 to 17: vehicle alone (n=2) and treated groups with low dose (LD) (n=4) and high dose (HD) (n=6). The three *dKO* groups were compared to wild-type (WT) mice (n=9) by serial fundoscopy, optical coherence tomography (OCT) both within and outside the treated retinal area, and histological sectioning. In 10-week-old mice, terminal A2E measurements were analyzed in the three *dKO* groups and age-matched WT mice at 50 days after subretinal injection using a reversed phase chromatography (RP-HPLC) method with analyte detection at a wavelength of 430 nanometers. To assess ABCA4 expression and genomic integration, we administered the same DNA/RNA co-encapsulated LNP by subretinal injection in African Green monkeys (n=2). Immunohistochemical (IHC) studies co-staining for ABCA4 and Arrestin-3, digital droplet polymerase chain reaction (ddPCR), and quantitative reverse transcription (RTqPCR) were used to show *ABCA4* integration and photoreceptor localization. **Results:** There were no detectable differences in retinal thickness by OCT outside the treated area in 10-week-old *dKO* mice compared to WT mice. Within the treated area, LD and HD treated *dKO* mouse groups showed no differences in OCT retinal layer measurements compared to the untreated controls, implying good tolerability. Untreated *dKO* mice showed a significant ($p<0.0001$) increase in A2E levels compared to age-matched WT mice. At 50 days post LNP injection, *dKO* HD treated mice showed a 40% reduction in A2E compared to untreated controls ($p=0.03$). NHP retinal IHC staining showed ABCA4 protein co-localization with the cone photoreceptor marker, Arrestin-3, and evidence of integration by ddPCR. **Conclusions:** Fifty days after treatment, *dKO* mice exhibited a reduction in A2E levels compared to untreated *dKO* mice suggesting correction of the Stargardt phenotype. NHP studies confirmed expression of *ABCA4* in photoreceptors and the interspecies translatability of Gene Coding technology. This non-viral integrative gene therapy represents a potentially promising, one-time treatment for STGD1.

12. RNA-Based Gene Writer and Lipid Nanoparticle (LNP) Delivery Enables Generation of Functional Chimeric Antigen Receptor (CAR) T Cells with *In Vitro* and *In Vivo* Anti-Tumor Activity and T Cell-Specific Genome Engineering *In Vivo*

Kartika Venugopal, Michael Magee, Alberto De Iaco, Jason Rodriguez, James Rottman, Donghui Li, Giulia Schiroli, Yu Cao, Rebecca Levy, Travis Schute, Mohit Gupta, Christina Ferren, Michael Monte, Jane Wang, Bill Querbes, Cecilia Cotta-Ramusino, Madhusudan V. Peshwa, Michael C. Holmes

Tessera Therapeutics, Somerville, MA

Autologous Chimeric Antigen Receptor (CAR) T-cell therapies have added an effective tool to the treatment options for relapsed and refractory leukemias and lymphomas. However, significant challenges limit patient access to these therapies, including eligibility, cost, scalability, and manufacturing. These limitations highlight the critical unmet need for effective same day treatment options. We are combining our Gene Writing and delivery platforms to enable T cell specific genome engineering, in an effort to reduce the challenges associated with conventional autologous CAR-T approaches. RNA Gene Writers are designed to introduce a broad range of alterations to the genome, from writing in a transgene, inserting an exon, or introducing single nucleotide changes, providing an attractive toolkit for sophisticated cell engineering. RNA Gene Writers are inspired by the biology of non-LTR retrotransposons, a class of mobile genetic elements that can edit the genome through target-primed reverse transcription, or TPRT. RNA Gene Writers are designed to be delivered as all-RNA composition, which is intended to facilitate in vivo delivery by lipid nanoparticles, or LNP. LNP-RNA delivery of RNA Gene Writers resulted in the integration and expression of green fluorescent protein (GFP) transgenes in >70% of primary human T cells in vitro. We achieved successful LNP delivery of RNA to T cells in vivo in both mouse and non-human primate (NHP) models, with approximately 80% and 40% GFP reporter expression observed, respectively. We demonstrated that LNP delivery of one of our RNA Gene Writers achieved the integration and CAR expression in an average of ~21% of primary human T cells in vitro. We did not observe detriment to cell viability or proliferative capacity in these CAR-T generated cells, and robust tumor cell killing was also observed in vitro. Moreover, RNA Gene Writer derived CAR-T cells demonstrated antigen specific tumor clearance in vivo when introduced into mouse xenograft models. We also demonstrated an average of ~6% GFP reporter expression in T cells after in vivo administration of LNP-RNA delivery into a humanized mouse model, potentially opening up the possibility of using RNA Gene Writers for in vivo CAR-T treatment. In addition, the modularity of our RNA Gene Writers delivered in an LNP potentially allows for multiplex editing to co-introduce multiple genetic changes in a single step using all-RNA compositions. In preclinical studies, we achieved >90% knockout of the TRAC and B2M genes, individually, in T cells, that when multiplexed led to ~88% of T cells with knockout of both TRAC and B2M. We also demonstrated T cells containing 3 edits, including TRAC and B2M knockout, and either GFP or CAR writing, showing that rewriting and writing can be multiplexed. Further, we

observed approximately 30% B2M knockout by rewriting following in vivo administration in a humanized mouse model. We believe our RNA Gene Writers are uniquely positioned to drive a broad range of genetic alterations, including multiplex editing, and the ability to deliver RNA Gene Writers as all-RNA compositions packaged in a LNP potentially offers greater simplicity and flexibility compared to conventional lentiviral based CAR-T therapies.

13. Language Models Generate Novel Genome Editors from Scratch

Jeffrey A. Ruffolo, Stephen Nayfach, Aadyot Bhatnagar, Joseph Gallagher, Joel Beazer, Jennifer Yip, Riffat Hussain, Jordan Russ, Alexander J. Meeske, Peter Cameron, Ali Madani

Profluent Bio, Berkeley, CA

Gene editing has the potential to cure disease yet requires complex molecular machinery for precise manipulation of cellular genetic material. Editors derived from microbial CRISPR systems, while powerful, often face tradeoffs in key attributes like size, activity, and immunogenicity when used in non-native environments. AI-based protein engineering is a powerful alternative that has the potential to bypass evolutionary constraints and generate proteins with optimal properties. Here, we present the first programmable gene editor designed 'from scratch' using artificial intelligence models. Our models were trained on massive-scale biological diversity, including a dataset of over one million CRISPR operons systematically identified from 26 terabases of assembled genomes and meta-genomes. Our models generate thousands of diverse CRISPR-associated proteins never before seen in nature, including highly functional Type II nucleases that are >400 mutations away from any known natural protein. We also used AI models to generate fully synthetic single-guide RNAs that were compatible with their cognate gene editors and boosted activity. Our most performant AI-generated editor, which we denote OpenCas, exhibited up to 95% editing efficiency across a variety of cell types, has a low off-target rate, is compatible with base editing, and is predicted to have reduced immunogenicity. We intend to release OpenCas to the public in order to democratize the ethical usage of gene editing across research and commercial applications. Collectively, our results demonstrate the power of AI to build customized gene editing proteins and will be a foundation for AI-based protein engineering in the future.

14. Retron Mediated Exon-Sized Genome Insertion Using an All- RNA System

Kyong-Rim Kieffer-Kwon, Jacob Layer, Zhongxia Yi, Socheata Ly, Elise Keston-Smith, Shailesh Gurung, Elizabeth Narayanan, Sierra Harken, Abril Fleitas, Aisha A. Aljanahi, Dawn Ciulla, Matthew Pandelakis, Hari Jayaram, Vlad Presnyak, Inna Shcherbakova

ReNAgade Therapeutics, Cambridge, MA

While exogenous DNA has been instrumental in advancing precise genome editing technology, effective delivery of donor DNA remains a substantial hurdle in therapeutic applications. DNA is frequently packaged in viral vectors, which presents challenges such as non-specificity, liver toxicity, immunogenicity, and high manufacturing costs. There is a need for a non-viral "all-RNA" gene editing modality that enables *in vivo* targeted insertions and potential re-dosing. Retrons, which hail from bacterial retroelements involved in bacteriophage defense, are minimally two-component RNA systems comprising a non-coding RNA (ncRNA) and an associated reverse transcriptase (RT) which together can be harnessed to create ssDNA templates required for targeted insertion when coupled with a CRISPR nuclease or nickase. We have screened and optimized retrons that show vastly improved editing efficiency of exon-sized gene insertions in cell lines and primary cells. Starting with an *in silico* retron prediction algorithm we identified >7000 novel retrons. After screening a phylogenetically diverse subset of hundreds of these for precise insertion activity we identified >50 retrons with up to a 45-fold better performance than our Eco1 benchmark. We have utilized a combination of retron RT engineering, ncRNA minimization, chemical modification, and homology-dependent repair manipulation to improve retron-mediated target insertion. Retron system optimization using *in silico* modeling-based RT engineering achieved 2-fold higher 305 base pair insertion. These combinations of ncRNA engineering and retron system optimization has led to ~40% editing efficiency in hematopoietic stem cells and T cells. Together, our initial data demonstrates retron capabilities to produce repair templates for exon-sized insertions using an all-RNA system in mammalian cells.

15. Reduction in Triglycerides through a Novel Ultracompact CRISPR System: Efficacy in Mouse Models and NHP Studies

Lucas B. Harrington, Janice Chen

Mammoth Biosciences, Brisbane, CA

In the last decade, CRISPR-based therapies have made tremendous progress in cell therapy applications and selected diseases that can be targeted in the liver. However, correcting the vast majority of genetic diseases outside the liver has remained challenging due to the large size of the original CRISPR systems, such as Cas9 and Cas12a. Ultracompact CRISPR systems present an opportunity to overcome the challenge of *in vivo* delivery, enabling single-AAV delivery of genome editors, including next-gen editing techniques such as epigenetic editing, reverse transcriptase editing, and base editing. Although many compact CRISPR systems have been described, their application to therapeutic targets has been limited due to challenges in matching the efficacy of larger systems. Here, we describe the discovery of a novel ultracompact CRISPR platform and show it is a potent genome editor *in vivo* in mouse and nonhuman primate models. Through high-throughput screening of natural variants, extensive protein engineering, and guide optimization, we optimize this platform for robust editing with both AAV and LNP delivery. This ultracompact CRISPR system was effectively used to target the APOC3 gene, a key player in triglyceride metabolism, offering promising therapeutic potential for durable treatment of severe hypertriglyceridemia. *In vivo* studies in humanized mouse models demonstrated an 80% reduction in triglyceride levels. Complementing these findings, our nonhuman primate studies further confirmed significant genetic editing of APOC3 with no major safety concerns observed. In addition, we provide an update on leveraging these ultracompact CRISPR systems for single AAV delivery of epigenetic editing for muscular dystrophy. These results underscore

the potential of ultracompact CRISPR systems for precise and efficient gene editing applications in diverse conditions and to enable single-aav compatible next-gen editing approaches such as epigenetic editing.

16. Selective Repression of C9ORF72 Repeat Expansion-Containing Sense and Antisense Transcripts in an ALS Mouse Model

Amrutha Pattamatta[1], Mohammad Samie[2], Awdhoot Godbole[3], Chen Ting-Wen[1], Anagha Sawant[1], Susan Ping[4], Alexandra Srnka[1], Siobhan Ford[4], Lujain Al-Sowaimel[1], Ricardos Tabet[4], Robert Bell[1], Lisa R. Keyes[5], Sheila Rao-Dayton[5], Rob Moccia[3], Claudia H. Huichalaf[4], David Shivak[6], Bryan Zeitler[2], Amy Pooler[2], Christine Bulawa[1]

[1]Previous affiliation, Pfizer Inc, Cambridge, MA,[2]Sangamo Therapeutics, Inc., Richmond, CA,[3]Pfizer Inc, Cambridge, MA,[4]Genomic Medicine, Alexion AstraZeneca Rare Disease, Cambridge, MA,[5]Genomic Medicine, Alexion AstraZeneca Rare Disease, Durham, NC,[6]Previous affiliation, Sangamo Therapeutics Inc, Richmond, CA

Amyotrophic lateral sclerosis (ALS) is a progressive disease characterized by degeneration of motor neurons in the brain and spinal cord, leading to paralysis and early death. The most frequent genetic cause of ALS is the expansion of the hexanucleotide GGGGCC (G4C2) repeats in the first intron of C9ORF72 gene. Healthy individuals carry up to 30 hexanucleotides repeats, but expansions of this region to hundreds or even thousands cause familial ALS and frontotemporal dementia. There are several mechanisms that may contribute to the disease progression as a result of this expansion. G4C2 repeats undergo bidirectional transcription generating sense and antisense expansion containing RNA and RNA foci as well as repeat-derived peptide translation products, suggesting a pathological, gain-of-function mechanism. To decrease the levels of expansion-containing transcripts in cells, while maintaining expression of healthy C9ORF72 mRNA levels, we previously identified an engineered transcription factor comprised of a zinc finger protein (ZFP) specifically targeting the G4C2 repeat region fused to a DNA-binding repressor protein (KRAB), named ZF-R. We showed >90% reduction of both sense and antisense G4C2 containing transcripts in patient-derived fibroblasts and motor neurons from patient-derived iPSCs carrying 1200 G4C2 repeats with minimal detectable off-target activity as measured by global transcriptomics analyses. To evaluate the potential use of this approach therapeutically, we studied the effect of ZF-R in neonate and adult $C9^{500}$-BAC transgenic mice. ZF-R was delivered via ICV in neonates, and 5 weeks post injection animals were euthanized for molecular analysis. Using this approach, we reached ~70-80 % reduction of expanded repeat RNA (sense and antisense) in the cortex, associated with 40% of C9ORF72 protein reduction. We also observed ~50% decrease in sense and antisense RNA foci in the same brain region. The repression of sense and antisense containing RNA in the cortex was not associated with any changes in the molecular markers of neuroinflammation or neuronal loss. For the adult study, five month old $C9^{500}$-BAC mice were injected via ICV with the same construct. Five months post injection animals were euthanized, and molecular analysis was performed. Similar to the neonate study, we found a strong reduction of expanded repeats and also ~60% decrease in Dipeptide repeats (DPR) proteins in the Cornu Ammonis (CA) of hippocampus. Overall, these findings strongly support the use of ZF-Rs for the treatment of familial ALS.

17. Systematic Discovery, In Vivo Delivery, and DNA Repair Mechanism of Single-Strand Annealing Protein for Precision Integration of Large DNA Sequences

Le Cong[1], Di Yin[2], Guangxue Xu[3], Yuanhao Jerry Qu[1], Chengkun Wang[1], Xiaotong Wang[2], William Arthur Johnson[4], Gabriel Filsinger[2], Tim Wannier[5], George M. Church[6], Lai Yee Phoon[7], Boya Gao[7], Li Lan[7]

[1]Pathology, Genetics, Stanford University, Stanford, CA,[2]Stanford University, Stanford, CA,[3]Stanford University, Palo Alto, CA,[4]Stanford University Laboratory for Cell & Gene Medicine - Palo Alto, CA, Stanford, CA,[5]WildMicrobes, NA, CA,[6]Harvard Medical School, Boston, MA,[7]Duke University, Durham, NC

The challenge of accurately inserting large DNA sequences is a significant limitation in current genome editing tools, particularly for in vivo gene therapy applications. Our project explores the potential of microbial recombination systems, focusing on phage-encoded Exonuclease and Single-Strand Annealing Proteins (Exo-SSAPs), for efficient, cleavage-free gene editing in mammalian cells. Building on prior work demonstrating large DNA integration via SSAPs directed by catalytically inactive dCas9, we investigated a large range of microbial recombination systems, identifying over 26,000 recombination systems originating from both prokaryotic and eukaryotic sources. Via this systematic survey, we identified top candidates with three-fold efficiency compared to reported SSAP editors. This improved system is shown to be compatible with in vivo delivery using AAV vectors, successfully integrating knock-in sequences in primary mouse cells. Further, we explored Exo-SSAP mediated integration and DNA repair mechanisms in human cells, particularly with dCas9 genome targeting to avoid activation of double-stranded break (DSB) repair pathways. Our genetic and chemical perturbation results revealed that the Mismatch Repair pathway (MMR) proteins could play a pivotal role in the Exo-SSAP mediated DNA insertion. Specifically, by targeting MMR enzymes, we could further enhance the efficiency of Exo-SSAP integration in human cells, indicating room for future improvements. Overall, our study highlights the significant potential of microbial recombination systems for large DNA sequence integration in human cells. The mechanistic part indicates that mismatch repair proteins could be a previously under-appreciated mechanism that influences DNA integration when DSB is absent during the editing process. These results open new avenues for understanding and applying large DNA insertion in gene therapy, setting the stage for in vivo therapeutic applications.

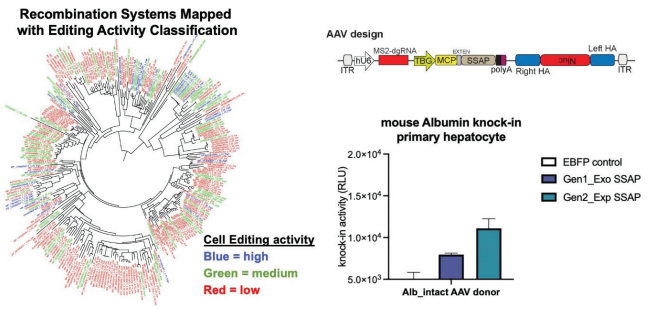


Figure. Left: mining of over 26,000 Exo-SSAP systems reveal differential cellular activities distributed across evolutionary tree. Right, proof-of-concept demonstration of large insertion (800bp) in primary hepatocyte using improved Exo-SSAP system.

AAV Vectors - Preclinical and Proof-of-Concept: Therapy Focus

18. Breaking Ground in CMT1B Treatment: AAV9-Mediated Dual RNAi and Gene Replacement Therapy Targeting Schwann Cells Improves Myelination and Peripheral Nerve Function in Mice

Daniella Munezero[1], Jingting Zhu[1], Arun Paripati[1], Russell Chuah[1], Lauren Taylor[1], Afrooz Rashnonejad[1,2]

[1]Center for Gene Therapy, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, OH,[2]Department of Pediatrics, The Ohio State University, Columbus, OH

Charcot-Marie-Tooth (CMT) encompasses a diverse group of hereditary sensory and motor neuropathies resulting in progressive disability and pain. Lacking a cure for these debilitating diseases, current patient care strategies involve symptom management, surgery, rehabilitation, and occupational therapy. CMT1B, a demyelinating subtype, is associated with over 200 different mutations in *Myelin Protein Zero* (*MPZ*) gene. Most mutations in *MPZ* gene are autosomal dominant and exhibit toxic gain-of-function effects, resulting in the aggregation of mutant MPZ protein within Schwann cells (SCs) followed by endoplasmic reticulum (ER) stress, defective myelination, reduced nerve conduction, failure of peripheral nerve and subsequent muscle wasting and weakness. Creating a single, comprehensive therapy for CMT1B is challenging due to the diverse mutations throughout the MPZ gene. In response, we developed a novel, universal AAV gene therapy approach termed AAV9-KDAR (**K**nock**D**own **A**nd **R**eplacement). This strategy involves knocking down mutated MPZ using RNAi to reduce the aggregate burden in SCs, and concurrently replacing it with a functional RNAi-resistant MPZ (rMPZ) gene provided by the same vector to promote re-myelination. Given that MPZ constitutes over 50% of proteins in the myelin sheath, its replacement is essential for enhancing myelination. Twelve micro-RNAs targeting MPZ gene (miMPZ) were designed and cloned. To identify lead miRNAs capable of targeting both mutant and wild-type MPZ (mut/wtMPZ) mRNA without affecting rMPZ expression, we co-transfected HEK293 cells with mut/wtMPZ plasmids, rMPZ, and each miMPZ, followed by luciferase assays, western blot, and qRT-PCR analyses. Then, the effects of AAV-based MPZ knockdown, rMPZ replacement, and MPZ-KDAR were investigated separately in an ex-vivo model of severe early-onset CMT1B-R98C's DRG/ SCs co-cultures. Reduction of mutant MPZ, decreased ER stress, and increased myelination were assessed using IF staining, western blot, and qRT-PCR. Off-target effects of miMPZ were investigated by RNA-seq analysis in human Schwann cells. Next, we tested the in vivo efficacy of AAV9-KDAR therapy at two-time points: pre and early onset of CMT1B disease. We administered 2.5E+11 or 5E+11 vg of AAV9-miMPZ-rMPZ via intracerebroventricular or intrathecal injections on neonatal and adult CMT1B-R98C mice, respectively. Molecular, behavioral, and electrophysiological assays were performed at 6 months post-injection. Lead miMPZs successfully reduced mut/wtMPZ levels by > 85-fold in both ex vivo and in vivo models without affecting rMPZ expression. Interestingly, silencing MPZ alone worsened demyelination symptoms, and replacement of MPZ alone was insufficient to reverse ER stress and restore myelination. In contrast, CMT1B-DRG/SCs co-cultures treated with KDAR approach exhibited enhanced myelination, increased numbers of MBP + internodes, and reduced ER stress biomarkers. Similarly, we detected a significant reduction in mut/wtMPZ and a 60-fold increase in rMPZ expression in treated sciatic nerves accompanied by improvement in treadmill and rotarod motor performance by 50% , as well as CMAP and NCV electrophysiological outcomes in treated pre- and post-onset CMT1B-R98C mice compared to untreated controls. Furthermore, no significant off-target effects were detected by RNA-seq analysis on lead miRNA-treated human SCs. This study marks a groundbreaking development in gene therapy for CMT1B, providing proof of concept for treating CMT1B through AAV9-KDAR approach. Nonetheless, additional research is imperative to optimize AAV doses, enhance efficacy, and assess in vivo safety thoroughly.

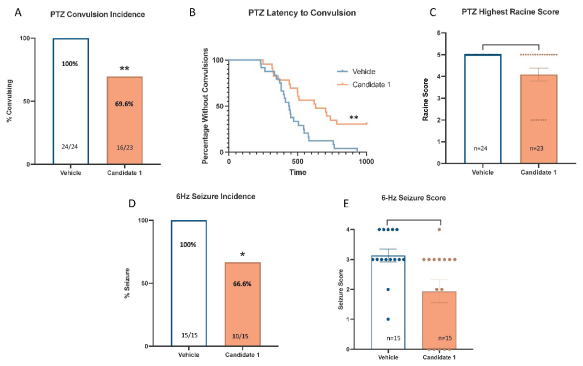
19. GABA Selective AAV-Mediated Gene Therapy Provides Durable Seizure Protection in Multiple Refractory Epilepsy Models

Brooke Babineau, Sheila Sears, Jennifer Su, Chao Tai, Keiko Takahashi, Adriana Gonzalez-Sandoval, Annie Tanenhaus, Pingping Jia, Rangoli Aeran, Tselmeg Amarlkhagva, Ming Chen, Dixon Hoffelt, Jason Le, Keith Place, Sammy Liang, Mark Elegue, Suresh Poda, Stephanie Tagliatela

Encoded Therapeutics, South San Francisco, CA

Rationale: Epilepsy affects approximately 1% of the population, equating to 3 million individuals in the U.S. Nearly a third of epilepsy cases fail to respond to first- and second-line treatment, leaving patients with persistent and potentially disabling seizures. Antiseizure medications often exhibit a 'honeymoon period' of initial efficacy that diminishes over time and are associated with significant side effects, including organ toxicity, drowsiness, dizziness, and cognitive impacts. Consequently, there is an urgent unmet need for novel treatments to address refractory epilepsies. Genetic targeting of GABAergic neurons to enhance inhibition in an activity-dependent manner has the potential to restore excitatory-inhibitory balance in the brain and

reduce seizure burden. **Methods:** Using Encoded's vector engineering platform, we identified regulatory elements capable of driving cell-selective transgene expression within GABAergic inhibitory neurons. We coupled these GABA-selective AAV expression cassettes with effector genes with known roles in regulating neuronal excitability and/or neurotransmission. Candidates or vehicle were administered to wild-type mice via intracerebroventricular (ICV) injection on postnatal day 1 and seizure protection was evaluated 7-8 weeks later after chemical or electrical induction. In the 6-Hz model of focal seizures and refractory epilepsy, mice were administered an electric shock to induce seizures and protection was measured within 30 seconds of electrical stimulus. In the Pentylenetetrazole (PTZ) model for tonic-clonic seizures, mice were given a subcutaneous injection of PTZ to induce seizures and protection was measured over 30 minutes. **Results & Conclusions:** The top candidate (Candidate 1) showed promising results in the 6-Hz and PTZ models, reducing both seizure incidence and severity. In the PTZ assay, Candidate 1 also prolonged the latency to first convulsive seizure. Our novel AAV-mediated GABA-selective gene therapy approach demonstrated *in vivo* efficacy across multiple epilepsy models, supporting its potential to provide durable seizure reduction in genetically heterogenous epilepsy populations. **Figure 1.** Efficacy of gene therapy Candidate 1 in the PTZ and 6Hz electroshock seizure assay: PND1 ICV delivery of Candidate 1 significantly reduced seizure incidence (Panel A; p=0.0156, Wilson Signed Rank Test), increased latency to first tonic-clonic convulsion (panel B; p=0.0025, Mantel-Cox test), and reduced seizure severity score (Panel C; p=0.039, Mann-Whitney) in the PTZ assay at 7 weeks of age. Additionally, PND1 delivery of Candidate 1 in the 6Hz assay at 8 weeks of age resulted in a significant reduction in both seizure incidence (Panel D; p=0.0143, Chi-square) and seizure severity (Panel E; p=0.0128, Mann-Whitney) when compared with vehicle controls.

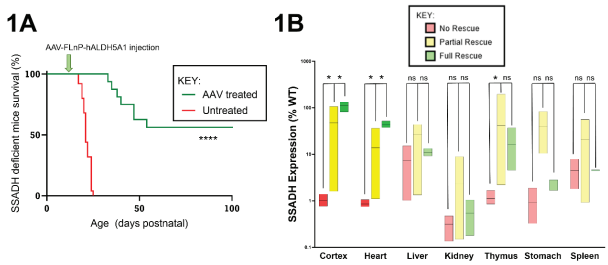


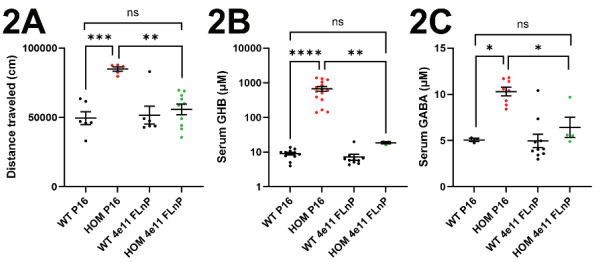
20. Curable Hyperactive Behaviors and Serum Biomarkers upon Gene Replacement Therapy in Succinic Semialdehyde Dehydrogenase Deficiency in Mice

Henry Lee[1], Gabrielle McGinty[1], Amanda Liebhardt[1], Bjoern Welzel[1], Alex Zhang[1], Clifford Woolf[1], Guangping Gao[2], Alexander Rotenberg[1]

[1]Neurology, Boston Children's Hospital, Boston, MA,[2]UMass Chan Medical School, Worcester, MA

Introduction: Succinic semialdehyde dehydrogenase deficiency (SSADHD) is a rare metabolic neurodevelopmental disorder due to loss of function mutations of *ALDH5A1* gene. SSADH is essential for degradation of the inhibitory neurotransmitter γ-aminobutyric acid (GABA). In SSADHD, pathologic accumulation of GABA and its metabolite γ-hydroxybutyrate (GHB) results in early life encephalopathy with autism, refractory epilepsy, and high mortality rate from seizures. Targeted drug treatments for SSADHD are ineffective. Gene replacement therapy is a potential cure for SSADHD but is unavailable. As a step toward clinical translation of SSADH gene replacement, we developed and tested the therapeutic efficacy of a novel vector encompassing a human *ALDH5A1* full length native promoter (FLnP) tethered with a human *ALDH5A1* coding sequence, namely AAV-FLnP-hALDH5A1, and tested its efficacy using SSADH deficient mice. **Methods:** We packaged AAV-FLnP-hALDH5A1 into PHP.eB capsid and injected into homozygous (HOM) SSADH deficient mice and wild-type (WT) littermates via intraperitoneal (IP) injection at high dose (~6-8x10^{13} gc/kg) at P14 symptomatic stage. Survival was recorded and tracked until P100. Automated native exploratory behavioral tracking was performed using a high resolution ventral view camera at P100. Whole blood, brain tissues, and internal organs were collected at P100 for serum metabolites and protein analyses. **Results:** AAV-FLnP-hALDH5A1 administration resulted in significant increase in survival at P100 (55%) compared to 0% (p=0.0002, Fisher Exact Test) in untreated HOM mice (1A). Western blot data revealed that SSADH was restored to 70-95% WT levels in the brain of treated mutants, and up to 50% in other vital organs (1B). Importantly, the level of restored SSADH in brain and myocardium correlated with HOM mice survival. At a behavioral level, we found hyperactive behavior (distance travelled) before treatment (WT=495±46m, HOM=848±16m, p=0.0043, Mann-Whitney test) is reversible upon AAV-FLnP-hALDH5A1 treatment (WT+AAV=517±64m, HOM+AAV=557±38m, p=0.4278, Mann-Whitney test) (2A). These behavioral results corroborate serum biomarkers GHB and GABA demonstrating phenotypic reversibility upon AAV-FLnP-hALDH5A1 treatment: GHB (in μM) - WT=8.99±0.80, untreated HOM=672.17±108.99, AAV-treated HOM=18.60±1.32 (p=0.00001, One-way ANOVA) (2B); GABA (in μM) - WT=5.07±0.17, untreated HOM=10.31±0.47, AAV-treated HOM=6.43±1.10 (p=0.0003, One-way ANOVA) (2C). **Conclusions:** We demonstrated hyperactive behaviors and disease-associated serum biomarkers in SSADH deficient mice are reversible upon treatment of AAV-FLnP-hALDH5A1 at symptomatic stage. AAV-FLnP-hALDH5A1 therapeutic effects directly correlate with the amount of restored SSADH in brain and myocardium, providing insights into tissue targeting, delivery route, AAV serotype selection, and relevant biomarker for eventual clinical translation of our findings into a first-in-class therapy for SSADHD.





21. From Darkness to Light: *CIB2* Gene Delivery Rescues Age-Related Macular Degeneration-Like Phenotype in a Preclinical Model

Sehar Riaz[1,2], Saumil Sethna[3], Saima Riazuddin[1], Zubair M. Ahmed[3]

[1]Otorhinolaryngology Head & Neck Surgery, University of Maryland, School of Medicine, Baltimore, MD,[2]Center of Excellence in Molecular Biology, University of the Punjab, Lahore, Pakistan,[3]Department of Otorhinolaryngology - Head & Neck Surgery,, University of Maryland, School of Medicine, Baltimore, MD

Worldwide, nearly 200 million people suffer from irreversible vision impairment due to age-related macular degeneration (AMD), a multifactorial neurodegenerative disorder. Prior studies have identified risk alleles in over 30 genes associated with AMD. However, the exact mechanism behind AMD remains largely elusive. AMD comes in two flavors: neovascular (wet-AMD) and slower, irreversible dry-AMD. Recently, the FDA has approved two drugs targeting complement pathways to treat geographic atrophy (GA), the most advanced stage of dry-AMD. Previously, we reported that deficiency of calcium and integrin binding protein 2 (CIB2), specifically in the RPE, leads to progressive vision impairment and dry-AMD-like phenotype in mice. We also showed that *CIB2* regulates autophagy in RPE via the mTORC1 signaling pathway. Herein, we packaged h*CIB2* in two different Adeno-associated virus (AAV) serotypes (AAV-Anc80L65 and AAV2.7m8), along with a control GFP-expressing vector, and performed gene augmentation studies in *Cib2* mutant mice via subretinal delivery. Interestingly, *hCIB2* subretinal delivery in mutant mice, at 1 month of age, using either capsid rescued the visual function as ascertained by electroretinograms (ERG), which persisted until 9 months (the latest timepoint tested) post-injection. We also found improved photopic visual acuity and contrast sensitivity levels in *hCIB2*-injected mice. Transmission electron microscopy (TEM) studies revealed a reduction of sub-RPE deposits while immunolabeling followed by confocal imaging demonstrated significantly reduced levels of drusen proteins such as Apolipoprotein E, beta-amyloid, C3, and C5B-9, and lipids such as unesterified cholesterol in *hCIB2* injected mice, indicating improved autophagy, which was confirmed through immunoblotting of LC3-II and p62/SQSTM1 levels. Finally, significantly reduced levels of pS6K and p4E-BP1, suggesting that the delivery of *hCIB2* results in the downregulation of mTORC1 and, consequently, an upregulation of autophagy. Taken together, these findings support the notion that AAV-mediated *CIB2* delivery could be a promising therapy for individuals suffering from dry-AMD and other mTORC1 hyperactivity-associated disorders and warrants further safety and efficacy studies in larger species.

22. Prevention of High-Risk Allogeneic Corneal Transplant Rejection by AAV-Mediated Transfer of Novel Anti-Inflammatory Immunomodulator

Tomoko Hasegawa[1], Brian C. Gilger[2,3], Sutton B. Roger[3,4], Jacquelyn Bower[1,5], Chengwen Li[3,6,7], Matthew L. Hirsch[1,3,6]

[1]Department of Ophthalmology, University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Department of Clinical Sciences, North Carolina State University, Raleigh, NC,[3]Bedrock Therapeutics, Raleigh, NC,[4]Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX,[5]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC,[6]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC,[7]Department of Pediatrics, University of North Carolina at Chapel Hill, Chapel Hill, NC

Corneal blindness affects over 5 million individuals globally, with over 180,000 allogeneic corneal transplantation (CT) performed worldwide annually. However, high-risk CT encounter a challenge as almost all grafts are rejected within 10 years, despite the current standard of care. In this study, adeno-associated virus (AAV) ex vivo gene therapy was investigated to establish immune tolerance in the corneal allograft to prevent graft rejection. Human leukocyte antigen G (HLA-G) is present in ocular tissues and contributes to immune privilege by inhibition of immune cells as well as by preventing neovascularization. Optimal HLA-G function relies on beta 2 microglobulin (B2M) association and then binding to another HLA-G/B2M heterodimer generating a 4 protein complex, the formation of which has been deemed rate-limiting. Although our previous work demonstrates that AAV-HLA-G gene addition treats uveitis, ocular graft versus host disease, corneal neovascularization and fibrosis, concerns remain as to the sequestration of host B2M altering tissue homeostasis. Therefore, a novel protein, termed single chain immunomodulator (scIM), was engineered that mimics the structural conformation of the 4 protein HLA-G/B2M complex. Investigations in cell lines demonstrate that scIM is a stable protein that exists intracellular and is also secreted. To investigate function, AAV8-scIM was administered via subconjunctival injection to a murine model prior to an alkali-induced corneal burn. One-week post-injury, AAV8-scIM treated animals demonstrated a significant reduction in corneal vascularization and decreased fibrosis compared to untreated controls. Next, ex vivo AAV8 gene delivery to rabbit corneal allografts was optimized which resulted in tissue retention of 3.0e6 vg following a 15 min tissue incubation with AAV8-scIM. To generate a high-risk CT rejection model, 4 sutures were placed in the recipient rabbit cornea 14 days before CT using vehicle or AAV8-scIM treated corneal allografts. Ocular inflammation and central corneal thickness steadily increased for both groups 14 days post-surgery, at which point the data diverted, with the vehicle group steadily increasing for both metrics while the scIM treated corneas returned to baseline levels over a 42 day period. Based on rejection index, 5/6 vehicle treated corneas were rejected while all of the AAV8-scIM allografts

were remarkably well tolerated with minimal vascularization and central corneal clarity. Histological analysis of rabbit central corneas revealed many CD3 positive infiltrating T cells in control group eyes while none were observed in scIM treated group. Consistent with the clinical analyses, neovascularization and fibrosis at the central cornea was obviously observed in control group eyes but not in scIM treated group. Biodistribution studies demonstrated viral genomes were restricted to the corneas that underwent CT with no induction of AAV8 neutralizing antibodies. The collective data demonstrate that scIM gene therapy prevents corneal neovascularization, reduces trauma-induced corneal fibrosis, and prevents allogeneic CT rejection in a high risk large animal model warranting further investigations to prevent allogeneic organ rejection in humans.

23. Neonatal AAV9 Gene Therapy Prevents Hepatic Mitochondrial Dysfunction in a Mouse Model of DGUOK Deficiency

Nandaki Keshavan[1], Simon Waddington[2], Rajvinder Karda[2], Shamima Rahman[1]

[1]Genetics and Genomic Medicine Programme, University College London, London, United Kingdom,[2]University College London, London, United Kingdom

Background and Aims: The mitochondrial DNA depletion syndromes are severe debilitating monogenic disorders of childhood. Mutation of the gene encoding deoxyguanosine kinase (*DGUOK*) is one cause, presenting with an infantile-onset hepatocerebral phenotype leading to death by 2 years. There are no curative treatments. Liver transplantation is unsatisfactory because it does not reverse or prevent the development of neurological disease. Effective disease-modifying treatments are urgently needed. We aimed to establish proof-of-principle for a neonatal intravenous (IV) AAV9 gene therapy approach to treating a mouse model of DGUOK deficiency that has recently been shown to recapitulate the human hepatocerebral disease phenotype. **Methods:** AAV9-CAG-*hDGUOK* vectors were generated and injected intravenously to *Dguok* KO mice at P1 for efficacy studies. A dose escalation of 8e13 vg/kg, 8e14 vg/kg and 8e15 vg/kg was undertaken to determine the minimum effective dose. **Results:** Excellent long-lasting liver transduction was demonstrated at both the 8e13 vg/kg and 8e14 vg/kg doses as indicated by high hepatocellular targeting (>99% transduction in vector copy number studies), and reconstitution of *DGUOK* RNA expression to endogenous levels. Immunohistochemical findings suggested that transduced hepatocytes in knock-out animals had a repopulation advantage over untransduced cells. Efficacy was clearly demonstrated by prevention of liver mtDNA depletion (Figure 1a), complete rescue of liver transaminitis to wild-type levels (Figure 1b), a dose-dependent improvement in survival (Figure 1c) and rescue of liver Complex I, III and IV activities (Figure 2a-c). Administration of 8e15 vg/kg, however, resulted in early mortality (Figure 1c).

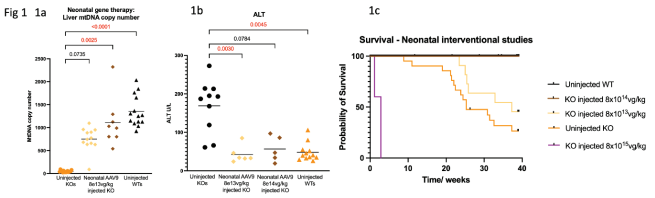


Figure 1 Liver mtDNA copy number, ALT and survival in gene therapy treated and control groups. The numbers above the brackets represent p values (Kruskal-Wallis test with multiple comparisons. p values <0.05 are considered statistically significant and are indicated in red).

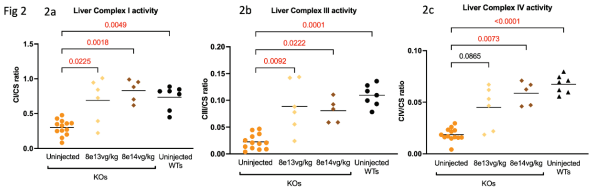


Figure 2 Liver Complex I, III and IV activities as a ratio to citrate synthase activity in gene therapy treated and control groups. The numbers above the brackets represent p values (Kruskal-Wallis test with multiple comparisons. p values <0.05 are considered statistically significant and are indicated in red)

Conclusions: Intravenous neonatal AAV9 gene therapy doses up to 8e14 vg/kg prevented liver mitochondrial dysfunction. This study suggests that AAV9-based gene therapy is a viable gene therapy approach to treating liver manifestations of DGUOK deficiency.

24. AAV9 Delivered Artificial microRNAs Effectively Improves GFAP Pathology and Motor Impairment in an Alexander Disease Rat Model

Wassamon Boonying, Guangping Gao, Jun Xie

Horae Gene Therapy Center, University of Massachusetts Chan Medical School, Worcester, MA

Alexander disease (AxD) is a type of rare neurodegenerative disease known as leukodystrophies. AxD is caused by dominant gain-of-function mutations in the glial fibrillary acidic protein (*GFAP*) gene and occurs in approximately one in one million births. The disease is progressive and fatal, ranging from infancy to adulthood. GFAP is mainly present in astrocytes of the central nervous system (CNS) and plays an important role in controlling myelination. Mutations in *GFAP* result in the overproduction and accumulation of the mutant form of GFAP, which causes the formation of Rosenthal fibers, the histopathological hallmark of AxD throughout the CNS. Currently, there is no therapy available to treat AxD. Thus, silencing mutant *GFAP* could be an attractive therapeutic approach. We have previously developed a scAAV9 vector (scAAV9-GfaABC1D-amiR-*GFAP*) expressing an artificial microRNA (amiR) to target the conserved *GFAP* sequences among mice, rats, NHPs and humans. The amiR expression directed by the native promoter of the human *GFAP* gene (GfaABC1D) was able to downregulate *GFAP* gene expression and effectively reduce Rosenthal fibers in the AxD mouse brains. However, the mouse model does not show the apparent leukodystrophy and the severity of the human disease phenotypes. Thus, we tested our scAAV9-GfaABC1D-amiR-*GFAP* in an AxD rat model which recapitulates the whole spectrum of severe features of the AxD syndrome, including high amounts of GFAP accumulation, widespread Rosenthal fibers

formation throughout the brain and spinal cord, myelin deficits, motor impairment, failure to thrive and increased mortality. We injected scAAV9-GfaABC1D-amiR-*GFAP* into three-week-old AxD rats (*Gfap*$^{+/R237H}$) by intravenous administration at a dose of 2.0 x e14 genome copies/kg. We found that the treated AxD rats started gaining body weight from three weeks post-injection compared to the untreated AxD rats. Four weeks later, the treated *AxD* rats showed significant improvements in the rotarod, grip strength and performance in the horizontal ladder test when compared to the untreated animals. In addition, histopathologic analysis showed significant reductions of Rosenthal fibers in whole brain of treated *AxD* rats. Reduced hyperintense signals (i.e. signs of brain edema) on T2 MRI imaging were observed in the treated AxD rat brains as well. Western blot revealed that GFAP protein levels were reduced after four weeks post-injection. Furthermore, the treated AxD rats continue to show improved performances in motor function tests at 12- and 36-weeks post-injection. Taken together, our data indicates that astrocyte-restricted gene silencing is a promising strategy for efficient and sustainable treatment of AxD and potentially other gain-of-function diseases that affect astrocytes. G.G. and J.X. are corresponding authors.

AAV Manufacturing I

25. Production of Different AAV Serotypes Using a Novel Plant-Based Viral Vector Manufacturing Platform

Pranav Dinesh Mathur, Qingqing Xie, Kevin Wong, Terri Grider, Finley Burns, Katarina Dumar, Matthew Schafer, Connors Jake, Daniel Gibbs

Cirsium Biosciences, San Diego, CA

Introduction: The manufacturing gap in Gene Therapies is emerging as the primary bottleneck in the field. The expensive production of AAV vectors in mammalian or insect cells results in prohibitively high costs for approved AAV-based gene therapies. With single doses priced in the millions of dollars, these crucial treatments are inaccessible to many patients. In addition, with more than 3000 pre-clinical and clinical cell and gene therapies currently under development, and with the existing scale-up process taking anywhere from 9-18 months, manufacturing facilities are finding it harder to keep up with the growing demand. While the concept of producing biologics in plants has existed for decades, with several plant-based biologics demonstrating effectiveness and gaining regulatory approval, manufacturing viral vectors in these systems had not been previously attempted. Here we report for the first time, successful production of highly potent AAVs using whole plants as modular bioreactors. Methods: Using a transient plant-based expression methodology, we delivered the necessary components for making AAV2 or AAV9-CMV-GFP viruses (Rep, Cap, GOI and helper genes) to *Nicotiana Benthamiana* plants. In this method, plants were infiltrated using *Agrobacterium* containing engineered plasmids of interest. Five days later, harvested leaf matter was homogenized, and AAV was purified from the lysate using post-clarification downstream methods identical to conventional AAV purification. This involved TFF for crude lysate concentration, followed by affinity capture and Iodixanol gradient purification to enrich full AAVs. In parallel, we transfected adherent HEK293 cells with plasmids containing Rep, Cap, GOI and helper genes to produce AAV2 or AAV9-CMV-GFP. Cells and media were harvested and mammalian-AAV-CMV-GFP was purified using the identical downstream protocol that was used to purify the plant-AAV-CMV-GFP virus. Next, we characterized and compared the mammalian and plant produced AAV-CMV-GFP using dPCR, ELISA, TEM, SDS page, Sterility testing, Endotoxin and Mycoplasma testing to name a few. Further, we tested the infectivity of plant-AAV-CMV-GFP against the mammalian-AAV-CMV-GFP in-vitro, ex-vivo and in-vivo. Results: We found that Plant produced AAVs purified with similar recovery percentages to that of the HEK293T derived AAV, suggesting that the existing downstream methods for AAV purification works well for the plant derived AAVs. Plant-AAV-CMV-GFP demonstrated VP1, VP2 and VP3 banding ratios and purity by SDS-PAGE similar to the mammalian-AAV-CMV-GFP vector, and both sources produced ~90% full capsids post purification, as observed by TEM and ELISA. We were able to obtain ~1e14vgs from 1Kg biomass of plant and from 1L of mammalian culture. Both AAVs were free of any contaminants, mycoplasma and had lower than FDA recommended Endotoxin levels. When compared in vitro, ex vivo, and in vivo, both plant and mammalian AAVs showed potent infectivity and comparable tropism. Conclusion: Here we report for the first-time, transient production of two different AAV serotypes using plants as modular bioreactors. Manufacturing AAVs by this method drastically (>10-fold) reduces the cost of goods compared to the traditional mammalian-based systems. Further, this method significantly reduces the scale up time and because individual plants act as a single reproducible unit of production, this method is highly reliable during the scale up process. This unique upstream method of AAV production in plants still provides identical downstream purification options as used for current mammalian-AAV production methods, making this technology easy to adapt by any existing AAV manufacturing facilities. We believe that our manufacturing method can solve some major challenges that the field of Cell and Gene therapy is currently facing.

26. A Novel Proviral Plasmid Reduces Cross-Packaging and ITR Promoter Activity in AAV Vector Preparations

Noah Taylor, Matthew John Guggenbiller, Pranali Mistry, Scott Q. Harper

Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH

Background: AAV preps largely contain correctly packaged genomes, with the intended ITR-flanked, therapeutic gene expression cassette inserted within the AAV capsid. However, achieving 100% AAV genome purity is challenging. A recent survey suggested AAV preps contain ~3.5% packaged DNA contaminants (N=30; range <1 - 13%), mostly derived from mispackaging of proviral plasmid backbone sequences instead of the intended payload. Thus, AAV contaminants can include sequences required to propagate plasmids in bacteria, such as antibiotic resistance genes and bacterial sequences with immunostimulatory CpGs and/or potential open reading frames (ORFs). Such AAV contaminants are often referred to as cross-packaged material. Potential risks of cross-packaged bacterial sequences in AAV vectors were highlighted in a recent study in non-human primates, where AAV contaminants were toxic to monkey brain (PMID: 34663988). Toxicity was linked to ITR-driven expression of transcripts arising

from cross-packaged bacterial sequences in the plasmid backbone and *not* the intended payload. These results suggested that improved production methods are needed to (1) increase correctly packaged AAV payloads, and (2) decrease ITR-driven transcription of cross-packaged material to avoid expressing potentially toxic bacterial sequences. Methods: *Interfering with ITR-driven transcription.* We generated a dual luciferase reporter plasmid containing promoterless *Renilla* or Firefly luciferase cDNAs outside each ITR (i.e. in the plasmid backbone). We then inserted insulator sequences in the plasmid backbone between the ITR and luciferase genes or flanking the ITR on both sides, and assessed ITR-driven gene expression via dual luciferase assay. *Reducing cross-packaging.* We designed a novel AAV proviral plasmid for vector production. We inserted 3 modifications into a basic scAAV proviral plasmid containing an AAV2 ITR-flanked miRNA payload and stuffer sequence, and plasmid backbone containing a Kanamycin resistance gene and bacterial origin of replication (5.6 kb total size). Specifically, we (1) flanked ITRs with insulator sequences to decouple ITR-driven transcription from adjacent ORFs; (2) inserted large human intronic sequences in the plasmid backbone adjacent to the ITRs (11.1 kb total size), so that any cross-packaged material would more likely contain human, and not bacterial DNA; (3) mutated all ATG start codons in the human intronic sequences to ensure that protein translation could not be initiated from any remaining cross-packaged plasmid backbone sequences. We then used this plasmid to generate 7 different AAV serotypes, and assessed vector titer, gene expression, and presence of cross-packaged material. Results: Insulators reduced *Renilla* and firefly luciferase activity by 55% (insulators in plasmid backbone only) or 90% (ITR-flanking insulators), supporting this design may be useful for inhibiting ITR promoter activity. Average percentage of cross-packaged material was 1.35% using our first-generation scAAV proviral plasmid, while our optimized scAAV proviral plasmid significantly reduced cross-packaging by half (0.65% avg), with improvements for every serotype tested (1, 2, 5, 6, 9, rh10, rh74). The new plasmid had no impact on viral titers or expression from correctly packaged genomes. Conclusions: We achieved the goals of our study by reducing cross-packaged DNA in AAV preps, and decoupling ITR promoter activity from adjacent DNA sequences, thereby reducing the potential for expression of deleterious ORFs. This plasmid could be used to improve yield of correctly packaged AAV genomes and reduce the potential for toxic gene expression from remaining cross-packaged material.

27. Removal of Empty and Partial AAV Capsids Using Cu Ions and Oversaturated Loading on Multicolumn AEX Chromatography

Ohnmar Khanal, Vijesh Kumar, Mi Jin

Technical Development, Downstream, and Drug Product Development, Spark Therapeutics, Philadelphia, PA

A common platform adeno-associated virus (AAV) purification process comprising filtration and two-step chromatography operations is costly and can impact product quality and efficacy. A current challenge in the downstream purification of recombinant Adeno-associated virus is the separation of transgene-containing capsids from empty and undesired capsid variants. Anion exchange chromatography (AEX) resin is often used below its saturated binding capacity to separate empty and full AAV particles on a single column. However, the outcome of the traditional single-column operation can be impacted by variability in load composition, sensitivity to buffer composition, and fractionation strategies. Furthermore, the removal of empty and partial capsids with a high yield is rarely demonstrated in literature. This talk will present three-stage separation strategies for two rAAV constructs and show how to overcome the yield/purity trade-off. The first is the selection of resin chemistry (Poros XQ, Poros HS, Poros I) with a concoction of kosmotropic agents such as ammonium sulfate or sodium sulfate to promote capsid stability at low ionic strength. Metal ions (Cu, Mg, Ca) were used to promote desired capsid selectivity. Using Cu ions improved the resolution between empty and full capsid separation and helped remove other product-related impurities. The second is selecting an appropriate resin/media structure and geometry to exclude AAV from smaller resin pores such as Poros XQ, minimizing dispersion and improving resolution. Monolith and membrane chromatography showed better peak resolution than traditional resin based on favorable transport behavior. The third is the transition from single column to multiple columns in series with a loading of 1.5x the resin saturation capacity to allow for > 40% reduction of partial capsids and > 95% removal of empty capsids. Using these strategies, we provide the best modality to achieve maximum empty and partial capsid removal with a yield > 80%.

28. Novel Mechanism to Increase rAAV Yield through Blocking rAAV Transduction of the Manufacturing Hek293 Cells During rAAV Production

Xiaofei E[1], Anmol Kotian[2], Isabella Pajevic[2], Bisma Ajaz[2], Jennifer Marlowe[1], Sharon McGonigle[1]

[1]R & D, Solid Biosciences, Charlestown, Boston, MA,[2]A & D, Solid Biosciences, Charlestown, Boston, MA

Recombinant adeno-associated virus (rAAV)-mediated gene therapy is a promising gene delivery strategy that allows for broad clinical application. With recent clinical success, there is rising demand for improved rAAV production processes to increase yield, enhance quality, and reduce costs. In this study, we first demonstrated that extracellular rAAV being produced in HEK293 cells by transient transfection can transduce the manufacturing cells. Protein expression from two different rAAV-delivered transgenes was detected in lysates from HEK293 cells sampled at various timepoints during rAAV production. Supernatant transfer experiments further confirmed that extracellular rAAV does transduce HEK293 cells during rAAV production. From this observation, it follows that a lower rAAV yield will result from loss of rAAV to transduction of HEK293 cells. We then explored whether inhibitors of endocytosis could block AAV transduction of the production HEK293 cells. Two small molecule inhibitors of endocytosis (compounds A and B) were identified for their ability to limit this transduction. When added to HEK293 cells during rAAV production, both compounds resulted in a significant reduction in rAAV transduction as measured by transgene protein expression. Reduced transduction translated into increased rAAV yield of 2-3-fold, presumably since this rAAV was no longer being lost to transduction and returned to the media supernatant. The impact of compound A treatment on rAAV yield and quality was further characterized. The optimal timing of addition to HEK293 cells was identified with an observed ~3-fold increase in rAAV yield, as compared to low or no effect when administered at earlier or later timepoints. A dose-

responsive effect on rAAV yield was observed for the compound with no impact on HEK293 cell viability or growth. Following iodixanol purification of rAAV produced in the presence or absence of compound A, the viruses were assessed in various quality assays, where compound A-produced rAAV yielded similar or marginally improved quality attributes. Significantly, rAAV produced in the presence of compound A showed consistently higher transgene expression in a cell-based assay. Increased yield through transduction inhibition by Compound A has been extensively studied with rAAV-SLB101 (a novel capsid with AAV9 backbone) production; moreover, a similar effect has also been observed during rAAV rh74 manufacture. These data highlight that loss of rAAV to transduction during manufacture does occur and is significant. Addition of endocytosis inhibitors limits this transduction and consequently improves rAAV yield and quality via this previously unreported mechanism. Studies are underway to better understand how broadly applicable this novel finding may be across various rAAV manufacturing processes and platforms.

29. Enhancing Product Quality: Utilizing Salt-Tolerant Anion Exchange Chromatography in Flow-Through Mode as a Polishing Step for Advanced Process-Related Impurity Removal

Johanna Weizenegger, Regina Staffler, Julia Trommer, Sinisa Petrik, Matthias Boscher, Johanna Wagner, Jared Babic, Beatriz Laurena Carnio, Anita Heinlein, Christine Zach, Sebastian Ritter, Matthias Gora, Tobias Kloetzler, Alexandra Schoberth, Ahmed Youssef, Karl Heller, Markus Hoerer, Melanie Langhauser

Ascend, Planegg, Germany

Recombinant adeno-associated virus (rAAV) is among the most widely used viral vectors for gene therapy. However, vector manufacturing remains a challenge to demand for **high quality AAV vectors**. In our modular AAV **platform purification process** as polishing step for further unpacked impurity removal an **Anion Exchange Chromatography (AEX) in Flow-Through (FT) mode** can be applied after affinity capture chromatography. Since higher salt concentrations during the affinity chromatography elution reduced the aggregation level for some AAV serotypes, this study explores the applicability of a salt tolerant serotype independent AEX step in FT mode. Usually, an AEX step is performed at low salt concentrations to receive unpacked impurity removal. Therefore, the goal of this study was to assess the effectiveness of AEX membranes and resins to remove impurities despite a higher level of conductivity. We performed a comprehensive screening study with **four AEX resins/membranes at varying conductivities**, assessing their performance in terms of recovery and the removal of aggregation, host cell proteins (HCPs) and non-packed DNA. To challenge the purification capacities and to determine impurity removal factors, the load material was spiked with a mixture of HCPs and non-packed DNA. The step recoveries were not affected by the conductivity of the load material, but differences were observed between the tested AEX resins/membranes. Across all conditions, regardless of the resin/ membrane or salt concentration used, non-packed DNA was consistently reduced below the detection limit of the applied analytical method. Moreover, all conditions proved effective aggregate removal as determined by DLS. The most notable difference among the tested resins/ membranes was observed for the HCP removal. Two out of four tested AEX exhibited a remarkable efficacy with one of them demonstrating a consistent performance across a wide range of salt concentrations, showcasing its robustness. The second AEX resin displayed a salt-dependent trend, where higher conductivities correlated with a decreased ability to remove aggregates. However, given that the impurity level in spiked samples was significantly higher than in our standard Downstream Process, the removal capacity was sufficient. Despite an excellent performance in aggregate and non-packed DNA removal for all tested resins and membranes, we selected one specific AEX resin for our modular platform process due to its most desirable properties with respect to step recovery, HCP removal and scalability. **This serotype independent AEX step in FT mode improves product quality and can be applied under high-conductivity conditions if needed.** High product quality and safety is at the core of our research and platform offering. This AEX step can be used to deliver high quality AAVs. It is an optional process step which can be implemented based on client needs.

30. Optimizing Affinity Chromatography Purification for Enhanced Quality and Stability of Adeno-Associated Viral Vectors (rAAVv)

Thuy Linh Do, Zeynep Çukurçeşme, Susanne Konrad, Thomas Bissinger, Chris Williams

Gene Therapy Technical Research & Development, Roche, Penzberg, Germany

The production and purification of Adeno-associated viral vectors (rAAVv) is a complex process that requires simultaneously careful consideration of numerous factors to ensure optimal yield, purity, potency and stability. Specifically, during the Affinity Chromatography purification step, there are potential product quality risks such as loss of viral vector particles, contamination, non-specific binding of impurities, and potential impact on the integrity and potency of the final product. To ensure comprehensive management of product quality risks, it is important to holistically examine the entire Affinity Elution/ Neutralization phase, taking into account factors such as buffer pH, buffering components, stabilizers, pooling parameters, and the timing of neutralization. To determine the optimal elution conditions, a comprehensive investigation was conducted. Initially, a negative linear gradient using an acidic buffer solution was employed to determine the range of elution pH. The elution profile revealed a suitable pH range for subsequent experiments. Subsequently, affinity chromatography purification of rAAVv with the elution buffer solutions from more mild to more acidic pHs was performed to assess the impact of different elution pH values on the yield and purity of the eluted product. To further explore the stability of rAAVv under optimal elution conditions, an acidic hold time study was conducted. The eluted rAAVv product was held at two pH levels for various periods, ranging from 0 up to 16 hours before neutralization. The stability study aimed to evaluate changes in yield, purity, aggregation, and potency over time. The results demonstrated that selection of the most appropriate elution pH can confer superior rAAVv stability in terms of yield and purity, even over extended periods. Here correlations were observed by product loss and the formation of aggregated particles. We also observed a time-dependent formation of post-translational modifications which, if left uncontrolled, may contribute to variability in product quality

and performance. These findings have significant implications for the development of rAAVv purification processes in gene therapy applications. Optimizing elution pH, particularly by employing the newly identified pH range for elution, can improve the yield, purity, and stability of rAAVv vectors. By carefully selecting elution conditions, it is possible to obtain high-quality rAAVv vectors while minimizing the detrimental effects of low pH on the capsid structure. In summary, this study offers valuable insights into the influence of elution buffer components and neutralization timing on the stability of rAAVv during affinity chromatography purification. These findings are significant for the development of strategies aimed at improving the efficiency and quality of rAAVv purification processes, thereby supporting the progress of gene therapy applications. Further research is necessary to investigate additional factors that may impact rAAVv stability and to further optimize the purification process.

31. Not Created Equal: Impact of Deamidation on Capsid Heterogeneity and Anion Exchange Chromatography (AEX) Performance

Alex Meola, Alice Aguiar, Carlos Chong, Zaid Junaid, Thomas Thiers, Luke Mustich, Kayla Friscia
Oxford Biomedica (US) LLC, Bedford, MA

Post-translational modification of Adeno-Associated Virus (AAV) capsid proteins is becoming a focus in the gene therapy process and analytical development space. In particular, deamidation of capsid protein VP1 specific residues including N57 has been linked to a loss of potency in vivo. Temperature, pH, and storage time have been identified as key factors causing this phenomenon. As a result, controlling strategies in the upstream and downstream processes should be studied and defined at the early stage of process development to limit onset of deamidation in order to minimize product related impurities and loss of vector functionality. Besides process control to prevent deamidation, here we also discuss our approach to removing deamidated AAV capsids in the downstream process. We hypothesize that extrusion of VP1 and deamidation of N57 are related and result in capsid heterogeneity of empty, partial, and full capsids which significantly influences the Anion Exchange Chromatography (AEX) process' ability to remove empty capsids and deliver functional AAV. Currently, charge driven separation techniques do not provide adequate resolution required to differentiate between deamidated and non-deamidated capsid species. Here we highlight our novel approaches that can be employed in the AEX process to tackle this complex challenge in removing not only empty capsids but also deamidated intact capsids.

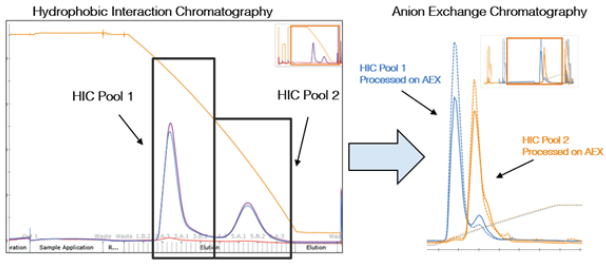


Figure 1: Hydrophobic Interaction Chromatography was first utilized to separate out to distinct peaks with near baseline resolution. Each peak was isolated and reprocessed on AEX individually.

We present how non-orthodox AAV purification techniques were utilized to isolate and further characterize the different AAV capsids that are difficult to resolve on a typical anion exchange chromatography (AEX) gradient. Surprisingly, we discovered that Hydrophobic Interaction Chromatography (HIC) resolved two different species of AAV capsids with near baseline resolution. Each species was reprocessed on AEX and all product quality attributes were assessed for the intermediate peaks that were generated. We found that the capsids that were more hydrophobic were also more negatively charged. LC-MS data demonstrated the capsids that were more hydrophobic and more negatively charged had significant levels of VP1 specific N57 deamidation which has been linked to a loss in gene expression. We hypothesize that the capsids are more hydrophobic due to VP1 extrusion which exposes the hydrophobic PLA2 domain on VP1u. This phenomenon would make VP1 N57 residue solvent exposed and susceptible to deamidation. We demonstrated that hydrophobic interaction chromatography can be used to separate non-deamidated capsids from deamidated capsids. We also present a novel, patent-pending approach to simultaneously remove deamidated species and enrich for full capsids on anion exchange chromatography.

Neurologic Diseases I

32. Promoter Influences Acute Liver Toxicity and Long-Term Hepatic Genotoxicity in rAAV SMA Gene Therapy in Mice

Xiupeng Chen, Qing Xie, Hao Liu, Veena Kumanan, Hong Ma, Phillip WL Tai, Guangping Gao*, Jun Xie*
Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA

The use of recombinant adeno-associated viruses (rAAVs) for human gene therapy has been a significant breakthrough, demonstrating therapeutic efficacy in an increasing number of clinical trials. However, liver toxicity is one of the major safety concerns. Onasemnogene abeparvovec (Zolgensma®) is an approved rAAV gene therapy for infants with spinal muscular atrophy (SMA). Currently, over 3,000 patients have been treated with Zolgensma. The high systemic dose at 1.1×10^{14} vector genomes/kg is required for efficacy but is associated with safety concerns as warned by a black box warning for severe liver injury and acute liver failure which is likely caused by strong ubiquitous expression of SMN1. We previously developed a second-generation SMA gene therapy expressing a codon-optimized *hSMN1* transgene driven by a promoter derived from the native *hSMN1* gene. This vector restored SMN expression close to physiological levels in the central nervous system and other major tissues of a severe SMA mouse model. In a head-to-head comparison between the 2nd-generation SMA gene therapy (SMA GTx) and a benchmark vector with a design identical to Zolgensma, the 2nd-gen. SMA GTx showed improved efficacy including a longer life span; increased motor, cardiac, and respiratory functions; and minimal peripheral tissue manifestations. Here, we investigated

those two vectors for acute liver toxicity in SMA mice and long-term hepatic genotoxicity in C57BL/6 mice. We first assessed transaminitis by measuring ALT and AST levels in SMA mice on Day 30 after treatment with the benchmark or 2nd-gen vector. SMA GTx vector at a dose of 3.3E+14 vg/kg by facial vein injection at P0. We detected significant elevations of ALT and AST in the benchmark vector-treated mice (150.4 U/L and 290.8 U/L respectively) but not in the 2nd-gen. vector-treated mice (35.2 U/L and 93.3 U/L, respectively, $p < 0.01$ for both comparisons) which were statistically indistinguishable from healthy carrier littermates ($p > 0.96$ for both comparisons). We next compared liver toxicities of the two vectors after intracerebroventricular (ICV) administration to C57BL/6 mice at P0 with a dose of 1.0E+14 vg/kg. In a 20-month study duration, we found ~44% (8/18) of mice died in the benchmark group, and 6% (1/17) of mice died in the 2nd-generation vector group. Among the surviving mice, all the animals (10/10) from the benchmark group developed hepatocellular carcinoma (HCC). In contrast, only one mouse (1/16) in the 2nd-gen vector group detected liver tumors. In addition, significant elevations of ALT and AST were found in the benchmark vector-treated mice (620.2 ± 410.7 U/L and 1518.2 ± 1097.0 U/L respectively) but not in 2nd-generation vector-treated mice (20.6 ± 3.0 U/L and 49.4 ± 6.1 U/L, respectively, $p < 0.05$ for both comparisons). We further analyzed the expressions of *Dlk1*, *Rian*, *Mir543*, *Mirg*, *Mir300*, *Mir134*, *Mir1188*, *Mir382*, and *Mir487b* which are associated with AAV genome integration-related hepatic genotoxicity. Our RT-ddPCR data revealed significant increases in the expression of these 9 genes in the liver tumors as compared to that of the tumor-adjacent tissues and livers from untreated C57BL/6 mice. Importantly, ICV delivery of 2nd gen. vector to SMA mice at P1 achieved significantly better therapeutic efficacy as compared to the benchmark vector. In sum, endogenous hSMN1 promoter-mediated SMA expression holds a promise to not only enhance therapeutic efficacy but also mitigate acute and long-term liver toxicity in AAV gene therapy. * **G.G., and J.X are co-corresponding authors**

33. Intra-CSF AAV9-GBA Delivery Produces Species and Route of Administration Differences in Safety and Efficacy

Neeta Abraham, Ana Amaral, Shawn Kyle Bourque, Yi Chen, Pete Clarner, Jim Fikes, Peter-Joseph De Giovanni, Davide Gianni, Kelly E. Glajch, Branka Grubor, Stefan Hamann, Warren Hirst, Melissa Kirkland, Lee Koetzer, Merilla Michael, Prasad Nadella, Kalyani Nambiar, Ed Plowey, Jennifer Sebalusky, Junghae Suh, Eric Tien, Patrick Trapa, Dominic Walsh, Shanqin Xu, Weike Zeng

Biogen, Cambridge, MA

Previous studies have demonstrated AAV delivery of lysosomal enzymes to the brain is efficacious in rodent models of disease and this approach is currently being tested in clinical trials for several indications. Unfortunately, limited biodistribution, expression and safety data exist from non-human primate (NHP) studies testing AAV brain delivery, and very little using AAV9, which is a common capsid for this approach. In this study, we used AAV9 to deliver *GBA1*, the gene encoding the lysosomal enzyme glucocerebrosidase (GCase), to mice and NHPs. Loss of function mutations in GCase cause Gaucher's disease and are a major risk factor for Parkinson's disease. In mice, early postnatal intracerebroventricular (ICV) administration of AAV9-GBA1 produced significant brain viral biodistribution and a dose dependent increase in functional protein (as indicated by increased GCase activity) in several brain regions. Furthermore, histological analysis did not show any signs of inflammation (microglial reactivity) in the brain and no in-life clinical signs were detected. These findings allowed us to identify a target dose for scaling to NHPs that produced a sufficient increase in expression and activity without any histological signs of toxicity. Next, we performed two separate NHP studies to administer AAV9-GBA1 into the brain via intracisterna magna (ICM) or intraparenchymal (IPa) delivery. ICM delivery led to modest biodistribution and very minimal transduction and expression (RNA and GCase activity) in the brain. However, there was a significant transduction of dorsal root ganglia (DRG) neurons along with adverse microscopic findings in DRGs as well as in the spinal cord, dorsal nerve roots, and peripheral nerves. In contrast, IPa delivery into the thalamus and/or putamen produced significant biodistribution and transduction along with increased GCase expression and activity in several brain regions. While no DRG toxicity was observed with IPa delivery (in line with the limited transduction in this region), there were significant adverse microscopic findings in several brain regions and clinical signs that were procedure and AAV9-GBA1 related. In summary, these findings demonstrate that AAV9-GBA1 delivery in NHPs (via ICM or IPa) did not achieve the desired biodistribution and expression levels in the brain without concomitant safety findings, in contrast to our results in mice. Further technological advances are needed to obtain robust transduction and expression with AAV delivery to the NHP brain without significant safety findings.

34. AAV1.NT-3 Gene Therapy Improves Function, Electrophysiology, and Histopathology in *Sh3tc2*$^{-/-}$ Mouse, a CMT4C Model

Burcak Ozes[1], Lingying Tong[1], Kyle Moss[1], Morgan Myers[1], Lilye Morrison[1], Zayed Attia[1], Zarife Sahenk[1,2,3]

[1]Center for Gene Therapy, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, Columbus, OH,[2]Department of Pediatrics and Neurology, The Ohio State University, Columbus, OH,[3]Department of Pathology and Laboratory Medicine, The Ohio State University, Columbus, OH

Charcot-Marie-Tooth disease (CMT) is a group of heterogeneous inherited polyneuropathies. CMT4C is an autosomal recessive demyelinating subtype of CMT, caused by mutations in the SH3 domain and tetratricopeptide repeats 2 (*SH3TC2*) gene. Neurotrophin 3 (NT-3) is an autocrine factor supporting Schwann cell (SC) survival and differentiation and stimulates axon regeneration and myelination. NT-3 also has a role in the maintenance of neuromuscular junction (NMJ) integrity. In this study, we investigated the efficacy of AAV1.tMCK.NT-3 gene therapy in a CMT4C model, *Sh3tc2* $^{-/-}$ mouse. *Sh3tc2* $^{-/-}$ mice received 1x10^{11} vg dose of rAAV1.tMCK.NT-3 vector via intramuscular injection at four weeks of age (n=15, even sex distribution). Age- and sex-matched *Sh3tc2*$^{-/-}$ mice were used as untreated (UT) controls (n = 16). The mice were sacrificed six months post-treatment. Outcome measures included rotarod and grip strength functional tests, electrophysiology, quantitative histological assessment

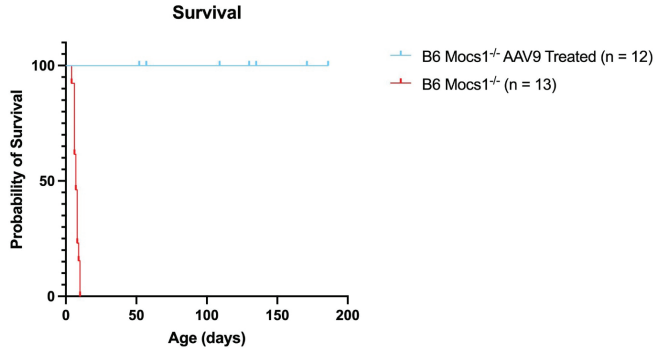
of nerve and muscle and innervation status NMJs. Functional studies showed that NT-3 treated mice preserved function with time and performed better than the UT counterparts at the endpoint by 17% for both rotarod and grip strength (rotarod, p=0.0381; grip strength, p=0.0297). Electrophysiology performed at the endpoint also showed improvement in the mean nerve conduction velocity in the treated cohort by 31% compared to UT mice (NT-3: 29.7 ± 2.47 vs. UT: 22.7± 2.01 m/s; n=15 in each cohort, p=0.0256). G ratio quantified from the sciatic nerve images of the treated mice decreased significantly compared UT cohort indicating improvements in the myelin thickness. (p=0.0492). Quantification of the density of small myelinated fibers (MFs) and the SC nuclei associated with Remak bundles showed an increase in both sciatic and tibial nerves of NT-3 treated group suggesting that NT-3 promoted SC proliferation and regeneration associated myelination. A significant increase was observed in the total MF density in the tibial nerve (p= 0.0084) although this increase was not observed in the sciatic nerves indicating that treatment improved the length dependent axon loss. Moreover, percent of innervated NMJs were higher in the treated mice compared to UT counterparts in the lumbricals (p= 0.048). Fiber size analysis of gastrocnemius, and tibialis anterior muscles following succinic dehydrogenase stain indicated an increase in both muscles in females, being prominent in the type II fibers (gastrocnemius, p=0.045; tibialis anterior, p= 0.0199). *Sh3tc2*$^{-/-}$ model for CMT4C is the fourth CMT subtype model that we showed the efficacy of AAV1.NT-3 gene therapy, highlighting the potential for clinical applicability of this surrogate gene therapy approach to a large group of CMT patients.

35. AAV9-rMOCS1 Gene Therapy, a Bicistronic Gene Delivery, Fully Rescues Neonatally Lethal Mouse Model of Molybdenum Cofactor Deficiency Type A

Claire E. Shamber[1], Edwin Alyea[2], Guenter Schwarz[3], Pazhanichamy Kalailingam[1], Casey A. Maguire[4], Albert Misko[2], Patricia L. Musolino[5]

[1]Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA,[2]Neurology, Massachusetts General Hospital, Boston, MA,[3]Cologne University, Cologne, Germany,[4]The Massachusetts General Hospital, Charlestown, MA,[5]Center for Genomic Medicine, Neurology, Massachusetts General Hostpial, Boston, MA

Objective: To show the feasibility and efficacy of a bicistronic gene therapy for Molybdenum Cofactor type A using a construct that recapitulates the complex expression of the endogenous MOCS1 gene. Molybdenum cofactor (MOCO) deficiency (MOCOD) is a devastating genetic, neurodegenerative disease characterized by neonatal intractable seizures and neurological disability that leads to death in the first three years of life. MOCOD affects one in 200,000 newborns globally. MOCOD Type A stems from mutations in the conserved bicistronic MOCS1 gene that produces proteins MOCS1A and B which form a complex in the MOCO pathway. Loss of activity abolishes production of MOCO and inactivates MOCO dependent enzymes. Among these enzyme deficiencies, the loss of sulfite oxidase, which metabolizes toxic sulfite into non-toxic sulfate, is necessary and sufficient to cause the neurodegenerative phenotype of MOCOD. Current available treatments are not well tolerated or fail to prevent disease progression. We designed an AAV compatible recombinant version of the endogenous human MOCS1 gene. In vitro validation of MOCS1 expression and restoration of sulfite oxidase was performed by transfection of WT and MOCS1 knockout HEK293 cells. Expression of MOCS1A and MOCS1B was measured by western blot. We compare survival, behavioral, tissue metabolism and histological characteristics of the Mocs1 knock out mouse with WT and Mocs1 heterozygote. Biodistribution and efficacy studies were performed at 7 days and 8 weeks following post-natal day 1 (P1) retroorbital AAV9-rMOCS1 injections. Molecular read-outs at these time points include protein expression, enzymatic activity, IHC and vector genome copies. Long term survival studies are ongoing with weekly weights and behavioral testing. Our designed rMOCS1 construct expressed MOCS1A and MOCS1B and successfully restored sulfite oxidase enzymatic activity in MOCS1 KO HEK293 cells. Untreated Mocs1 KO mouse showed established severe phenotype characterized by progressive spasticity, stunted growth, hyperkeratosis, and death before 10 days of life. Biodistribution studies confirmed liver expression of MOCS1A and MOCS1B five days after P1 injection of AAV9-rMOCS1. Treated mutant mice show no significant difference in neonatal neurological development when compared to control littermates. Long term studies have shown survival extending beyond 8 months after a single dose of AAV9-rMOCS1. Mutant and control treated animals show no apparent toxicity or significant abnormalities in growth, neurological examination or behavior. We demonstrated in vivo successful endogenous splicing and protein expression of a bicistronic human gene delivered by a single AAV vector. We completed the Mocs1 mouse model phenotypic assessments and confirmed survival and severe neurological phenotype seen in patients. Single dose systemic AAV-mediated gene delivery of our rMOCS1 construct fully rescued neurological and systemic disease long term. Our study serves as a proof of concept for a single dose AAV9 gene therapy with long-lasting effects enabling a first in human study for children suffering from MOCOD Type A.



36. Developing an AAV-Based Gene Replacement Therapy for Mitochondrial Alanyl-tRNA Synthetase 2 (*AARS2*) Leukodystrophy

Ruxiao Xing[1], Lingzhi Ren[1], Nan Liu[1], Chen Zhou[1], Jialing Liang[1], Jiaming Wang[1,2], Guangping Gao#[1,2], Dan Wang#[1,3]

[1]Horae Gene Therapy Center, University of Massachusetts Chan Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, University of Massachusetts Chan Medical School, Worcester, MA,[3]RNA Therapeutics Institute, University of Massachusetts Chan Medical School, Worcester, MA

The nuclear *AARS2* gene encodes mitochondrial alanyl-tRNA synthetase 2 that is responsible for attaching alanine onto the cognate tRNA in mitochondria. The resulting alanyl-tRNA participates in mitochondrial translation that produces the key proteins constituting the mitochondrial respiratory complexes. *AARS2* mutations have been implicated in two recessive diseases: infantile cardiomyopathy and late-onset leukodystrophy. Currently, there is no treatment available for *AARS2* related diseases.Animal models that recapitulate human disease are of paramount importance to AAV gene therapy development. However, homozygous *Aars2* knockout (KO) mice are embryonically lethal. Therefore, we sought to engineer an AARS2 leukodystrophy patient-derived mutation in the mouse endogenous *Aars2*, and successfully generated mice homozygous for the M146T mutation. The *Aars2*$^{M146T/M146T}$ mice showed 50%-80% reduction in AARS2 protein (but not mRNA) in multiple tissues compared with WT littermates. We are currently characterizing the phenotype of the *Aars2*$^{M146T/M146T}$ mice as they age. As another approach to overcome embryonic lethality due to constitutive *Aars2* knockout, we generated a mouse line harboring a loxP-flanked human *AARS2* transgene, which is expected to rescue endogenous *Aars2* KO and enable conditional AARS2 depletion after crossing with a Cre driver line. Resulting mouse strains will allow us to dissect the impact of AARS2 deficiency in specific tissue and cell types, and to test targeted AAV-based gene therapy.To develop a potential gene replacement therapy, we generated recombinant AAV9 vectors expressing human *AARS2* driven by a ubiquitous promoter (rAAV9.hAARS2), and injected wild-type (WT) mice to test the safety of AARS2 over-expression after birth. We found that systemic delivery to neonatal mice via facial vein injection caused animal death within a week, whereas intracerebroventricular injection was well tolerated. TUNEL assay revealed apoptotic hepatocytes in the animals receiving facial vein treatment, suggesting liver toxicity. Therefore, we modified the expression cassette to include miR122 binding sites that enable liver-specific, miR122-mediated transgene silencing (rAAV9.hAARS2.miR122BS). Indeed, the modified vector design prevented hepatocyte apoptosis and animal lethality following facial vein delivery. Together, our preliminary results support the safety of brain-directed, liver-detargeted *AARS2* gene replacement therapy. Studies on disease pathomechanisms and *in vivo* gene therapy efficacy involving various mouse models will be presented and discussed. (#Co-corresponding authors)

37. Extracellular Vesicles as the Underlying Mechanism for Cross-Correction with rAAV-Based Gene Therapy for GM3 Synthase Deficiency

Sophia Liu[1], Jonathan Valeros[2], Max Zinter[3], Travis Thomson[3], Jessica Spinelli[2], Guangping Gao[1,4], Phillip Wl Tai[1,4]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Program in Molecular Medicine, UMass Chan Medical School, Worcester, MA,[3]Department of Neurobiology, UMass Chan Medical School, Worcester, MA,[4]Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA

GM3 synthase deficiency (GM3SD) is a neurodevelopmental disorder caused by a loss-of-function mutation in the *ST3GAL5* gene. *ST3GAL5* encodes for GM3 synthase and loss of this enzyme results in perturbation of the ganglioside biosynthesis pathway. These gangliosides are important for cell-signaling pathways and dysfunction leads to various neurological deficits, including brain atrophy and cognitive issues. These biochemical and physiological phenotypes are observed in GM3SD mouse models. Treatment with recombinant adeno-associated viral vectors (rAAV) packaging an *ST3GAL5* expression cassette was able to reverse these effects. Not only was the rAAV able to reverse disease phenotypes, but widespread correction of gangliosides across the cortex suggests that some brain structures may be cross-corrected by a mechanism that extends beyond the direct effects of the transduced cells. We hypothesize that cross-correction is possible through the use of extracellular vesicles (EVs) as trafficking vehicles for gangliosides across cells. Based on preliminary data from transwell assays, we show that gangliosides can be trafficked between cells without direct cell-to-cell contact. We also show that *ST3GAL5* expression is linked to EV biogenesis. These results strongly suggest that EVs are associated with the correction of *ST3GAL5* in GM3SD model systems. Thus, GM3SD presents a valuable opportunity to explore cross-correction mechanisms associated with rAAV treatments in the brain. Ongoing investigations into EVs as a part of this mechanism is expected to reveal new methods for increasing rAAV efficiency for the treatment of GM3SD and other disorders. G.G. and P.T. are corresponding authors.

38. AAV Gene Therapy Corrects Neurological Phenotypes with Clinically Relevant Doses in a Mouse Model of *STXBP1*-Related Developmental and Epileptic Encephalopathy

Wu Chen[1,2], Joo Hyun Kim[1,2], Alysee Michaels[1,2], Armando Rivera[1,2], Yusuf Sham[1,2], Catalina Elwonger[1,2], Jose Paulo Carneiro[1,2], Shawn Nguyen[1,2], Zain Moin[1,2], Sheldon Zou[1,2], Kevin Jin[1,2], Amy Wang[1,2], Nikita Dhar[1,2], Peter Park[1,2], Jaimie Vega[1,2], Alice Chen[1,2], Brooke Horist[2,3], Hongmei Chen[1,2], Alberto Lopez[4], Allison Knoll[4], Nicholas Flytzanis[4], Mingshan Xue[1,2,3]

[1]Department of Neuroscience, Baylor College of Medicine, Houston, TX,[2]The Cain Foundation Laboratories, Jan and Dan Duncan Neurological Research Institute at Texas Children's Hospital, Houston, TX,[3]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX,[4]Capsida Biotherapeutics, Inc., Thousand Oaks, CA

Developmental and epileptic encephalopathies (DEEs) are a group of devastating pediatric neurological disorders, manifesting with aggressive seizures and significant neurological comorbidities. *De novo* heterozygous pathogenic variants in *STXBP1* encoding syntaxin-binding protein 1 are one of the most frequent genetic causes of DEEs. The treatment options for patients with *STXBP1* encephalopathy are very limited, as the current antiepileptic drugs are often ineffective and there are no treatments for other neurological impairments. Furthermore, abnormal brain activity during early development is believed to contribute to the pathogenesis of *STXBP1* encephalopathy, presenting a great challenge for developing clinical treatments that can still be effective later in life. We previously demonstrated that *Stxbp1* haploinsufficient mice recapitulate all key neurological phenotypes of *STXBP1* encephalopathy and restoring Stxbp1 protein to the wildtype level in adult *Stxbp1* haploinsufficient mice rescued most of the phenotypes including epileptic seizures, motor, cognitive, and psychiatric impairments. To generate investigational new drug application enabling data, we tested intravenous delivery of an engineered AAV capsid expressing human *STXBP1* with low, medium, and high dosages in rescuing adult *Stxbp1* haploinsufficient mice. The vector biodistributions and mRNA/protein levels were quantified and correlated with the outcomes examined by health monitoring, video-electroencephalogram (EEG) recording, and neurobehavioral tests. We found that AAV mediated gene replacement therapy rescues most of the phenotypes of adult *Stxbp1* haploinsufficient mice in a dose-dependent manner with long lasting effects including abnormal hindlimb clasping, epileptic seizures, intellectual disability, motor deficits, and impaired innate mouse behaviors. The high dose rescues the most phenotypes whereas the medium and low doses rescue or partially improve some of the phenotypes. Importantly, our candidate, CAP-002, which consists of an engineered AAV capsid that is a product of our high-throughput, non-human primate (NHP)-based AAV engineering platform packaging the human *STXBP1* gene, can effectively cross the blood-brain barrier in NHPs and achieve a brain-wide distribution at levels expected to provide therapeutic benefit. Thus, gene replacement achieving widespread neuronal transduction by engineered capsids has the potential to be a promising gene therapy for *STXBP1*-related developmental and epileptic encephalopathy.

Genetically Modified Immune Cells for Malignant and Non-Malignant Diseases

39. An Allogeneic Engineered Tissue Treg Approach for the Treatment of Acute Ischemic and Inflammatory Diseases

Payam Zarin[1], Maegan Hoover[1], Yash Agarwal[2], Priya Saikumar[1], Madison Milazewski[1], Nicole Reed[1], Guoping Deng[3], Carlos Frias[1], Abigail Doherty[1], Dalia Gaddis[4], Marko Repic[1], Xiaoming Hu[5], Jun Chen[5], Tiffany F. Chen[6], Thomas Wickham[6]

[1]Discovery Research, GentiBio, Cambridge, MA,[2]GentiBio, Cambridge, MA,[3]Gentibio, Cambridge, MA,[4]Gentibio, Cambridge, CA,[5]University of Pittsburgh, Pittsburgh, PA,[6]GentiBio Inc., Cambridge, MA

FOXP3+ Regulatory T cells (Tregs) play a critical role in the prevention of fatal autoimmunity and maintaining tissue homeostasis. A specialized population of these cells within non-lymphoid organs, dubbed Tissue Tregs, modulate a diverse set of regenerative and homeostatic functions in addition to niche-adaptive immune surveillance. The function of these cells is imparted by their ability to respond to distinct survival factors, along with specific tissue homing and anchoring capabilities. Life-threatening acute inflammatory and ischemic diseases including acute kidney injury (AKI), ischemic stroke and acute respiratory distress syndrome (ARDS) are often associated with poor prognosis stemming from rapid-onset runaway inflammation and misguided tissue-repair, making off-the-shelf Tissue Tregs a fitting therapeutic approach for multiple disease modalities. We report for the first time, the generation of allogeneic Tissue Engineered Tregs (Tissue EngTregs) through a gene editing approach of PBMC isolated CD4+ T cells, leading to stable *FOXP3* expression and a rapamycin-activated signaling complex that provides tunable IL-2 signal combined with the expression of an alarmin tuned transgene. Tissue EngTregs express hallmark Treg proteins including FoxP3, CD25 and were shown to suppress conventional T cells in a Treg suppression assay. Tissue EngTregs recapitulated key aspects of naturally occurring Tissue Tregs, showing greater activation (based on Ki67 and HLA-DR) and an enhanced ability to modulate an acute inflammatory milieu (e.g. higher expression of CD39, TIGIT) and sequestration of pro-inflammatory cytokines (e.g. IL-2, IL-33 and TNFα). Further, Tissue EngTregs are enabled with rapid response to acute inflammation as a result of enhanced expression of key homing receptors (e.g. CCR4, and CCR8) enabling inflammation driven homing and anchoring. Finally, we show Tissue Tregs gain the ability to express repair mediators such as Osteopontin relevant to indications such as ischemic stroke. As a proof of concept, we made use of a surrogate allogeneic polyclonal murine Engineered Treg (mEngTreg) system in several models of acute inflammatory and ischemic diseases. Briefly, mEngTregs were generated by Cas-9 mediated editing of MND in the *Foxp3* locus of C57BL/6 (autologous) or CB6F1 (allogeneic) CD4 splenocytes and transferred following disease induction in models of AKI, LPS mediated acute lung injury and transient middle cerebral artery occlusion (tMCAO) model of ischemic stroke. mEngTregs showed efficacy in the aforementioned models based on key disease parameters. In models of AKI, mEngTreg treated animals showed significant reduction in serum kidney function (SCr, BUN), and inflammatory (Kim-1) parameters

while also showing significantly reduced kidney damage as assessed by H&E. LPS induced acute lung injury mice treated with allogeneic mEngTregs showed restored blood oxygen saturation, reduced immune infiltrates as assessed by flow cytometry, lower levels of BAL inflammatory cytokines and reduced tissue damage based on H&E. Finally, tMCAO mice treated with allogeneic poly mEngTregs showed enhanced sensori-motor, memory and learning function based on several key readouts including accelerated rotarod, and foot fault tests. Overall, Tissue EngTregs display enhanced Tissue Treg properties that positions them as an exciting therapeutic option for acute ischemic and inflammatory diseases.

40. Myelin-Specific Engineered Treg Exhibit Therapeutic Benefit in a Murine Model of Multiple Sclerosis

Travis Drow[1], Brock McKinney[1], Noelle Dahl[1], Peter J. Cook[1], David J. Rawlings[1,2]

[1]Center for Immunity and Immunotherapies and the Program for Cell and Gene Therapy, Seattle Children's Research Institute, Seattle, WA,[2]Departments of Pediatrics and Immunology, University of Washington, Seattle, WA

Regulatory T cells (Treg) represent a promising avenue for treating tissue-specific autoimmune diseases including multiple sclerosis (MS). Strikingly, in comparison to polyclonal Treg, antigen-specific Treg display superior therapeutic efficacy in animal models of autoimmunity and are predicted to exhibit similar benefits in human subjects. However, expansion of rare naturally occurring antigen-specific Treg represents a major hurdle in bringing therapeutic cell products into the clinic. To overcome this challenge, we previously developed robust strategies to create antigen-specific, engineered regulatory T cells (EngTreg) from $CD4^+$ T cells via homology directed repair (HDR)-based gene-editing and demonstrated efficacy in mouse and non-clinical human type 1 diabetes studies[1-5]. Expanding on this previous work, here, we describe studies assessing the efficacy of EngTreg in an Experimental Autoimmune Encephalomyelitis (EAE) model, a robust animal model of MS. First, we optimized our engineering strategy using $CD4^+$ T cells expressing a myelin oligodendrocyte glycoprotein (MOG)-specific TCR. HDR at the mouse *Foxp3* locus was used to precisely insert a strong, ubiquitous promoter upstream of the first coding exon of endogenous *Foxp3;* thereby converting antigen-specific $CD4^+$ T cells into antigen-specific Tregs *en masse*. Using a GLP compliant MaxCyte platform, we delivered *Foxp3* targeting RNPs to 100 million 2D2 $CD4^+$ T cells with minimal impact on cell viability. Co-delivery of rAAV donor template was combined with a DNA-PK inhibitor to enhance HDR, resulting in significant improvements in editing rates. Cryopreserved products exhibited uniform Treg phenotypic and functional properties and excellent post-thaw viability. Using these cell populations, we modelled MS therapy in a chronic progressive EAE model. Immunocompetent C57BL/6 mice were immunized with MOG_{35-55} peptide in complete Freund's adjuvant (CFA). Varying doses of polyclonal vs. MOG-specific EngTreg were adoptively transferred at Day -1 prior to- or Day 7 post-immunization. Animals were evaluated daily for differences in clinical score using standardized EAE-scoring criteria. Delivery of MOG-specific EngTreg led to either prevention or a marked reduction in disease manifestation reflective of the EngTreg dose and timing of administration. Our combined data demonstrate the robust efficacy of antigen-specific EngTreg in an immunocompetent model of MS and highlight the importance of improving scalability for assessing gene-edited cell therapies in autoimmune disease models. 1. Honaker, Y., et al. (2020). Gene editing to induce FOXP3 expression in human CD4(+) T cells leads to a stable regulatory phenotype and function. Sci Transl Med 12(546), eaay6422. 2. Cook, P.J., et al. (2023). A chemically inducible IL-2 receptor signaling complex allows for effective in vitro and in vivo selection of engineered CD4+ T cells. Mol Ther 31(8), 2472-2488. 3. Hunt, M.S., et al. (2023). Dual-locus, dual-HDR editing permits efficient generation of antigen-specific regulatory T cells with robust suppressive activity. Mol Ther 31(10), 2872-2886. 4. Yang, S.J., et al. (2022). Pancreatic islet-specific engineered T(regs) exhibit robust antigen-specific and bystander immune suppression in type 1 diabetes models. Sci Transl Med 14(665), eabn1716. 5. Uenishi, G.I., et al. (2024). Development of GNTI-122, an autologous antigen-specific engineered regulatory T cell therapy for type 1 diabetes. JCI Insight, *in press*.

41. Development of Chimeric Antigen Receptor Macrophages Targeting Amyloid-β in Alzheimer's Disease

Mara Davis[1], Krystal Oon[1], Matias Porras Paniagua[1], Chris Bennett[2], Saar Gill[1]

[1]Center for Cellular Immunotherapies, University of Pennsylvania, Philadelphia, PA,[2]Division of Neurology, Children's Hospital of Philadelphia, Philadelphia, PA

Alzheimer's Disease (AD) is characterized by the presence of two disease hallmarks: amyloid plaques and neurofibrillary tangles (NFTs). Aggregation of Amyloid-β (Aβ) peptides leads to the formation of amyloid plaques and is thought to contribute to the production of NFTs[1]. While Aβ is an alluring therapeutic target, the development of Aβ-directed AD therapeutics is hindered by the physiological neuroprotective functions of monomeric Aβ[2]. Aducanumab is a monoclonal antibody with enhanced affinity for pathogenic Aβ aggregates over Aβ monomers[2], but in clinical trials it caused cerebral edema suggestive of unfavorable immune activation. To circumvent antibody-mediated Fc receptor activation in response to amyloid with Aducanumab, we engineered macrophages with a chimeric antigen receptor (CAR) lacking a signaling domain with Aducanumab's antigen binding domain. Aβ-CAR-macrophages (Aβ-CARMs) showed enhanced uptake of human Aβ in vitro, as well as the ability to clear Aβ following short-term exposure. The 5xFAD mouse model, which produces human Aβ, was used to assess the efficacy of the Aβ-CAR in vivo. 5xFAD mice were exposed to myeloablative busulfan conditioning before receiving a bone marrow transplant with CAR-expressing mouse hematopoietic stem cells (HSCs). Analysis of blood draws revealed reconstitution of the immune system of mice transplanted with CAR-positive cells, although live CAR macrophages were undetectable in the brains of mice 3 months post-bone marrow transplant. Despite being unable to detect CAR-positive cells in the brains of these mice, immunohistochemical staining revealed exacerbated amyloid plaque production in Aβ-targeting versus control CAR-HSC transplanted and untransplanted cohorts. We hypothesize that macrophage dysfunction and subsequent death are accelerated by enhancing Aβ-uptake via a CAR, and this dysfunction likely explains the CAR's contribution to the increased plaque deposition seen in vivo. Recent studies suggest

that, while brain macrophages are the primary phagocytes of Aβ, their ability to clear Aβ deteriorates over prolonged exposure. These macrophages adopt an exhausted-like, inflammatory phenotype, that is thought to contribute to their death and plaque seeding in mice[4], and this macrophage signature of impaired ability to respond to persistent stimuli are also observed in human AD patients[5]. To understand the contribution of our Aβ-CAR to plaque production, we evaluated Aβ-associated toxicities in Aβ-CARMs in vitro. Evaluating CARM response to their cognate antigen will further elucidate their potential efficacy in an in vivo model. References: 1. Ismail R, et al. J Neuroinflammation. 2020. doi:10.1186/s12974-020- 01820-6 2. Karisetty BC, et al. Front Mol Neurosci. 2020. doi:10.3389/fnmol.2020.577622 3. Vaz M, et al. Clin Interv Aging. doi:10.2147/CIA.S325026 4. Baik SH, et al. Glia. 2016. doi:10.1002/glia.23074 5. Millet A, et al. Immunity. 2024. doi:10.1016/j.immuni.2023.12.001

42. Base Editor Mediated CAR Integration with Simultaneous Multiplex Knockout for Enhanced Cancer Immunotherapies

Joseph G. Skeate[1], Nicholas J. Slipek[2], Walker S. Lahr[1], Joshua B. Krueger[3], Erin M. Stelljes[1], Alexandria K. Gilkey[1], Cara-lin Lonetree[3], Prateek P. Thenge[3], Mitchell G. Kluesner[4], Beau R. Webber[3], Branden S. Moriarity[3]

[1]University of Minnesota - Moriarity Lab, Minneapolis, MN,[2]University of Minnesota, Saint Paul, MN,[3]University of Minnesota, Minneapolis, MN,[4]Fred Hutchinson Cancer Research Center, Seattle, WA

With the November 2023 FDA bulletin highlighting T cell malignancies occurring in patients receiving commercial chimeric antigen receptor (**CAR**) T therapies, it's becoming clear that random integration of tumor-specific receptors poses safety risks in cell therapy products (fda.gov). Furthermore, as the circuitry of immune function becomes more clear, increasingly complex engineering is being pursued to improve therapy efficacies. We previously implemented base editors for multiplex gene knockout (**KO**) in CAR T cells to enhance efficiency and reduce genotoxicity associated with Cas9 nuclease. Here, we leverage the nickase activity of adenine base editor (**ABE8e**) to stimulate homology-directed repair (**HDR**) at targeted loci in support of an all-in-one platform for concurrent multiplex KO and knockin (**KI**) in primary T cells. Through a novel gRNA design strategy and a rAAV template, we enhanced the efficiency of ABE8e-stimulated HDR by >2-fold, achieving transgene KI rates of >30% in human T cells. We then combined KI of CD19, CD33, or mesothelin targeting CARs with >95% quadplex gene KO in T cells (*B2M*, 98% *TRAC*, 97% *PDCD1* 98%, *CISH* 95%). ABE8e multiplex-engineered CAR T cells showed enhanced functionality against multiple tumor lines. Specifically, in serial co-culture challenge assays, checkpoint KO CAR-T cells resisted exhaustion and sustained tumor cell killing when compared to controls and showed enhanced production of effector cytokines in later rounds of serial stimulation. In vivo, these cells controlled and improved survival in a Burkitt's Lymphoma xenograft model engineered to overexpress hPD-L1. Concurrent KI and multiplex KO using ABE8e in a one-step manufacturing process establishes a safe, scalable platform for complex therapeutic cellular manufacturing with broad applications across oncology, autoimmunity, and the treatment of rare disease.

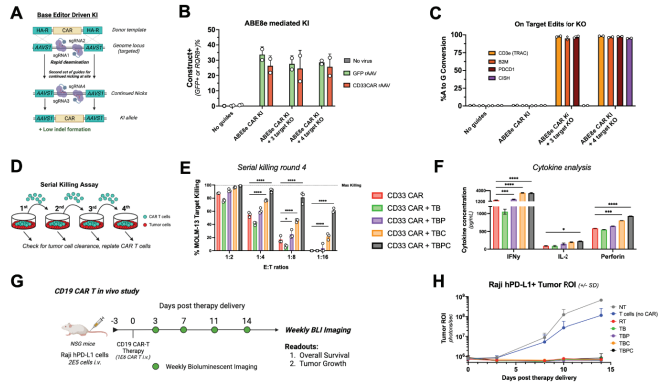


Fig 1. Adenine Base Editor (ABE8e) can carry out site-specific KI of a CAR and multiplex KO of target genes simultaneously in primary T cells to create functionally enhanced therapies. (A) Schematic of site-specific gene knockin using ABE8e and multiple guide strategy to induce HDR. (B) KI efficiency is not impacted by simultaneous addition of gRNA designed to KO target genes. (C) Multiple gene KO is achievable alongside site-directed cargo KI. (D) Diagram of serial killing assay to test long term functionality. (E&F) ABE8e multiplex engineered CD33 CAR T with CD3e (T), B2M (B), PDCD1 (P), and CISH (C) KO show functional enhancements at low Effector:Target (E:T) ratios (up to 1:16) in 4th round killing and enhanced cytokine production. (G) *In vivo* study design for testing CD19 CAR T cells. (H) ABE8e multiplex engineered CD19 CAR T cells show control of Raji-hPD-L1+ tumors *in vivo*. Images made with BioRender.

43. Peptide-MHC-Targeted Retroviruses Expand and Deliver Therapeutic Cargoes to Rare Populations of Anti-Tumor T Cells

Ellen J. K. Xu[1,2], Blake E. Smith[2,3], Michael J. Walsh[4,5], Winiffer D. Conce Alberto[3,5], Birkley Lim[5], Jiayi Dong[3,6], Caleb R. Perez[1,2], Andrea Garmilla[2,3], Qingyang Henry Zhao[1,2], Stephanie Gaglione[2,7], Connor S. Dobson[1,2], Michael Dougan[4,8], Stephanie K. Dougan[3,5], Michael E. Birnbaum[1,2,9,10]

[1]Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA,[2]Koch Institute for Integrative Cancer Research, Cambridge, MA,[3]Department of Immunology, Harvard Medical School, Boston, MA,[4]Division of Gastroenterology, Massachusetts General Hospital, Boston, MA,[5]Department of Cancer Immunology and Virology, Dana-Farber Cancer Institute, Boston, MA,[6]Division of Hematology-Oncology, Boston Children's Hospital, Boston, MA,[7]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA,[8]Department of Medicine, Harvard Medical School, Boston, MA,[9]Ragon Institute of MGH, MIT and Harvard, Cambridge, MA,[10]Singapore-MIT Alliance for Research and Technology Centre, Singapore, Singapore

Engineered T cell therapies have introduced a new class of effective and durable cancer treatments. Current strategies to produce these "living drugs" for the clinic require *ex vivo* T cell manipulations that rely on lentiviral vectors with broad tropism for activated T cells. More precise fine-tuning of T cell phenotype could be achieved with more specific gene delivery methods. While progress has been made to design viral vectors to specifically transduce CD3+, CD4+ or CD8+ T cell

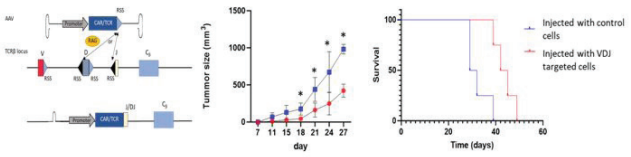
populations, no targeting method for antigen-specific T cells has been validated *in vivo*. Our lab has previously demonstrated that lentiviruses pseudotyped with single chain peptide-major histocompatibility complex (pMHC) in conjunction with a mutated form of the vesicular stomatitis virus glycoprotein (VSVGmut) allows antigen-specific infection of T cells. Here, we have developed pMHC pseudotyped gammaretroviruses to enable transduction of primary murine T cells with the goal of determining whether specifically engineered T cells can control tumors in an immunocompetent, syngeneic model. These pMHC-targeted gammaretroviruses were able to specifically transduce antigen-specific T cells in polyclonal populations while simultaneously activating and expanding those cells. After demonstrating specific targeting, we tested a tethered version of IL-12 as a therapeutic cargo that would be desirable to deliver in an antigen-specific manner given that systemic delivery induces adverse toxicities. In adoptive transfer experiments where cells were transduced *ex vivo* prior to injection into tumor-bearing mice, cells transduced by pMHC-targeted viruses delivering IL-12 (pMHC-IL12) were able to extend survival compared to cells transduced by pMHC-targeted virus delivering only ZsGreen or by traditionally pseudotyped gammaretroviruses. Furthermore, we hypothesized that the enhanced specificity of our targeted viruses could safely enable *in vivo* modification of antigen-specific cells, circumventing long and labor-intensive *ex vivo* manufacturing protocols. Preliminary experiments indicate that our pMHC-targeted viruses are able to target antigen-specific T cells at an initial frequency of 2-3% of the total CD8 population, expanding those anti-tumor T cells to 10-35% of CD8 T cells and transducing up to 15% of on-target cells. These data suggest that pMHC-targeted viruses have broad applications for engineering T cells both *ex vivo* and *in vivo.* While initial efforts have been focused on using pMHC-targeted viruses for cancer therapies, these viral vectors could also be applied to other applications where antigen-specific immune cell manipulation would be desirable, such as autoimmunity.

44. T Cell Engineering Using V(D)J Recombination

Miriam Horovitz-Fried[1], Carlo Cipriani[2], Natalia Gritsenko[1], Iris Dotan[1], Ayelet Dangot[3], Carmel Pundak-Mintz[1], Daniel Nataf[1], Tal Veig[1], Lee Reicher[3], Katia Beider[4], Arnon Nagler[4], Daniela Cesana[2], Adi Barzel[1]

[1]Tel Aviv University, Tel-Aviv, Israel,[2]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy,[3]Ichilov Medical Center, Tel-Aviv, Israel,[4]Shiba Medical Center, Ramat Gan, Israel

Autologous T cell therapies have shown clinical success in haematological malignancies, but they rely on prolonged and expensive ex vivo manipulations. Often applied as a late line of treatment, the efficacy of autologous therapies for both liquid and solid tumours is hampered by early T cell exhaustion, promoted by the prolonged culturing required for the scarcely retrieved cells. Conversely, "Off the shelf" allogeneic therapies reduce timelines and costs and may have favorable phenotypes, but they require disruption of the endogenous T cell receptor (TCR) to prevent graft vs. host disease (GVHD). We and others have demonstrated that nuclease mediated TCR disruption is associated with high rates of chromosomal aberrations and thus poses own challenges. Here, we propose "VDJ targeting": a novel immunotherapy approach targeting AAV-delivered immune genes into the genome using V(D)J recombination in developing T cells. In particular, we use adeno-associated viral (AAV) vectors coding for a transgenic TCR/CAR gene followed by a recognition signal sequence (RSS) for the recombination activating gene (RAG) complex, which is active only during T cell development. VDJ targeting is thus nuclease-free and produces potent naïve T cells that, compared to the products of conventional engineering, may be transplanted in lower numbers and have increased persistence in both autologous and allogeneic therapies. In addition, Targeted T cells express only the desired receptors, due to allelic exclusion. Finally, in younger patients, VDJ targeting could further be applied in vivo by direct intra-thymic vector administration, thus increasing efficacy and revolutionizing scalability. We have demonstrated VDJ targeting in ex vivo differentiated T cells of both human and mouse. Hematopoietic stem and progenitor cells (HSPCs) from mouse bone marrow were grown on OP9DL1 feeder cells in IL-7 and FLT-3 containing medium for 5 days, and then transduced with 1E6 vector genomes per cell of an AAV-DJ vector coding for a CAR gene against CD19. 13 days following transduction up to 40% of the CD3+ cells were found to express the CAR, but only when an RSS was coded on the CAR vector. Co-culture of the CAR-T cells with syngeneic CD19 expressing Eμ-ALL cancer cells led to high levels of IFNγ secretion. Concordantly, ultrasound-guided intrathymic injections of AAV8 vectors in immunocompetent mice allowed for in vivo CAR gene integration by VDJ targeting. Similarly, human cord blood derived HSPCs were grown on similar feeder cell with a SPEM-2 medium for 5 days and then transduced with an 7E5 vector genomes per cell of an AAV6 vector coding for either a CAR anti CD19 or a TCR targeting an NY-ESO1 peptide presented on HLA2-a2. 13 days post transduction, the receptor genes were detected on up to 36% and 12% of the CD3+ cells for the CAR and TCR respectively, and only when an RSS was coded on the vector. Co-culture with respective target cells allowed high levels of IFNγ secretion. As few as 200K Human T cells, engineered by VDJ recombination to express the TCR, were adoptively transferred into immunodeficient mice bearing the A375 melanoma cell line expressing NY-ESO-1. Importantly, we demonstrate tumor growth inhibition and prolonged survival (Figure 1). VDJ targeting is thus an efficient T cell engineering technology applicable in the autologous, allogeneic and in vivo settings.

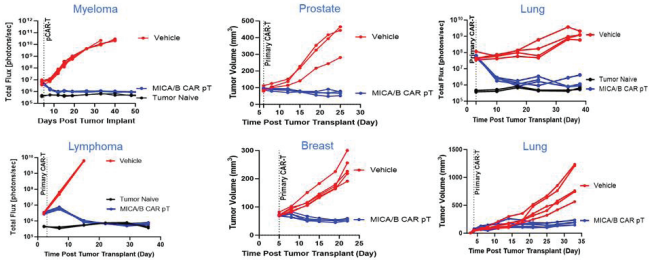


45. Novel α3-MICA/B-Specific CAR T-Cell Immunotherapy Demonstrates Ubiquitous Targeting of Cancer Cells and Resistance to Immune-Surveillance Evasion

Alex Garcia[1], Samad Ibitokou[1], Spas Markov[1], Bryan Hancock[1], Carissa Dege[1], Mochtar Pribadi[1], Yijia Pan[1], Lauren Fong[1], Thomas Dailey[1], Tom Lee[1], John Goulding[1], Jode Goodridge[1], Kai W. Wucherpfennig[2], Martin Hosking[1], Bob Valamehr[1]

[1]Fate Therapeutics Inc., San Diego, CA,[2]Dana Farber Cancer Institute, Boston, MA

MHC class I related proteins A and B (MICA/B) are surface ligands induced by cellular transformation and genotoxic stress, and their expression is reported in a variety of diseases, including cancer. NKG2D, expressed on NK and T cells, targets the highly polymorphic, membrane-distal α1 and α2 domains of MICA/B. However, NKG2D is susceptible to immune cell evasion by proteolytic cleavage and shedding of MICA/B, resulting in antigen escape and disease progression. We have developed a novel chimeric antigen receptor (CAR) targeting the highly conserved membrane-proximal α3 domain of MICA/B (3MICA/B CAR) that is resistant to MICA/B shedding, to attenuate immune evasion, restore MICA/B immune surveillance and direct the targeting and elimination of a broad range of diseased cells. Specific targeting of the α3 domain by 3MICA/B CAR was confirmed in several experiments, including targeting of α3 domain-only expressing transgenic cells and protease induced MICA/B cleavage cytotoxicity assay, comparing 3MICA/B CAR to NKG2D CAR T cell activity. Specific to α3 domain targeting, only 3MICA/B CAR T cells could recognize α3-only transgenic cells, confirming their unique ability to recognize α3 domain compared to NKG2D CAR T cells (98.5% ± 0.5% vs. 8.9% ± 4.2% cytotoxicity). In the induced cleavage assay, short-term exposure to trypsin selectively eliminated the membrane distal α1 and α2 domains of MICA/B and resulted in limited cytotoxic efficacy of NKG2D based CAR-T cells, while most of 3MICA/B CAR-T cell activity was unaffected. *In vivo,* primary 3MICA/B CAR T cells demonstrated robust anti-tumor control in a broad range of liquid and solid tumor settings: remarkably achieving an average of 92.3% ± 12.8% tumor growth inhibition (TGI) in mouse xenograft models of lymphoma, multiple myeloma, breast, prostate and lung cancer (Fig. 1). Importantly, this pan-tumor control seen with 3MICA/B CAR T cells was comparable to CAR-T cells targeting conventional tumor associated antigens such as HER2 (4D5) or CD19 (SJ25C1), underscoring the novelty and broad applicability of targeting the α3 domain of MICA/B for pan-tumor targeting applications (3MICA/B CAR vs HER2 CAR, 92.3% ± 12.8% vs 84.6% ± 15.9%; 3MICA/B CAR vs CD19 CAR, 92.3% ± 12.8% vs 93.5% ± 7.9%). To generate an off-the shelf and pan-cancer targeting cellular immunotherapy product, induced pluripotent stem cells (iPSC) were engineered to express 3MICA/B CAR under the transcriptional control of the T cell receptor alpha constant promoter and differentiated into TCR-less CD8 αβ T (iT) cells that express high and homogenous levels of 3MICA/B CAR. *In vitro* 3MICA/B CAR iT cells demonstrated robust and sustained control of both liquid and solid tumor targets of diverse MICA/B expression. In vivo, 3MICA/B CAR iT cells demonstrated potent and durable control of subcutaneous prostate cancer xenografts (96.7% ± 1.3% TGI). Together, these data demonstrate that α3-MICA/B-specific CAR T-cell immunotherapy has the unique ability to target stress ligands while being resistant to antigen shedding and escape and represent a novel pan-cancer targeting immunotherapy with potential therapeutic application in a variety of cancers. **Fig. 1: α3-MICA/B CAR primary T cells provide in vivo efficacy against multiple human hematopoietic and solid tumor xenograft models.**



Lysosomal Storage Disorders

46. Hematopoietic Stem Cell Gene Therapy for Hurler Syndrome: Interim Skeletal Outcome and Skeletal Cross-Correction Mechanisms

Francesca Tucci[1], Giulia Consiglieri[1], Maurizio De Pellegrin[1], Matilde Cossutta[1], Chiara Filisetti[1], Marina Sarzana[1], Stefano Scarparo[1], Cristina Baldoli[1], Renata Mellone[1], Ludovica Santi[1], Stefania Crippa[1], Rossella Parini[1], Alessandro Aiuti[2], Maria Ester Bernardo[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Milan, Italy

Introduction: Skeletal dysplasia is a prominent feature of Mucopolysaccharidosis type I Hurler syndrome (MPSIH) which is not fully addressed by allogeneic haematopoietic stem cell transplantation (HSCT). Autologous *ex vivo* hematopoietic stem cell gene therapy (HSC-GT) is a promising therapeutic strategy for MPSIH since it is capable of producing supraphysiologic IDUA levels, leading to extensive metabolic correction. Here we report interim results from a Phase I/II clinical trial (NCT03488394) on the skeletal outcome of 8 MPSIH patients treated with HSC-GT (OTL-203) up to a median follow-up of 4 years. As there is limited knowledge of the pathogenesis of skeletal defects and skeletal correction mechanisms in MPSIH after HSC-GT, we have characterized *in vitro* the key players of skeletal remodelling before and after HSC-GT. *Methods:* Skeletal outcomes of the 8 MPSIH patients were assessed at baseline and at specific timepoints after treatment by clinical (growth), functional (motor performance and joint range of motion) and radiological parameters (X-Rays and MRI for the hip and X-Ray and MRI for the spine) up to a median of 4 years after HSC-GT. Specific skeletal measures were retrospectively compared with an external cohort of patients who underwent HSCT in Monza. Patient-derived osteoclasts (OCs), mesenchymal stromal cells (MSCs) and osteoblasts (OBs) were isolated and characterized before and at several timepoints after HSC-GT. *Results:* IDUA activity in peripheral blood reached supraphysiologic levels in all patients after HSC-GT resulting in extensive substrate depletion, which was maintained at the latest follow-up. All patients exhibited longitudinal

growth within expected reference ranges according to age and gender, with a median height gain greater than the one observed in Monza HSCT patients. Regarding functional outcome, all HSC-GT patients progressively acquired motor skills, as shown by the stability of Total Motor Quotient score over time, and experienced a complete and earlier normalization of joint mobility as compared with HSCT patients. Both hip X-ray and hip MRI parameters improved after HSC-GT, representing an amelioration of hip dysplasia up to the latest follow-up. A specific spine MRI score showed stabilization of the typical spine MRI features *(i.e.* dorsolumbar kyphosis, foramen magnum and spinal stenosis) in all HSC-GT patients at last follow-up.In the HSC-GT scenario, we hypothesized that OCs, deriving from genetically corrected haematopoietic precursors, could deliver the missing enzyme in the bone microenvironment as mediators of cross-correction of non-haematopoietic cells. We demonstrated that patient-derived OCs after HSC-GT and their supernatant show supraphysiologic IDUA activity which can be taken up by patient MSCs and OBs, thus rescuing GAGs engulfment in MPSIH-OBs. Osteomedullary biopsies of MPSIH patients obtained at baseline showed foamy appearance of the cytoplasm of the soft tissue cells adjacent to bone cells (i.e., entheses) which fully resolved after HSC-GT. *Conclusions:* Clinical, functional and radiological parameters indicate an early beneficial effect of HSC-GT on MPSIH-typical skeletal features. A longer follow-up and a direct comparison with HSCT are needed to draw definitive conclusions on the impact of HSC-GT on skeletal dysplasia. Biological studies aimed at unveiling skeletal correction mechanisms after HSC-GT will be key to advance the treatment of MPSIH patients.

47. Adeno-Associated Virus-Based Gene Therapy Delivering Combinations of Two Growth-Associated Genes

Shunji Tomatsu[1], Estera Rinz[1], Betul Celik[2], Shaukat Khan[1]

[1]Nemours/Alfred I. DuPont Hospital for Children, Wilmington, DE,[2]University of Delaware, Newark, DE

Background: Mucopolysaccharidosis type IVA (MPS IVA) is caused by a deficiency of the GALNS enzyme responsible for the degradation of specific glycosaminoglycans (GAGs), keratan sulfate (KS) and chondroitin-6-sulfate. The progressive accumulation of GAGs leads to various skeletal abnormalities (short stature, knock knees, hypoplasia, tracheal obstruction). To date, no treatment is effective for patients with bone abnormalities. Approach: To improve bone pathology, we propose a novel combination treatment with the GALNS enzyme gene therapy and a C-type natriuretic peptide (CNP) as a growth-promoting agent for MPS IVA. MPS IVA knockout mice were treated at 4 weeks old via tail vein injection with the AAV vector, followed for 12 weeks. The experiment involved injecting two separate vectors (AAV9-hGALNS and AAV9-hNPPC) or a single vector expressing both transgenes (AAV9-hGALNS-hNPPC). Results: An equal dose of two AAV vectors (AAV9-hGALNS and AAV9-hNPPC) was administered. The high dose of both transgenes increased the expression of both GALNS enzyme and CNP peptide, resulting in the sustained elevation of GALNS activity in the bloodstream and notable GALNS activity in crucial target tissues such as bone, heart, and liver. A significant decrease in the accumulation of KS was seen both in the blood and tissues, leading to improvements in bone pathology and the promotion of bone growth in a dose-dependent manner. However, the experiment was terminated at 13 weeks of age due to the overexpression of the CNP peptide, which led to excessive growth in the mice. The mice faced difficulties in movement, indicating that CNP-related adverse effects are dose-dependent. The low-dose group of the CNP-expressing vector minimized the mouse growth. With the middle dose of the CNP, the growth was linear without adverse effects. We also tested the effect of the growth pattern in the mice after injecting one vector with two transgenes. The growth was linear without overgrowing in this group. However, we did not detect the GALNS enzyme activity in bone. We observed the same decrease in the KS level as in other groups. We also detected high anti-GALNS activity, which may neutralize the GALNS activity. The GALNS protein level in liver was lower than in the other combination groups. The micro-CT assessment did not reveal significant differences in most parameters among the various groups. However, the group receiving the highest dose of the NPPC-expressing vector in combination exhibited the most noticeable changes, which correlated with the visible alterations in the skeleton. We performed toluidine blue staining for the knee joints to evaluate the effectiveness of AAV treatment. Bone pathology revealed significant differences in the score calculation of chondrocytes. The untreated MPS IVA chondrocytes were vacuolated with a disorganized structure. After combination therapy of two AAV vectors with CNP and GALNS, we observed marked improvements in growth plate organization and decreased vascularization of treated chondrocytes. Conclusions: Combination therapy induces bone growth with increased enzyme activity in tissues (bone, liver, heart, lung) and plasma and shows significant changes in bone morphology with increased CNP activity in plasma. Combining CNP and GALNS gene therapies enhances bone growth in MPS IVA mice more than in GALNS gene therapy alone. Our findings indicate that combination therapy is a potential alternative since the enzyme would not easily penetrate the growth plate region in the bone. Overall, Our study has offered valuable insights into the potential therapeutic use of CNP for progressive skeletal dysplasia in MPS IVA. Thus, more extensive studies are necessary to validate the effectiveness of CNP delivered through an AAV vector expression cassette in combination with the GALNS enzyme as a potential combination therapy.

48. CAMPSIITE™ Phase I/II/III: An Interim Clinical Study Update of RGX-121, an Investigational Gene Therapy for the Treatment of Neuronopathic Mucopolysaccharidosis Type II (MPS II)

Laura Pisani[1], Roberto Giugliani[2], Can Ficicioglu[3], Paul Harmatz[4], Deepa Rajan[5], Joseph Hagood[1], Michele Fiscella[1], Lin Yang[1], Michelle Gilmor[1], Yoonjin Cho[1], Caroline Mulatya[1], Dawn Phillips[1], Nidal Boulos[1], Paulo Falabella[1]

[1]REGENXBIO Inc., Rockville, MD,[2]Hospital de Clinicas de Porto Alegre, Porto Alegre, Brazil,[3]Children's Hospital of Philadelphia, Philadelphia, PA,[4]UCSF Benioff Childrens Hospital Oakland, Oakland, CA,[5]University of Pittsburgh, Pittsburgh, PA

Purpose: MPS II is an X-linked lysosomal storage disease caused by deficiency of iduronate-2-sulfatase (I2S) leading to accumulation

of glycosaminoglycans (GAGs). Neuronopathic MPS II (nMPS II) results in irreversible neurodevelopmental decline not addressed by intravenously administered enzyme replacement therapy. RGX-121 is an investigational gene therapy that uses the adeno-associated virus serotype 9 (AAV9) vector to deliver a human iduronate-2-sulfatase (IDS) gene to the central nervous system (CNS). RGX-121 is designed to provide a permanent source of secreted I2S, potentially correcting neurologic and systemic disease manifestations. **Design/Methods:** CAMPSIITE™ is a phase I/II/III, open-label, 104-week trial enrolling boys 4 months up to 5 years of age with nMPS II (NCT03566043) who receive one image-guided RGX-121 injection to the CNS. Assessments include safety and tolerability; cerebrospinal fluid (CSF), plasma, and urine biomarkers; neurodevelopmental scales; and imaging. Participants are encouraged to enroll in a long-term follow-up study. **Results:** As of January 3, 2023, 15 participants were enrolled in the phase I/II portion of CAMPSIITE in 3 dose cohorts ($1.3x10^{10}$, $6.5x10^{10}$, and $2.9x10^{11}$ genome copies/gram brain mass). RGX-121 was reported to be well-tolerated with no drug-related serious adverse events. Longest post-administration follow-up was > 3 years. CSF GAGs showed dose-dependent reductions with D2S6 continuing to approach normal levels in cohort 3 at 48 weeks. Interim neurodevelopmental assessments demonstrated CNS activity up to 3 years after RGX-121 administration. Evidence of systemic enzyme expression and biomarker activity was present after CNS RGX-121 administration. Updated interim results and topline pivotal results will be presented. **Conclusions:** RGX-121 has the potential to provide sustained CNS clinical outcomes and additional systemic effects in MPS II patients.

49. Rescue of Lysosomal Acid Lipase Deficiency in Mice by AAV Liver Gene Transfer

Marine Laurent[1,2], Rim Harb[1,2], Christine Jenny[1,2], Julie Oustelandt[1], Francesca Landini[3], Guillaume Core[1,2], Anaïs Brassier[4], Laetitia Van Wittenberghe1[1,2], Dagmar Kratky[5], Giuseppe Ronzitti[1,2], Mario Amendola[1,2]

[1]UMRS_951 Genethon, Evry, France,[2]Genethon, Evry, France,[3]Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy,[4]Necker-Enfants-Malades University Hospital, Paris, France,[5]Division of Molecular Biology and Biochemistry, Medicine University of Graz, Graz, Austria

Lysosomal acid lipase deficiency (LAL-D) is an autosomal recessive disorder caused by mutation in *LIPA* gene resulting in fatal lipid accumulation in liver and spleen. The most severe form, if not treated, results in premature death within the first year of life due to hepatic insufficiency. Weekly administration of the enzyme replacement therapy (ERT) is the only available supportive treatment. Although life-saving, ERT is not curative and may be associated with development of neutralizing antibodies decreasing treatment efficacy. To develop a curative treatment for LAL-D, we first characterized a new $lal^{-/-}$ mouse model, generated by deleting the exon 4 of the *LIPA* gene, and then assessed an *in vivo* gene therapy. We confirmed the loss of LAL activity and the accumulation of lipids in liver and spleen which leads to an increase in organ weight, in liver transaminase and dyslipidaemia (HDL). $Lal^{-/-}$ mice display a dysregulation of the haematological compartment, with an inflammatory profile: increase of granulocytes, monocytes, and platelets. We also have new promising data using imaging flow cytometry to investigate the number of lipid droplets in blood cells as a novel method to easily assess disease burden. For gene therapy, we targeted the liver by systemically injecting 3-month-old $lal^{-/-}$ male mice using the AAV-8 serotype encoding the *hLIPA* gene under the control of hepatocyte-specific human α1-antitrypsin (hAAT) promoter. Transduced hepatocytes would both produce LAL to correct their own metabolic impairment and secrete the enzyme in bloodstream to cross-correct affected tissues. We conducted an 3-months follow-up study of 4 different AAV-8 doses: $5X10^{11}$ vg/kg; $1X10^{12}$ vg/kg; $3X10^{12}$ vg/kg; $1X10^{13}$ vg/kg. Starting from two weeks after injection, we increased LAL activity in plasma and we observed a therapeutic effect with decrease of hepatic transaminases and normalization of haematological parameter. At 3-months post-injection, we evaluated vector biodistribution, enzymatic activity and tissue correction by histological staining and cholesterol/triglycerides quantification. As expected, AAV-treated $lal^{-/-}$ mice displayed efficient hepatocyte transduction and LAL expression proportional to the injected doses. Noteworthy, all AAV doses were sufficient to reduce or correct hepatosplenomegaly and lipid accumulation with $3X10^{12}$ vg/kg being the best compromise between AAV dose and disease correction. With this minimal corrective dose, we assessed phenotype correction in $lal^{-/-}$ mice at 1-, 3-, and 8- months post-injection. Firstly, AAV administration extended survival, improved body weight, and stably normalize haematological parameters, hepatosplenomegaly, lipid accumulation and tissues architectures along the 8-months follow-up. Secondly, using liver transcriptomic analysis, we observed a previously unreported impairment of mitochondria, which was confirmed by biochemical analysis and fully corrected by gene therapy. To further decrease vector dose, we engineered a novel *LIPA* cDNA to improve LAL expression and secretion by optimizing the codon usage and changing its signal peptide sequence and we are currently evaluating its therapeutic benefit *in vivo.* Finally, we are testing this strategy in $lal^{-/-}$ human liver organoids. Overall, our *in vivo* gene therapy strategy represents an effective approach to achieve stable LAL expression and correct LAL-D disease opening the possibility of developing a curative treatment to improve patients' life expectancy and quality.

50. AAV-Ep+ in Preclinical Studies for Late Infantile Neuronal Ceroid Lipofuscinosis

Luis Tecedor[1], Yonghong Chen[1], David E. Leib[2], Paul T. Ranum[3], Bryan P. Simpson[2], Megan S. Keiser[4], Melanie McFadden[1], Alexis Mackiewicz[1], Brian Lewandowski[1], Elena Lysenko[1], Jang-Ho Cha[5], Beverly L. Davidson[1]

[1]Children's Hospital of Philadelphia, Philadelphia, PA,[2]Latus Bio, Philadelphia, PA,[3]Latus Bio, Saint Paul, MN,[4]Ohio State University Wexner Medical Center, Columbus, OH,[5]Latus Biosciences, Arlington, MA

Late Infantile Neuronal Ceroid Lipofuscinosis (LINCL) is a childhood neurodegenerative disease that appears between 2-4 years of age and progresses with visual, motor, and mental decline, with a life expectancy of less than two decades. Most cases of LINCL are caused by deficiency in the soluble lysosomal enzyme tripeptidyl peptidase (TPP1) as a result of mutations in the *CLN2* gene. We have previously demonstrated that gene therapy-mediated enzyme replacement of TPP1 produced significant clinical benefits in a LINCL canine model. In these experiments, therapeutic amelioration of LINCL pathology was achieved using wild-type AAV to target ependymal cells resulting in secretion of TPP1 into the cerebrospinal fluid. We hypothesized that

a more efficient delivery vector with improved tropism for ependymal cells and parenchymal neurons could enhance the efficacy of this TPP1 gene therapy strategy and help facilitate the translation of this successful therapeutic approach to humans. To achieve this, we applied a peptide-modified AAV vector screening methodology developed in our lab to identify new vectors with improved tropism for ependymal cells in non-human primates (NHPs). We found AAV-Ep+, which showed a cumulative rank enrichment of ~30,000 from the last rounds of screening. To characterize the transduction efficiency of AAV-Ep+ in ependyma cells of NHP brain, AAV-Ep+.mRuby3 was bilaterally injected in the lateral ventricles of a cynomolgus monkey. Ependymal cell transduction was highly efficient, with mRuby3 expression detected in 76-99% of cells across different regions of the brain ventricle system. Moreover, at this dose, AAV-Ep+ shows low levels of off-target transduction in peripheral tissues and dorsal root ganglia. We next tested AAV-Ep+ in a TPP1-deficient mouse model. Brain TPP1 levels in mice treated with AAV-Ep+ were up to 11-fold higher than mice treated similarly with wtAAV. Furthermore, extended lifespan and improved disease phenotypes were achieved to a significantly greater degree than the highest doses of WT serotypes tested. In summary, AAV-Ep+ results in broad and robust transduction of brain ependyma cells in non-human primates. Utilized as a vector to deliver gene therapy in a LINCL model, AAV-Ep+ increases recombinant TPP1 biodistribution and rescues phenotypes at doses significantly lower than conventional serotypes. AAV-Ep+ is a promising candidate for improving the translatability and applicability of gene therapy for LINCL and other diseases amenable to ependymal cell targeted treatments.

51. Long-Term Haematopoietic Stem Cell Gene Therapy in Mucopolysaccharidosis IIIB Mice Corrects Disease with No Evidence of Insertional Mutagenesis Despite High Vector Copy Numbers

Brian W. Bigger[1], Stuart M. Ellison[2], Shaun R. Wood[2], Rebecca Holley[2]

[1]Centre for Regenerative Medicine, University of Edinburgh, Edinburgh, United Kingdom,[2]Division of Cell Matrix Biology and Regenerative Medicine, University of Manchester, Manchester, United Kingdom

Mucopolysaccharidosis IIIB (MPSIIIB) is caused by a lack of the enzyme alpha-N-acetylglucosaminidase as a result of mutations in the *NAGLU* gene. Loss of NAGLU results in a build-up in heparan sulphate in cells and the extracellular matrix leading to cell damage and inflammation. Patients with MPSIIIB experience severe neurodegeneration and a progressive loss of acquired skills. There is no effective therapy. We designed a lentiviral-driven haematopoietic stem cell gene therapy (HSCGT), transducing with a CD11b-NAGLU lentiviral vector and transplanting corrected MPSIIIB haematopoietic stem cells into MPSIIIB mice. Use of this therapy resulted in significant delivery of NAGLU enzyme to the brain, due to the migration and engraftment of monocytes into the brain. Analysis of animals more than 1 year after transplant showed sustained NAGLU expression with brain enzyme activity of 26% of normal levels, increased over time compared to animals that were analysed at 6-months post-transplant (13% of normal). Widespread correction of heparan sulphate storage throughout the body was visible. Significantly, neuroinflammation which is widespread in untreated animals, was minimal in multiple brain regions. We also show, for the first time, prevention of cortical neuron loss in mucopolysaccharidosis IIIB mice following treatment. A single incidence of malignant lymphoma was seen in one mouse treated with LV-NAGLU HSCGT following histology, and was considered an incidental finding. However, this mouse exhibited high VCN and alongside animals transplanted with the same pool of HSCGTs was analysed for integration site analysis to rule out insertional mutagenesis. This mouse exhibited the relatively low number of IS (141 in leukocytes), suggesting a lower number of engrafting cells, but with no clonal expansion of any one integration site. Our data suggests a minimum brain enzyme level of 9.3% to enable behavioural correction, equating to approximately 1.07 vector copy numbers per bone marrow cell. These data make a strong case for rapidly moving haematopoietic stem cell gene therapy for mucopolysaccharidosis IIIB to clinical trial, providing hope for MPSIIIB patients and their families to alleviate the devastating neurological symptoms of this disease.

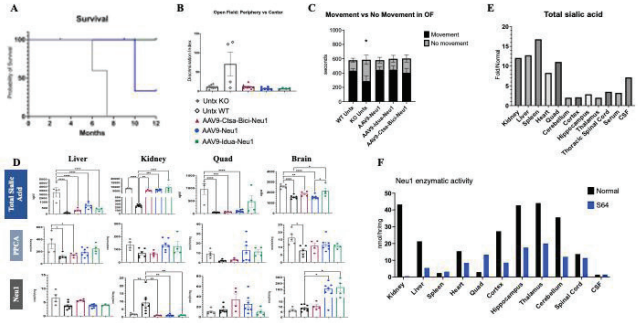
52. From Mouse to Sheep: Developing an AAV Gene Therapy for Sialidosis

Jillian Gallagher[1], Judith Gallant[2], Ping Xu[2], Rachael Gately[3], Stephanie Bertrand[3], Rachel Prestigiacomo[3], Abigail McElroy[1], Erin F. Hall[4], Hector Ribeiro Benatti[5], Leigh Fremuth[6], Diantha Van de Vlekkert[6], Camilo Toro[7], Cynthia J. Tifft[7], Scot A. Wolfe[5], Alessandra d'Azzo[6], Miguel Sena-Esteves[8], Yvette Lopez-Diaz[1], Heather L. Gray-Edwards[5]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Department of Pediatrics, UMass Chan Medical School, Worcester, MA,[3]Cummings School of Veterinary Medicine at Tufts University, Grafton, MA,[4]University of Massachusetts Chan Medical School, Worcester, MA,[5]UMass Chan Medical School, Worcester, MA,[6]St. Jude Children's Research Hospital, Memphis, TN,[7]National Institutes of Health, Bethesda, MD,[8]Neurology, UMass Chan Medical School, Worcester, MA

Sialidosis is a rare lysosomal storage disease caused by mutations in the *NEU1* gene that encodes the lysosomal sialidase neuraminidase 1 (NEU1). Deficiency of NEU1 causes accumulation of sialylated glycopeptides and oligosaccharides in tissues and body fluids, resulting in cell and organ dysfunction. Sialidosis has two forms the late onset type I or early onset type II. Type I patients have adolescent onset with myoclonus, ataxia, seizures, cherry-red spot, and vision loss with death in midlife. Type II infantile/juvenile patients are more severe, including hepatosplenomegaly, coarse facial features, dysostosis multiplex, developmental delay, and death in childhood. There are no approved therapies for sialidosis. We developed an AAV-mediated gene therapy strategy to treat sialidosis that includes three AAV9 vectors: encoding either *Neu1* alone (AAV9-Neu1), or in combination with its chaperone protein cathepsin A (AAV9-Ctsa-Bici-Neu1) or encoding modifications to enhance secretion (AAV9-Idua-Neu1). Neonatal Neu1-KO mice were injected via intracerebroventricular with 1E11vg of each AAV vector. Increased survival was noted for all three vectors compared to untreated Neu1-KO mice, however both AAV9-Neu1 treated WT (n=1) and NEU1-KO mice developed seizures at ~10 months of age (**A**). Behavioral testing revealed significant motor improvement for AAV treated Neu1-KO mice for all three vectors compared to Neu1-KO untreated mice with a trend toward decreased anxiety-like behavior

(**B&C**). Reduction of sialic acid and PPCA levels were observed in the liver, brain, and quad, while mild reduction was seen in the kidney of treated mice compared to untreated Neu1-KO mice (**D**). Increased Neu1 activity was observed in the kidney, quadriceps, and brain of treated mice versus untreated Neu1-KO mice (**D**). Pathology of the liver, spleen, kidney, and pancreas was markedly ameliorated as well as diminished astrocytosis in the hippocampus of treated mice compared to untreated Neu1-KO's. Overall, the most efficacious vector is AAV9-Ctsa-Bici-Neu1, which will be further tested in a large animal model of sialidosis. We have successfully generated the first sheep model of sialidosis type II. Using CRISPR-SpCas9 technology, we generated a founder sheep heterozygous for a 3 base pair deletion within exon 5 of Neu1(A320del) that is in the same loation as the amino acid change in patients (A319V). the A320del in sheep recapitulates many aspects of sialidosis including increased sialic acid levels (**E**), reduced Neu1 activity (**F**), neurological deficits, hepatosplenomegaly, hydronephrosis in the kidney, bone deformities, contracted tendons, and short lifespan. Further characterization of this model is currently ongoing. Altogether these studies in parallel with natural history studies in sialidosis patients will inform future clinical trials for this devastating disease.



Epigenetic Editing and RNA Editing

53. A High-Fidelity CRISPR-Cas13 System Improves Abnormalities Associated with C9ORF72-Linked ALS/FTD

Tristan McCallister

Bioengineering, University of Illinois Urbana Champaign, Urbana, IL

An abnormal expansion of a GGGGCC (G_4C_2) hexanucleotide repeat in the first intron of the C9ORF72 gene is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), two devastating disorders that are now thought to form a broad neurodegenerative continuum. One of the major mechanisms proposed to explain the pathogenicity of the hexanucleotide repeat expansion (HRE) is a gain-of-function toxicity originating from the bidirectional transcription of sense and antisense transcripts carrying it. Given the evidence in support of a role for the hexanucleotide repeat-containing RNAs in C9ORF72-linked ALS and FTD, hereafter referred to as C9-ALS/FTD, strategies silencing their production hold potential for treating the disorder. One emerging technology with the capabilities to enable this is the Cas13d nuclease from *Ruminococcus flavefaciens* XPD3002, known as RfxCas13d or CasRx, a class 2 type VI CRISPR effector protein that, when complexed with a crRNA molecule complementary to a target transcript, can cleave it via its intrinsic ribonuclease activity. RfxCas13d is compact enough to fit within a single adeno-associated virus (AAV) vector to enable its delivery to the nervous system and can be fused to nuclear localization signal sequences to target transcripts in the nucleus. Given these properties, we hypothesized that RfxCas13d could be used to silence expression of repeat-containing RNAs and influence the pathological hallmarks of C9-ALS/FTD. To facilitate the design of crRNAs to silence the G_4C_2 repeat-containing RNA, we searched the exon 1a and the intron 1 sequences flanking the HRE using the Cas13 Design Resource. We then developed a dual-reporter screen to determine the ability of the 15 highest ranking crRNAs to target their respective sequences. Through this screen, we identified several crRNAs that decreased the expression of the reporter transgene by up to ~90% and induced no apparent collateral effects in cells. We next evaluated the targeting capabilities of these lead crRNAs in HEK293T cells and SH-SY5Y cells, finding by qPCR that the most effective crRNAs decreased V3, a C9ORF72 transcript variant that carries the HRE, by 90-95% and had no effect on all-V, the reservoir of C9ORF72 transcript variants that consists most predominately of V2, a transcript variant that lacks the HRE. To evaluate targeting *in vivo*, we injected the motor cortex (MC) and hippocampus (HPC) of C9-BACexp mice with an AAV-PHP.eB vector encoding RfxCas13d with one of the three lead crRNAs. According to qPCR, our most potent crRNA reduced V3 mRNA by two-fold, both in the MC and HPC, and decreased the formation of RNA foci consisting of the G_4C_2 repeat-containing RNA by up to 37%. Because of their risk for inducing collateral effects, we next determined the ability for high-fidelity forms of RfxCas13d, which possess a reduced capacity to *trans*-cleave non-target RNAs, to target C9ORF72. In addition to decreasing V3 mRNA in HEK293T cells and SH-SY5Y cells by 80-95%, we found by RNA-seq that the most effective high-fidelity variant, RfxCas13d-N2V8, possessed improved transcriptome-wide specificity compared to the native protein, affecting the expression of nearly six-fold fewer genes. Given these improved capabilities, we determined if RfxCas13d-N2V8 could target C9ORF72 *in vivo.* Consistent with our earlier study, we measured a two-fold decrease in V3 mRNA in the MC and HPC of C9-BACexp mice injected with RfxCas13d-N2V8, as well as a ~37% decrease in RNA foci formation. Last, through an RNA-seq analysis of transduced nuclei from the MC of C9-BACexp mice and their wild-type littermates, we found that RfxCas13d-N2V8 reversed at least 50% of the transcriptional deficits that manifested in C9-BACexp mice. Altogether, our results illustrate the potential of CRISPR-Cas13 technology for C9ORF72-linked ALS/FTD.

54. Do Some Loci Have a Propensity to Retain Ectopic Methylation?

Henriette O'Geen[1], Alina Mihalovits[2], Benjamin D. Brophy[1], He Yang[1], Marketa Tomkova[2], David J. Segal[1]

[1]UC Davis Genome Center, Davis, CA,[2]Ludwig Institute for Cancer Research, University of Oxford, Oxford, United Kingdom

In this study, we aimed to identify if methylation hotspots exist, what pre-existing chromatin environments might enable long-lasting DNA methylation, and whether this could affect gene expression. Epigenetic editing is emerging as a safe way to treat genetic disorders. Indeed, great advances have been made recently to achieve long-term repression of

target gene expression. We and others have used a catalytically dead dCas9 tethered to a DNA-methyltransferase and a recruitment domain for a histone-methyltransferase to enable longer lasting methylation at the target locus. However, before epigenetic editors can be translated into clinical settings, we need to have a deeper understanding of longevity of gRNA-independent off-target events, how widespread off-target methylation really is, and if some loci have a propensity to retain ectopic methylation and therefore are considered ectopic methylation hotspots. Our study demonstrated that hundreds of CpGs can retain off-target methylation in cell culture even after transient delivery of dCas9-based epigenetic editors. We identify chromatin features that predispose genomic regions to not only gain, but also retain off-target methylation for at least 24 days. We identify a distinct subset of genes where retained CpG hypermethylation translates into long-lasting effects on gene-expression. Expansion of our analysis to other epigenetic targeting strategies and cell types corroborate our findings. In summary, we show how targeted DNA methylation can lead to unintended expression changes at genes with distinct epigenetic marks. Our results warrant further considerations for on-target efficiency and unintended off-target effects of epigenome editing.

55. Engineered Extrachromosomal Genetic Technologies for Persistent and Tunable Control of Therapeutic Human Cell Types

Daniel A. Brenner[1], Hailey Szadowski[2], Sofia Aguilera[1], Ana Naqvi[1], Isaac B. Hilton[1,2]

[1]Department of Bioengineering, Rice University, Houston, TX,[2]Systems, Synthetic, and Physical Biology Graduate Program, Rice University, Houston, TX

Retroviral vectors have seen clinical success with respect to delivering transgenes to primary human cells. For instance, these vectors are the gold standard for the delivery of chimeric antigen receptors (CARs) in T-cells and other cell types. However, retroviral vectors are prone to random genomic integration of payloads, which poses important clinical drawbacks, such as the disruption of endogenous genes, epigenetic silencing of integrated transgenes over time, and oncogene activation. These drawbacks converge to make the use of programmable non-integrating methods of transgene expression attractive. Current non-integrating viral expression systems used in therapeutic contexts are either very restricted in terms of payload capacities (e.g., adeno-associated virus) or are modified to eliminate any potential for virulence and/or immunogenicity (e.g. adenovirus). These modifications tend to result in transient payload expression. Further, both integrating and non-integrating cargo expression approaches lack facile tunability of transgene expression. To overcome these limitations, we developed a new class of technology that combines the strategies displayed by latent non-integrating viruses to maintain long-term gene expression with synthetic biology tools. In doing so, we created persistent and programmable extrachromosomal expression systems that allow user-defined tunability of payload expression and maintenance of genetic components. Specifically, we have built and optimized a class of programmable and stably maintainable non-integrating DNA systems containing modular cassettes that confer mitotic stability and host-mediated replicative ability. We hypothesized that this system would allow extrachromosomal or episomal DNA to persist in dividing human cells without genomic integration. We first constructed and tested an engineered tethering system that permitted us to site-specifically control the targeting and frequency of engagement between extrachromosomal DNA and the human genome in live cells. Using ChIP-qPCR, we confirmed that our tethering modules can bind to both the human genome and synthetic extrachromosomal DNA. We also used fluorescence in situ hybridization (FISH) and high-resolution microscopy to verify co-localization. Next, we transfected human cells with episomes containing EGFP, our tethering system, and an engineered replication module. We optimized the number of tether binding sites in the host genome and showed the long-term persistence of payload expression (EGFP) out to ~3 months post-transfection. We also isolated genomic DNA and used qPCR to verify that persistence was not due to off-target payload integration into the genome. To streamline constructs for more versatile viral and non-viral delivery modalities, we also created truncated versions of tethering and replication modules. Using these compact designs, we demonstrate delivery to primary human cell types, including T cells, and in turn, use these platforms to express high levels of therapeutics tunably and persistently. In summary, we developed a novel method of maintaining extrachromosomal DNA payloads in human cells and successfully demonstrated that we can maintain therapeutic gene expression in dividing primary cells without genomic integration. Our extrachromosomal genetic technology opens new possibilities for treatments that rely upon transgene delivery without the associated risks of altering the endogenous human genome.

56. Compact Epigenetic Modulators for CRISPR Mediated Persistent Gene Activation

Giovanni Carosso[1], Robin Yeo[1], T. Blair Gainous[1], M. Zaki Jawaid[1], Xiao Yang[1], James Y. S. Kim[1], Kavita Jadhav[1], Nina Juan-Sing[1], Siddaraju Valagerehalli Boregowda[1], Vincent Cutillas[1], Lei S. Qi[2], Alexandra Collin de L'Hortet[1], Timothy P. Daley[1], Dan Hart[1]

[1]Epicrispr Biotechnologies, South San Francisco, CA,[2]Stanford University, Stanford, CA

CRISPR-mediated epigenetic gene silencing is a powerful tool with significant therapeutic potential, due to its ability to induce long-lasting changes in gene expression without introducing mutations to genomic DNA. This holds great promise in the field of genetic medicine, potentially allowing for the treatment of complex human diseases. However, the lack of tools for robust and persistent activation of target gene expression remains a significant obstacle to epigenetic editing. To overcome this limitation, at Epic Bio we have established a screening platform to discover novel epigenetic activators. Through this platform, we have discovered multiple Gene Expression Modulation System (GEMS) activators capable of inducing persistent gene activation following transient delivery. In initial studies, GEMS activators containing a vIRF2 core domain (vCD) outperformed dCas-VPR (a fusion of VP16, P65 and Rta) and other potent activators in terms of gene activation potency, robustness, and persistence after transient delivery, in both immortalized and primary cells. This was observed when GEMS were transiently delivered to target genes in multiple cell types and chromatin states (from quiescent to highly expressed). Notably, novel hypercompact activators (64 to 98 amino acids) derived from vCDs demonstrated durable activation of IL-21, an interleukin crucial for enhancing CAR-T cell function, lasting for 41 days after

transient expression in Jurkat cells. These GEMS activators achieved superior potency when compared to a benchmark transcriptional activator VPR. Initial potencies of vCD and VPR were ~730-fold and ~4 fold respectively, indicating that vCD outperforms VPR by a factor of ~177-fold. Throughout this period of gene activation, the vCD maintained an IL-21 activation plateau of ~30-fold, whereas VPR, in contrast, was unable to sustain IL-21 activation beyond 11 days. In hepatocyte HepG2 cells, flow cytometry studies were performed to assess the ability of vCDs to activate LDLR (Fig.1 a), a gene of therapeutic significance as *LDLR* haploinsufficiency constitutes 85 to 90% of genetically confirmed cases of familial hypercholesterolemia. We compared the activation potential of vCDs against VP64 and found that vCDs, but not VP64, were able to activate LDLR to levels approaching those achieved by transfection of an LDLR-encoding ORF cDNA (Fig.1 b). This activation was sustained in HepG2 cells for at least 16 days post-transient plasmid delivery. We then performed a direct comparison between vCDs and the potent activator VPR and demonstrated that vCDs were able to induce LDLR activation that persisted for 70 days post transient delivery in HepG2 cells (Fig.1 c). *In vivo* testing of vCD activators further demonstrated their efficacy and persistence. Modulating the expression of LDLR in primary human hepatocytes within a humanized mouse liver model, we achieved two-fold activation of LDLR, sustained for up to 5 weeks post-single dose administration of vCD GEMS activators encapsulated in lipid nanoparticles (LNPs), far beyond any previous report of *in vivo* CRISPR-mediated epigenetic gene activation (Fig.1 d-g). These results validate proof of concept for CRISPR-mediated persistent gene activation *in vitro* and *in vivo*, showcasing the potential of epigenetic activation of disease-modifying genes *in vivo*.

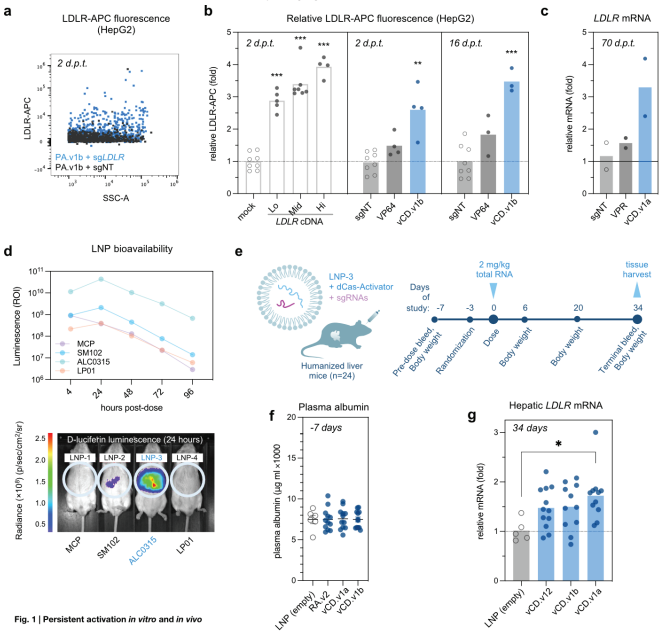


Fig. 1 | Persistent activation *in vitro* and *in vivo*

57. >95% Targeted RNA Editing in Brain with High Durability and Specificity Enabled by Systemic Injection of AAV-Packaged ADAR Guide RNAs

Alison VanSchoiack, Eric M. Chadwick, Yiannis A. Savva, Lina R. Bagepalli, Scott A. Rich, Henry Lee, Susan M. Byrne, Stephen M. Burleigh, Clay M. Patton, Robyn Laing, Robert Lloyd, Palmer Fliss, Ted Gniffke, Jana Henry, Jonathan H. Wang, Crystal G. Pontrello, Leah Helton, Evan E. Kania, Aditya Radhakrishnan, Thomas J. Long, Brian Booth, Yue Jiang, Ronald J. Hause, Melissa Works, Adrian Briggs, David J. Huss

Shape Therapeutics, Inc., Seattle, WA

Programmable RNA editing using guide RNAs (gRNAs) that recruit endogenous adenosine deaminase acting on RNA (ADAR) has great potential as a versatile gene therapy approach, without the need to deliver a protein or protein-encoding transgene. ADAR enzymes recognize double-stranded RNA substrates and deaminate adenosines into inosines. Due to structural similarity, inosines are read as guanosines by the cellular machinery, creating a functional A-to-G base change. RNA editing can be used to correct pathogenic G-to-A mutations, regulate RNA splicing, and modulate protein expression or function. The many molecular applications of RNA editing, combined with the ubiquitous expression of ADAR enzymes throughout all tissues of the body, enable vast therapeutic opportunities. We developed the RNAfix® platform to advance the therapeutic application of programmable RNA editing. RNAfix uses generative machine learning to design gRNAs that hybridize to a target RNA transcript, create an ideal double-stranded RNA substrate for endogenous ADAR recruitment, and drive efficient and specific RNA editing of the target adenosine. RNAfix gRNAs are delivered via AAV for sustained, high level expression that maximizes the durability and therapeutic impact of a single administration. Natural RNA editing is highly active in the central nervous system (CNS) and is critical for neuronal development and synaptic transmission; therefore, we hypothesized that RNAfix would be particularly well-suited to treat CNS disorders. Here we present preclinical data highlighting efficient and specific RNA editing in *in vitro* and *in vivo* disease-relevant CNS model systems. RNAfix gRNAs delivered *in vitro* demonstrate 85-97% target-specific RNA editing in mouse, non-human primate, and human neural cell models. In mouse studies, systemic administration of AAV-PhP.eB-encoded gRNAs at 1e11 viral genomes (vg)/mouse yielded efficient, specific, and durable RNA editing in disease-relevant brain regions from 4 to 16+ weeks. RNA editing was evaluated by Sanger sequencing on microdissected brain tissue, without sorting for gRNA+ cells. At 16 weeks post-treatment, we observed high efficiency editing in the mouse cortex (73%), hippocampus (75%), striatum (79%), substantia nigra (82%), and cerebellum (57%). gRNA expression, measured by ddPCR and *in situ* hybridization, strongly correlated with RNA editing efficiency in each brain region. Increasing the RNAfix AAV-PhP.eB dose to 1e12 vg/mouse increased RNA editing efficiency to 93-96% in bulk cerebrum. Importantly, RNAfix gRNAs are highly specific to the target adenosines of interest with no discernable off-target editing events across the entire transcriptome and no disruption to natural ADAR activity as measured by deep RNA-sequencing. For certain therapeutic targets, RNAfix was harnessed to edit translation

initiation sites (**A**UG>**G**UG) and inhibit protein translation. In both *in vitro* and *in vivo* model systems, RNA editing efficiency strongly correlated with target protein knockdown, highlighting an additional molecular application of the RNAfix platform. Collectively, these data highlight the cross-species conservation of ADAR-mediated RNA editing and provide confidence in the translatability of the RNAfix platform. Furthermore, these results demonstrate the highest *in vivo* CNS editing data reported to date and emphasize the potential of RNAfix to deliver durable, precise, and efficient programmable RNA medicine to the clinic.

58. Durable and Specific Silencing of Therapeutically Relevant Genes Using Epigenetic Editors is Reversible *In Vivo*

Frederic Tremblay, Yesseinia Anglero-Rodriguez, Kenneth Kelly, Shrijal Shah, Gabriel El Sebae, Chih-Wei Ko, Glen Acosta, Christine Waters, Rayman Choo-Wing, Liam Lofgren, Tess Sevetson, Caroline Mugambwa, Jhon Medina, Scott Clarkson, Ricardo N. Ramirez, Erica Hildebrand, Shane Wright, Kendra Bayne, Mary Morrison, Haley Sharp, Gabrielle Mitchell-Lee, Kara Allison, Songlei Liu, Sam Linder, Sahar Abubucker, Ari E. Friedland, Morgan L. Maeder, John Xiong, Vic Myer, Aron Jaffe

Chroma Medicine, Boston, MA

Epigenetic editing is a powerful approach designed to durably silence or activate genes by leveraging nature's endogenous cellular mechanism for gene regulation without the inherent genotoxic risks of other editing approaches that nick or cut the DNA. Our epigenetic editors have a distinct mechanistic advantage by utilizing DNA methylation and demethylation to silence or activate genes, respectively, a targeted approach for regulation of gene expression rather than indirect regulation through cutting or nicking the DNA, which can cause unintended chromosomal effects. We have identified epigenetic editors (EEs) that have demonstrated efficient silencing activity against human hepatitis B virus (HBV) and PCSK9 in primary human hepatocytes (PHH) and mice. To characterize the specificity of epigenetic editing, we profiled PHHs treated with either HBV-EE or PCSK9-EE by RNAseq, methylation array, and whole-genome methylation sequencing. No statistically significant differentially expressed off-target genes or methylated regions were identified, confirming the specificity of our EEs in PHHs. We then evaluated the durability of epigenetic editing in transgenic mice expressing HBV and showed potent reduction (>2-log) in circulating HBsAg with durability out to 5 months. Similarly, transgenic mice carrying the human PCSK9 genomic locus treated with PCSK9-EE showed >98% reduction in circulating PCSK9 over 300 days. In addition, we confirmed that the efficient PCSK9 silencing observed after treatment with PCSK9-EE was fully maintained following a 2/3 partial hepatectomy, an established model of liver regeneration. Furthermore, targeted CpG methylation at the PCSK9 promoter region was highly correlated with PCSK9 expression and faithfully maintained in the regenerated liver of partially hepatectomized mice. Finally, we explored the reversibility of epigenetic editing in vivo by using an epigenetic activator that was identified for its ability to restore PCSK9 expression in previously silenced cells in vitro via targeted CpG demethylation. Mice that had been previously silenced with PCSK9-EE were treated with a PCSK9 epigenetic activator, resulting in a near-complete restoration of PCSK9 levels to baseline levels within 14 days after treatment and maintained for at least 56 days. Taken together, we believe our data further support the utility of epigenetic editors to specifically, durably and reversibly silence therapeutically-relevant target genes in the liver without altering the DNA sequence.

59. Predicting CRISPR-Cas13 On-Target Efficiencies and Intrinsic RNA Off-Targets

Xiaolong Cheng[1], Zexu Li[2], Zihan Li[2], Teng Fei[2], Wei Li[1]

[1]Center for Genetic Medicine Research, Children's National Hospital, Washington, DC,[2]Northeastern University, Shenyang, China

CRISPR-Cas13 systems have emerged as versatile toolkits for RNA-related applications. However, mechanisms governing their on-target efficiencies and specificities are still not clearly known. Here we present two studies to understand and predict Cas13 efficiencies and off-target activities. In the first study, we conducted a systematic evaluation of several popular Cas13family effectors delivered via lentiviral vectors. Surprisingly, we uncovered distinct defects and characteristics among these systems. By employing RNA immunoprecipitation sequencing, transcriptome profiling, biochemistry analysis, high-throughput CRISPR-Cas13 screening, and machine learning techniques, we elucidated novel mechanisms through which Cas13 targets intrinsic RNA in mammalian cells, which differ from known RNA targeting activities of Cas13. Unlike traditional cis/trans effects, we discovered that intrinsic RNA cleavage mediated by Cas13 can exhibit crRNA-independent, transcript-specific, and dynamically tunable properties based on the various states of Cas13 nucleases. Furthermore, we identified host genes involved in viral processes that are intrinsically targeted by Cas13, contributing to lentiviral defects observed in our experiments. In the second study, we employ deep learning methods to accurately predict Cas13d guide-dependent on-target and off-target effects. We perform CRISPR-Cas13d proliferation screens and design a deep learning model, named DeepCas13, to predict the on-target activity from guide sequences and secondary structures. DeepCas13 outperforms existing methods to predict the efficiency of guides targeting both protein-coding and noncoding RNAs. Guides targeting non-essential genes display off-target viability effects, which are closely related to their on-target efficiencies. Choosing proper negative control guides during normalization mitigates the associated false positives in proliferation screens. We apply DeepCas13 to the guides targeting lncRNAs, and identify lncRNAs that affect cell viability and proliferation in multiple cell lines. The higher prediction accuracy of DeepCas13 over existing methods is extensively confirmed via a secondary CRISPR-Cas13d screen and quantitative RT-PCR experiments. DeepCas13 is freely accessible via http://deepcas13.weililab.org.

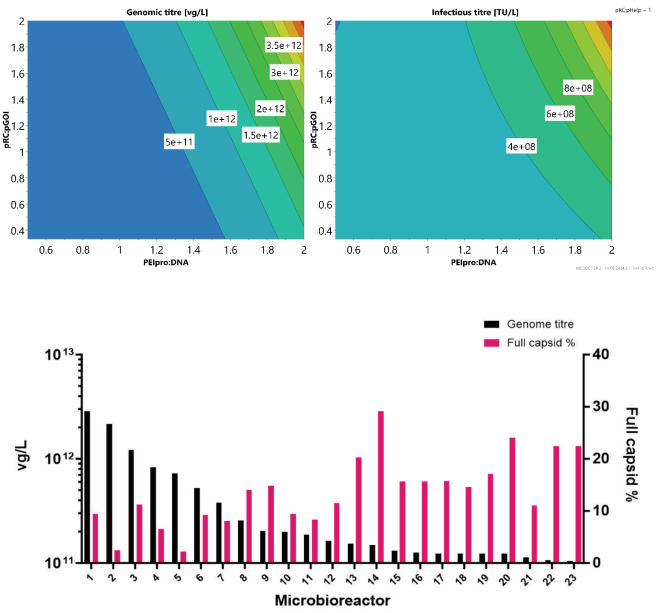
AAV Manufacturing II

60. pAAVing the Way to High Titres: Plasmid Ratio Optimisation for AAV Production Utilising the Ambr 15 Stirred-Tank Bioreactor

Sandeep Mangrati[1], Érica Schulze[2], Ulrike Jung[3], Stefano Boi[3], Sven Mathias[3], Yi-Hsuan Huang[3], Qasim Rafiq[1]

[1]Department of Biochemical Engineering, University College London, London, United Kingdom,[2]Sartorius Stedim Biotech GmbH, Goettingen, Germany,[3]Sartorius Stedim Cellca GmbH, Ulm, Germany

Background. Stirred-tank bioreactors (STBs) are the most promising candidates for large-scale rAAV vector manufacturing. However, rAAV bioprocess optimisation in bench-top STBs is challenging due to prohibitively high costs and resources. This is of particular concern when optimising plasmid ratios (pGOI, pRC, and pHelper plasmids) which requires running many vessels. Here, we investigated the impact of plasmid ratios on titres and full capsid percentages of an AAV2 vector, incorporating novel pAAV-GFP and pRC2 plasmids. In the literature, such optimisation has often been limited to shake flasks, however herein we utilised the Ambr® 15 high throughput microscale STB which has proven scalability and is more commercially relevant. **Method.** A design-of-experiment (DOE) was performed in MODDE® to evaluate a wide range of plasmid ratios (pRC:pGOI and pRC:pHelper) under PEIpro® and FectoVIR®-AAV transfection reagents. We selected a multilevel full factorial approach incorporating 83 microbioreactors using the Ambr® 15, a high throughput STB system with 48 microbioreactors (15 mL). Crude lysates were used to determine genomic, capsid, and infectious titres. **Results and conclusions.** We observed that increasing pRC:pGOI significantly improved genomic and infectious titres. Under PEIpro®, increasing pRC:pGOI from 0.33:1 to 2:1 improved genomic and infectious titres approximately 3-fold. By contrast, the effect of pRC:pHelper on titres was not significant. We also observed that when both pRC:pHelper and PEIpro:DNA were at low (or high) setpoints, full capsid percentage was negatively impacted. And notably, the standard 1:1:1 ratio resulted in infectivity below the limit-of-detection indicating the importance of plasmid ratio optimisation to evaluate functionality of new rAAV vectors. Under FectoVIR®-AAV transfection, there was a significant negative correlation between genomic titres and full capsid percentage, likely because the plasmid ratios had opposite effects on genomic titres and full capsid percentages (increasing pRC:pGOI improved genomic titres but lowered full capsid percentages). This indicated that a "sweet spot" may be needed and that it could be achieved by simply adjusting the plasmid ratios. Currently, the main challenge in AAV gene therapy is efficient and cost-effective production of rAAV vectors. This study demonstrated the applicability of the Ambr® 15 system in conjunction with a DOE approach for improving rAAV production and it highlighted the importance of plasmid ratio optimisation on CQAs. Such bioprocess improvements are important for ensuring successful commercialisation of rAAV drugs.



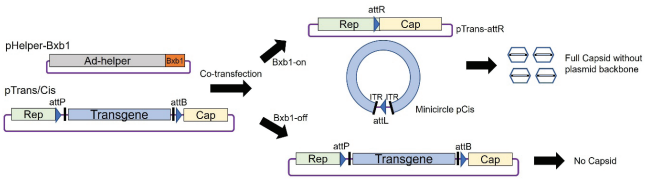
61. Producing High-Purity rAAV Vectors by Recombination-Dependent Minicircle Dual Transfection

Hao Liu[1], Lingzhi Ren[1], Nan Liu[1], Chen Zhou[1], Guangping Gao[1,2], Dan Wang[1,3]

[1]Horae Gene Therapy Center, University of Massachusetts Chan Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, University of Massachusetts Chan Medical School, Worcester, MA,[3]RNA Therapeutics Institute, University of Massachusetts Chan Medical School, Worcester, MA

Triple transfection of HEK293 cells is the most widely used method for producing recombinant adeno-associated virus (rAAV), a leading vector for human gene therapy. It involves three plasmids co-transfected to cells at roughly equal molar or mass ratio: a helper plasmid that delivers adenoviral helper genes (pHelper), a trans plasmid that expresses AAV rep and cap genes (pTrans), and a cis plasmid that harbors a therapeutic transgene cassette flanked by AAV inverted terminal repeats (pCis). Depside its tremendous success, this method still faces many challenges. One important concern is vector impurity, including plasmid backbone encapsidation that can not be removed by downstream processing and empty capsid that compromises vector potency. Here we developed a recombination-dependent minicircle dual transfection method that reduces the backbone DNA encapsidation by 20-40 folds and improves full capsid ratio by 2-3 folds compared with triple transfection, while generating comparable rAAV yield with reduced plasmid demand in the upstream rAAV production stage. This methodology consists of two plasmids, pHelper-Bxb1 that provides adenoviral helper genes and the recombinase Bxb1, and pTrans/Cis with the attP- and attB-flanked cis element inserted into the C-terminus of Rep in pTrans plasmid (See Figure). When pHelper-Bxb1 and pTrans/Cis are co-transfected into HEK293 cells, Bxb1 catalyzes the recombination between attP and attB sites in pTrans/Cis, generating a functional pTrans-attR that expresses Rep and Cap proteins, and a minicircle pCis that contains

only ITR-flanked transgene without any plasmid backbone sequences, which serves as the template for further vector genome amplification. In this way, the packaged AAV vectors are devoid of plasmid backbone DNA. When HEK293 cells receive only pTrans/Cis, the ITR-flanked transgene cassette embedded in the Rep gene serves as a disrupting insertion that abolishes Cap expression, thus avoiding empty capsid formation. Using EGFP as a model transgene, we demonstrated that this newly developed dual transfection method dramatically improved vector purity compared with triple transfection: (1) it reduced bacterial backbone encapsidation from 5.2% to 0.14% for rAAV2, and from 3.3% to 0.16% for rAAV9; (2) the full capsid ratios in crude lysate, as determined by ddPCR of genome titer normalized to ELISA capsid titer, increased from 11% to 23% for rAAV2, and from 14% to 46% for rAAV9. Mechanistically, we found that the asynchronous presence of pTrans and pCis contributes to high empty capsid levels in triple transfection. Intracellular genetic coupling of pTrans and pCis in triple transfection improves the full capsid ratio to similar levels of recombination-dependent minicircle dual transfection. Altogether, these results demonstrate that recombination-dependent minicircle dual transfection serves as a cost-saving and easy-to-implement manufacturing method that produces rAAV with significantly improved vector purity. In addition, it highlights the importance of spatial synchronization of pTrans and pCis plasmids in mitigating empty capsid formation. *G.G. and D.W. are corresponding authors.*



62. Elucidating the Function of Adenovirus Late Gene Products and Impact on AAV Production

Hao Pan, Qingliang Li, Huimin Na, Karen A. Vincent, Yingchao Nie

Predevelopment, Genomic Medicine Unit CMC, Sanofi, Waltham, MA

Recombinant adeno-associated viral vectors (rAAV) are being developed for a wide variety of gene therapy applications, but efficient production at large scale remains challenging. The producer cell line (PCL) platform is an economical and scalable approach for rAAV manufacturing. AAV Rep/Cap genes and gene of interest (GOI) elements are stably integrated in the producer cell line, and rAAV production is induced by infection with a wild-type, replication-competent Adenovirus (wtAd5). The adenoviral helper function in AAV production has been studied extensively, however, the interplay of the AAV and wtAd5 genomes is not yet fully understood. To better understand the helper function provided by wtAd5 in the PCL platform, we infected a PCL with a variant Adenovirus serotype 5 virus (Ad5ts149), which harbors a temperature-sensitive mutation in the Ad5 polymerase gene E2B. Suppression of Ad5ts149 replication and packaging at the restrictive temperature (39°C) resulted in a 3-to-50-fold reduction in rAAV production compared to that of wtAd5. However, replication of the AAV genome was comparable or higher in Ad5ts149-infected cells. AAV Rep/Cap protein expression was analyzed to assess the underlying mechanism. An altered Rep expression pattern was detected, accompanied by reduced expression of Cap protein. Orthogonal methods were employed to further understand transcription of AAV and adenoviral genes in the context of an Ad5ts149 infection at 39°C. Analysis of Ad5ts149 gene expression via RT-qPCR showed that expression of the minimal essential Ad helper genes were similar to wtAd5 while the expression of the Ad late gene products was decreased. Hypotheses were tested via siRNA knockdown in conjunction with wtAd5 infection to gain insight on the role of Ad5 late gene products. The outcomes reveal a novel role of an Ad5 late gene product in the regulation of AAV rep/cap gene expression and efficient production of rAAV in Sanofi's AAV producer cell line platform.

63. Process Development of HEK293-Based rAAV2 Manufacturing Platform

Alexander Burns

Bioprocessing, Keck Graduate Institute, Claremont, CA

The Adeno-associated virus (AAV) has been successfully used as a viral vector for gene therapy in its recombinant form, as seen by the relative commercial successes of AAV-based gene therapy products like Luxturna® and Zolgensma®. Nonetheless, these gene therapy products are significantly expensive, thus, making them relatively inaccessible to many patients who wish to get the therapy but choose not to get it due to financial reasons. One of the main reasons for the high cost of these therapies, besides the R&D expenditures that were necessary to get the therapy to commercialize, is the Cost-of-Goods-Sold (COGS) for manufacturing these therapies; especially the raw materials and labor to manufacture these therapies. There exist various technical gaps in the manufacturing and quality control of recombinant AAV (rAAV) particles that make these processes more challenging. A classic method for the production of rAAV particles is the triple transient (TT) transfection of HEK293 cells, where three vector DNA plasmids are required to be transfected in a HEK293 cell simultaneously to deliver all the necessary genes to the host cell to produce rAAV particles. Due to the inherently low efficiency of the transfection technique, vector DNA plasmids are not used effectively for rAAV production. Also, during anion-exchange chromatography, a chromatographic technique for AAV full-capsid enrichment, there is an inherently low resolution between full and empty AAV capsids; empty capsids are a product-related impurity of rAAV bioprocessing. The low chromatographic resolution makes it difficult to identify and quantify the empty and full AAV capsids, which adds to the challenge of quality control of rAAV products. Also, AAV particles are prone to aggregation during downstream and formulation bioprocessing due to the complex protein structure of AAV capsids; aggregation amount is a critical quality attribute (CQA) of rAAV as a final product for gene therapy. Thus, aggregation adds to the challenges of rAAV downstream and formulation bioprocessing. To improve the transfection efficiency of the TT transfection method of HEK239-based rAAV2 production, we have results of a significant increase in full-capsid AAV2 titer from TT transfection of HEK293 cells to produce AAV2 capsids, by exposing the cells to DMSO during the standard transfection protocol. To improve the resolution between empty and full AAV2 capsids in anion-exchange chromatography, an elution method of AAV2 capsids in anion-exchange HPLC (AAV2 AEX HPLC) was developed. Development of the AAV2 AEX HPLC elution method was

achieved by optimizing the loading conditions of the AAV2 sample to be loaded into the AEX HPLC column. To minimize aggregation of AAV2 capsids in solution, the effect of a combination of different types of salts and sugars was studied and an optimum combination of salt and sugar was found to minimize AAV2 capsid aggregation the most. Cryo-preservative agents (CPAs), such as DMSO, glycerol and certain sugars, normally used to extend the shelf-life and freeze-thaw cycles of cells and proteins, can also serve as transport modulators of macromolecules, such as proteins and nucleic acids, without affecting their structure and function. Thus, harnessing the full power of CPAs can help alleviate some of the technical gaps mentioned earlier for rAAV manufacturing and quality control. The following work will present how CPAs can be used for the process development of HEK293-based rAAV2 manufacturing platform; a gold-standard platform for rAAV manufacturing for gene therapy applications.

64. Transforming AAV Vector Production with the TruStableTM AAV Producer Cell Line

Sandhya Pande, Corey Johnson, Carla Villela, Tina Bi, Chris Abeles, Blake Engelbert, Ramsey Saleem, Ken Prentice

Shape Tx, Seattle, WA

We have developed the TruStable cell line, a serum-free suspension adapted human cell line, which is genetically engineered to incorporate all elements needed for stable recombinant adeno-associated virus (rAAV) production, enabling seamless scalability. At the heart of this system lies an inducible, recombination-based production circuit that silences cytotoxic viral elements during the preliminary expansion phase of the cells, subsequently enabling a simple activation for viral production when optimal culture conditions are attained. The development of stable producer cell lines marks a paradigm shift in the manufacture of AAV vectors as it circumvents transient transfection limitations (e.g. scale-up limitations, challenging supply chain). Further enhancing this system, we have introduced successive iterations of the core molecular circuit. We substituted native AAV P5, P19, and P40 promoters with alternative heterologous promoters while maintaining the overlapping genetic architecture of the Rep open reading frame. This allowed us to precisely adjust the expression levels of rep and capsid proteins to enhance AAV titer and quality while keeping a simplified plasmid design. These strategic modifications have culminated in the creation of highly productive polyclonal pools, exhibiting exceptional packaging efficiency. The result is a significant increase in volumetric titers and encapsidated genomes (~8e14 vg/L and ~70%, respectively) as measured from crude cell extracts. Additionally, monoclonal cell lines derived from these polyclonal pools exhibit exceptional productivity, routinely exceeding 100,000 viral genomes/cell. Our custom biphasic upstream process for TruStable cell lines enhances AAV vector production, merging a high cell density cell growth phase with a rAAV-synthesizing production phase. Advanced fed-batch and perfusion methods have fine-tuned N-1 seed train for maximum cell viability. After several rounds of screening, we've selected chemically defined media that bolster high AAV titers, with the process proven effective in 3L benchtop and scaled up in 10L bioreactors. This innovative approach — combining sophisticated molecular circuits, selective clone development, and a scalable suspension protocol — substantially improves upon prior rAAV production methods. TruStable lines are adept at packaging varied payloads across AAV serotypes, reliably yielding high-titer clones to accommodate manufacturing needs from small to large scales.

65. Developability Assessment of Novel AAV Capsids and Payloads at Early Preclinical Stage to Enable Development of Gene Therapies

Matteo P. Placidi[1], Himabindu Ramachandrareddy[2], Robert Vass[3], David Alvarez[1], Kumar Dhanasekaran[1]

[1]TechOps, Voyager Therapeutics, Lexington, MA,[2]Voyager Therapeutics, Inc. - Cambridge, MA, Sharon, MA,[3]Voyager Therapeutics, Lexington, MA

Developability assessments, similar to those performed on antibodies, refer to the likelihood that an adeno-associated virus (AAV) development candidate can be advanced towards the clinic by being a manufacturable, safe and efficacious drug. These attributes of clinical grade AAV material manufacturing are well established through the requirements of drug product safety, identity, strength, purity and quality (SISPQ) to enable release for use in the clinic. However, developability refers to the likelihood that an AAV development candidate will go smoothly through the chemistry, manufacturing and control (CMC) process. For this reason, it is important to consider this as part of a development candidate nomination criteria. Traditionally, preclinical studies, leading up to the nomination of a development candidate, involve very limited analytical testing or characterization. This delay in the application of advanced analytics can lead to issues that must be resolved in tandem with the necessary process development activities, impacting CMC development timelines and ultimately time to clinic. In this work we outline analytical methods that can be utilized early in assessing several critical quality attributes that may have an impact on developability. Here, the payload concentration and sequence, capsid occupancy, integrity, sequence, aggregation state and functional output were examined using multiple techniques to provide a holistic view of the different candidates during early development. The detailed analytical characterization approach distinguished key methods able to provide early indicators of loss of structural integrity. Specific assays indicated the impacts of different capsid/transgene combinations and storage conditions. When examined in conjunction with in vivo data, these methods should be carefully monitored due to their potential impact on performance in animal studies.

66. Comparative Analysis of Sterile Grade Filters in Adeno-Associated Virus (AAV) Manufacturing: Accelerating First-in-Human AAV Therapeutic Production with Quality by Design Principles

Morven McAlister[1], Alexander Lambropoulos[2], Limin Wang[3], Andreia Domingues[4], Melanie Sorensen[4], Angela N. Johnson[5,6]

[1]Global Scientific & Validation Services, Cytiva, New York, NY, MA,[2]Bioprocessing, Cytiva, Boston, MA,[3]Global Regulatory Science, Cytiva, Marlborough, MA,[4]Global Regulatory Sciences, Cytiva, Boston, MA,[5]Global Regulatory, Cytiva, Boston, MA,[6]School of Professional Studies, Regulatory Sciences, Northeastern University, Boston, MA

Background: Commercial sterile grade filters are important in producing adeno-associated virus (AAV) vector gene therapies, yet performance of filters in this context varies widely. As AAVs become more attractive for their ability to elicit robust and durable transgene expression, we have increased need to optimize transition from bench to production scale manufacturing. Identifying optimal sterilizing grade filters early in process development is essential to efficient and cost-effective manufacturing to meet clinical demand. **Methods:** The objective of this study was to evaluate commercial sterile filter characteristics in terms of performance at bench vs production scale, providing insights into early decision-making on process development and filter selection to enable first-in-human AAV therapeutic production. Applying a Quality by Design (QbD) framework, laboratory tests were completed at bench and production scale to assess the impact of sterilizing grade filter clarification (post-depth filtration; n = 20) and final sterile filtration of drug product (n = 32). Data were analyzed on filter type, differential pressure, throughput, and capacity. Filterability trials were conducted post-depth filtration on typical feed stream and AAV titers determined by ddPCR. **Results:** Differential pressure was a robust critical quality parameter (CPP) with minimal impact on AAV titer as a critical quality attribute (CQA; **Fig. 1**), and other AAV characteristics also impacted post-depth filtration and clarification. The resulting model (**Fig. 2**) is useful in selection of filters performing at bench scale and production scale, while considering AAV product characteristics that significantly correlated with filter performance, including cell line, suspension versus adherent, total cell density, viability after lysis, turbidity of harvest lysis, lysing agent & hold time, endonuclease agent / hold time, additives (e.g., NaCl, AAV serotype, titer, and filtration flux. **Conclusions:** This demonstrates the impact of selecting appropriate filters during bench development, considering process type, target removal and transport product. By employing QbD principles systematically, these results suggest we can improve the transition from bench to production scale AAC therapeutics via careful section of optimal filtration components, thereby contributing to reduced time and cost burden of manufacturing gene therapy drug candidates for the clinic.

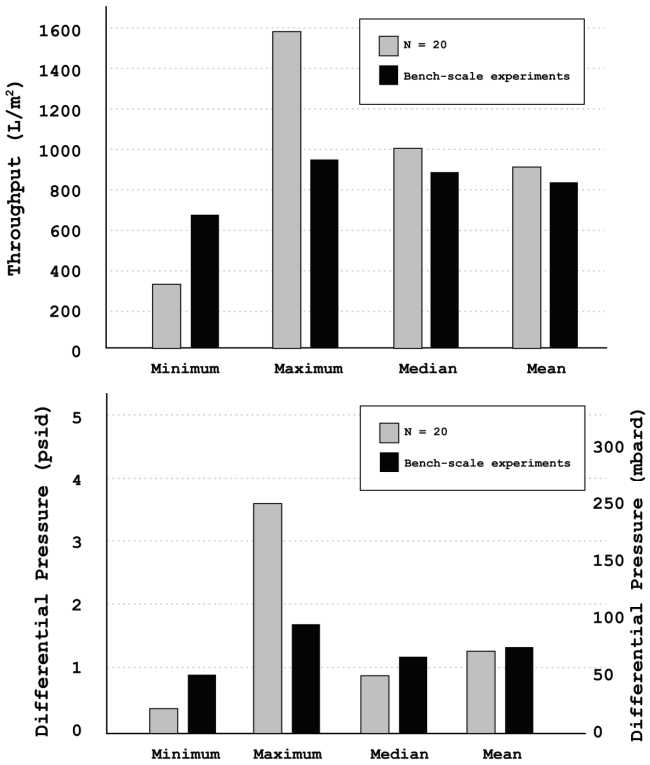


Figure 1 Impact of Sterilizing Grade Filter Critical Process Parameter (CPPs)/Critical Material Attribute (CMAs) on Clarification by Post-Depth Filtration

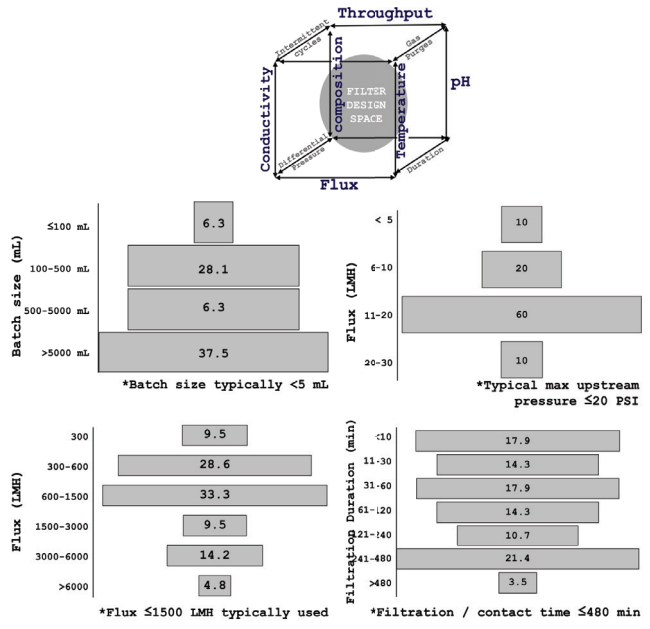


Figure 2 Impact of Analysis of Filter Validation Studies (n = 32)
Note: #s refer to % breakdown

AAV Vector Biology and Development I

67. Novel AAV8 and AAVrh39 ITRs and ITR-Proximal Regions Significantly Enhance Transgene Expression through Enhancer/Promoter-Like Activities

Suk Namkung[1], Elisabet Mandon[1,2], Jialing Yang Liang[1], Mengtian Cui[1], Fang Zhang[1], Jackson McGowan[1], Mitchell Yip[1], Thomas Leland[1], Sophia Liu[1], Tapan Sharma[1], Dan Wang[1], Guangping Gao[1], Phillip Tai[1]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]RNA Therapeutics Institute, UMass Chan Medical School, Worcester, MA

Advancements in adeno-associated virus (AAV)-based gene therapy engineering have greatly increased within the past decade. These trends have been mainly within the scopes of capsid engineering and regulatory cassette design. However, there has been an underwhelming amount of research focused on exploring genetic elements of wildtype AAV and their roles in transcription. Previous research has demonstrated that inverted terminal repeats (ITRs), the last viral elements in AAV vectors, have promoter-like activities. Furthermore, ITR-proximal regions (IPRs) at the 3' ends of wildtype AAV genomes also show tissue-dependent enhancer-like activities. These results have shown the potential for native elements within the AAV genome to further improve gene therapy vectors. To study the different contributions of AAV genetic elements towards vector transduction, we tested the ITRs and IPRs of AAV8 and AAVrh.39 for their potential to act as enhancers and/or promoters *in vitro* and *in vivo*. As previously reported, we found that the ITRs of AAV2, AAV8, and AAVrh.39 (ITR2, ITR8, and ITRrh.39 respectively) have very weak promoter-like activities on their own. However, we found that the IPRs of AAV8 and AAVrh.39 (IPR8 and IPRrh.39, respectively) have a stronger impact on transcription, conferring four-fold greater activity than achieved by the human phosphoglycerate kinase (*hPGK*) promoter. In addition, while AAV2's IPR (IPR2) only showed liver-specific activity, IPRrh39 was equally active in HEK293 and cultured hepatocytes. We next produced vectors carrying ITRs and IPRs from AAV8 or AAVrh.39. Vector production and sequencing of the vectors by third-generation sequencing showed that the these elements are fully compatible with standard triple-plasmid transfection production schemes using Rep proteins from AAV2. Surprisingly, we found that AAV vectors carrying ITR8 and ITRrh.39 showed a >10-fold enhancement in mouse liver transduction over vectors that carried conventional ITR2. We are currently working toward understanding the mechanisms that underpin ITR and IPR function. In conclusion, we demonstrate that there are still facets of AAV biology that remain to be uncovered and further studied in order to improve gene therapy vectors. G.G. and P.T. are corresponding authors

68. A Genome-Wide CRISPR/Cas9 Screen Identifies an Essential Role of SLC35A1 in rAAV Transduction

Xiujuan Zhang[1], Kang Ning[1], Siyuan Hao[1], Zehua Feng[2], Cagla Aksu Kuz[1], Shane McFarlin[1], Donovan G. Richart[1], Fang Cheng[1], Ziying Yan[2], Jianming Qiu[1]

[1]University of Kansas Medical Center, Kansas City, KS,[2]University of Iowa, Iowa City, IA

Recombinant Adeno-associated virus (rAAV) has become an important tool for delivering genetic material into cells, offering potential treatments for a variety of genetic disorders. Despite its significance, the mechanisms underlying rAAV transduction are not fully understood. GPR108 has been identified as an essential factor for the transduction of various rAAV vectors, excluding rAAV5. This dependence arises from the interaction between GPR108 and the N-terminal unique region of the large capsid protein (VP1u). Notably, VP1u region of AAV5 is divergent from that of other serotypes. To identify the essential host factors that are used by AAV5, we conducted a genome-wide CRISPR/Cas9 screen in suspension 293F cells transduced with rAAV5, employing the Brunello gRNA library. Following two round screens, the highly selected genes included the previously reported genes, *KIAA039L*, *TM9SF2*, and *RNF121*, along with a cluster of genes involved in glycan biogenesis. In this report, we focused on solute carrier family 35 member A1 (*SLC35A1*), a Golgi apparatus-localized CMP-sialic acid transporter, as AAV5 utilizes 2,3-linked sialic acid as a primary attachment receptor. Our study verified that knockout of *SLC35A1* reduced rAAV5 transduction to a level similar to that in *KIAA0319L* and *TM9SF2* knockout cells. Importantly, although *SLC35A1* knockout abolished 2,6-linked sialic acid expression on the cell surface, as detected by specific sialic acid-binding lections, the expression of 2,3-linked sialic acid, as well as rAAV5 binding to cells and internalization, was not affected. Moreover, the *SLC35A1* knockout significantly diminished the transduction of other rAAV serotypes, AAV2, AAV3B, and AAV13, which were previously reported not to use sialic acid for entry, suggesting that SLC35A1 plays a critical role in rAAV transduction after vector internalization. Overall, our study suggests that SLC35A1 serves as a crucial host factor facilitating the transduction of various AAV vectors beyond the initial steps of vector binding and endocytosis. This discovery unveils an unanticipated role of the CMP-sialic acid transporter in rAAV transduction, involving intracellular trafficking of vectors after internalization into the host cell.

69. Screening for Cellular Factors Underlying Mechanisms of AAV Tropism

Hagoon Jang[1], Qianhui Du[1], Mark A. Kay[2]

[1]Stanford University, Stanford, CA,[2]Stanford University School of Medicine, Stanford, CA

Understanding the intricate interplay between the AAV capsid and host cellular factors is crucial for optimizing AAV efficiency and achieving precise targeting. Our research group previously engineered the LK03 AAV capsid, demonstrating robust transduction in human liver but poor efficacy in rodent liver (Lisowski 2014). Further work established that differential transduction was not due to vector entry and nuclear uptake but rather related to the concentration of active epigenetic modifications, including histone acetylation (H3K27ac),

on the LK03 AAV genome in a species-dependent manner (Gonzalez-Sandoval 2023). Our studies went on to establish that the capsid protein can influence the epigenetic state of the vector genome in a species-dependent manner.To unravel the cellular key factors influencing LK03's epigenetic regulation, we employed two distinct approaches. Firstly, we established a Hepa1-6 mouse hepatoma cell line expressing the dCas9-p300 protein, designed to enhance site-specific histone acetylation guided by AAV genome-targeting guide RNAs. Following LK03-luciferase and DJ-luciferase transduction in Hepa1-6 dCas9-p300 stable cells, we introduced guide RNAs targeting the AAV genome promoter, gene body, and 3' region. Importantly, targeting the promoter region increased luciferase activity only in LK03 transduced cells and not in DJ transduced cells, indicating potential associations with histone acetylation events specifically linked to the AAV-LK03 capsid-delivered genomes.Secondly, we utilized histone deacetylase (HDAC) inhibitors (Panobinostat and Trichostatin A) to modulate the histone modifications in a non-sequence-specific manner. In this case, both LK03 and DJ-delivered genomes exhibited a two-fold increase in luciferase activity.We are currently determining the specific histone modifications associated with each of the dCas9-p300 guide RNA and HDAC-treated cells transduced with AAV-LK03 and AAV-DJ. Through these comprehensive approaches, our goal is to unravel the underlying molecular mechanisms driving the variation in LK03 AAV tropism between mouse and human hepatoma cells. The data here further supports a direct causal role linking the AAV capsid protein to histone modifications placed on the vector genome that ultimately causes differential transduction efficiencies observed with different capsids between different species and cell types.

70. Consequences of Human Genetic Variations of the Adeno-Associated Virus Receptor (AAVR) Gene (KIAA0319L) on AAV-Mediated Gene Transfer

Jenifer M. Vasquez, Hailey C. Lephart, Caner Günaydin, Mahboubeh R. Rostami, Philip L. Leopold, Ronald G. Crystal

Genetic Medicine, Weill Cornell Medical College, New York, NY

Vectors based on adeno-associated virus (AAV) mediate persistent gene expression with relatively low toxicity making them ideal vehicles for gene therapy. However, patients receiving identical doses of vector scaled to body weight yield variable levels of the therapeutic protein. Based on our prior report (Rostami et al. Mol Ther Methods Clin Dev 2023; 31:101136) of more than 69,000 human genetic variants among more than 60 genes encoding proteins that influence AAV expression levels, we hypothesized that some of these naturally occurring genetic variants are determential, mediating less receptor number and/or function, thus modifying the trafficking of AAV vectors, resulting in variable expression of the expressed gene. As an initial test of this hypothesis, we focused on AAVR, a cell surface receptor that mediates the initial steps in intracellular trafficking for most AAV serotypes . We assessed 10 naturally occurring human mutations of AAVR predicted to have deleterious effects on AAVR structure and function. HeLa-AAVR-knockout (HeLa-KO) cells were transfected with either wild-type human AAVR or one of the AAVR genetic variants. Following transfection, cells were modified with 1 of 6 different AAVR-dependent AAV serotype vectors (2, 5, 8, 9, and rh.10, representing 4 different AAV clades) coding for the nano-luciferase reporter gene. Luciferease expression levels were quantified 3 days post-infection. Four of the 10 variants encoding AAVR frameshift mutations mediated significant reduction in gene transfer for all serotypes (all $p<10^{-3}$). The 6 missense variants in AAVR also affected AAV-mediated gene expression, but did so differently depending on the AAVR domain containing the human genetic variant and the AAV serotype. For example, gene expression to serotypes in closely related clades E and F (AAV8, 9, and rh.10) were significantly decreased (all $p<10^{-3}$) by human variants in the N-terminal domain (Lys3Thr), PKD2 domain (Val440Ile), and in C-terminal domain (Lys959Glu, Gly1022Arg, and Ser1031Phe). In contrast, gene expression mediated by serotypes in clade E and F were not affected by a human mutation in the PDK3 domain (Ala563Val). More distantly related serotype AAV2 (clade B) was also mildly sensitive to Lys3Thr ($p<0.05$) but gene transfer was not affected by the variants in PKD2, PKD3 or C-terminal regions. AAV5, a serotype not assigned a clade, was distinct from other serotypes in that it was not sensitive to the human N-terminal, PKD2, or PKD3 variants but showed elevated gene expression in the context of C-terminal variants, Gly1022Arg and Ser1031Phe (both $p<10^{-2}$). In conclusion, naturally-occurring human genetic variations in AAVR influence the efficiency of AAV-mediated gene transfer and may be a factor in the variability that is observed when gene therapy treatments are administered to patients in clinical trials.

71. Epigenetic Blueprint: Understanding How the AAV Capsid Influences the Vector Epigenome

Eirini Vamva[1], Katja Pekrun[2], Yuqian Jiang[3], Adriana Gonzalez[4], Ningyang Gu[5], David A. Dean[5], Mark A. Kay[6]

[1]Genetics, Stanford University Laboratory for Cell & Gene Medicine, Palo Alto, CA,[2]Stanford University, Stanford, CA,[3]Stanford, Palo Alto, CA,[4]Stanford University, Burlingame, CA,[5]University of Rochester, Rochester, NY,[6]Stanford University School of Medicine, Stanford, CA

We recently showed that the AAV capsid plays a role in establishing the AAV-epigenome(Gonzalez-Sandoval, 2023). The AAV-LK03 capsid, which shows robust transduction in non-human primates and humans (Lindsey 2021), does not transduce rodents well. Insertion of a single Glycine into position 266 of the primate specific LK03 capsid, (266iG) named AAV-AM, changed the vector properties allowing efficient transduction in both mice and human cells. We found that vector-mediated transgene expression was unrelated to cell entry, nuclear transport, and double-strand vector DNA formation but related to the concentration of activating histone H3 epigenetic modifications (H3K4me3 and H3K27ac) present on the vector genome itself. We set out to understand how the different capsids interact with host proteins in the nucleus to set up the vector DNA epigenome. First, we performed direct intranuclear and intracellular AAV injections to study potential spatial differences in nuclear localization between the AAV-LK03 and AAV-AM delivered genomes and their effect on transgene expression. We discovered that intranuclear, but not intracytoplasmic vector injections led to successful transduction of mouse and human hepatoma cell lines. The intranuclear AAV-AM and AAV-LK03 injections led to transgene expression that followed the same pattern observed in natural transductions, supporting the notion that post nuclear entry events are responsible for setting up the vector

epigenome. Trans-complementation studies revealed that the effect of the AAV capsid on the vector epigenome occurs in *cis*. Changing and/or inserting various amino acids into the 260-270 hypervariable region of LK03 and AM revealed that maintaining a glycine in position 266 was the most important parameter to maintain pan-species transduction. To test this further, we found that insertion of a glycine (266iG) into AAV-NP59, another primate selective capsid, enhanced the capsid's ability to transduce both rodents and primates. Structural predictions suggest that this additional glycine in position 266 allows for a more flexible local structure that could have an influence on interactions between the capsid and the AAV genome and/or interactions of the vector genome with key host factor(s). To identify host factors that interact with the capsid and/or vector genome and are involved in this process, we performed anti-A20 immunoprecipitation and mass spectometry, followed by evaluation of candidate proteins on AAV-LK03 and AM transduction efficiency with siRNA silencing. Our siRNA screen identified the RPA complex as a novel host restriction factor critical for vector epigenome modifications. Using both siRNA technology and two small molecule RPA inhibitors, we showed that, RPA efficiently inhibited AAV-LK03 transduction in mouse but not in human hepatoma cell lines. Further analysis demonstrated that improvement of transduction efficiency facilitated by RPA inhibition was unrelated to cell entry but rather due to increased transgene mRNA expression. We are currently investigating the effect of RPA inhibition on the histone epigenetic modifications of the rAAV genome itself as well as the nature of the interaction between RPA and the rAAV genome. Our study supports the idea that the capsid sequence plays a major role in transgene expression by interacting with host nuclear proteins and by influencing the modified histone marks that bind to the vector genome. This mechanism may explain the variation in AAV-mediated transduction between species, tissues, and dose responses observed in clinical trials.

72. Lack of the Ability to Bind Heparin and a Y-to-F Mutation are Sufficient for AAP-Independent AAV2 Capsid Assembly

Anusha Sairavi[1], Yoya Vashi[1], Swapna Kollu[1], Hiroyuki Nakai[1,2]

[1]Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR,[2]Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR

The assembly of adeno-associated virus (AAV) capsids is primarily mediated by assembly-activating protein (AAP) and other host factors, which could potentially vary among different serotypes and production systems. AAP supports capsid assembly through a multitude of functions with the ultimate goal of stabilizing VP monomers and multimers, subsequently allowing them to assemble into capsids, the process of which is still not well understood. Although this mechanism applies to AAVs in the viral life cycle in nature, our group discovered AAP-independent (AAPi) serotypes capable of assembling into capsids in the absence of AAP in experimental settings. These include AAV 4, 5, 11 and rh32.33. We had previously reported that the AAPi serotypes exhibit a higher propensity for VP subunit association at the 2 and 5-fold axes compared to AAP-dependent (AAPd) serotypes, conferring AAPi capsids an equilibrium favorable for capsid formation. Understanding how and why the AAPi serotypes emerged would not only further our knowledge of AAV biology and evolution but also provide clues to exploring novel means to enhance AAV vector production for gene therapy purposes. Given this background, we have been seeking to identify mutants and their respective positions that could render AAPd serotypes independent of AAP for assembly (i.e., AAPd-to-AAPi phenotype conversion). Here, we report that we have successfully identified such mutants derived from AAV2, an AAPd serotype, through directed evolution, which has provided mechanistic insights into AAP requirements in AAV capsid assembly. Briefly, an ITR2-Rep2-AAV2Cap-ITR2 plasmid construct was generated with a codon-modified VP3 with the AAP2 ORF knocked out. The construct was subjected to error prone PCR to generate a library of mutants. AAV2 production was carried out in the absence of AAP2, applying stringent pressure, and the library was subjected to three rounds of directed evolution in HEK293 cells. The third-round AAV2 mutants were randomly selected and those that produced viable capsids were sequenced and found to commonly have a mutation at R585 and a mutation at position 370 from F to Y with other mutations variedly dispersed between mutants. A mutant, AAV2mt1, producing high vector yields, was selected for further analysis and its yields were compared to the wild-type (AAV2wt) and an AAV2 with AAP knockout ($AAV2^{AAPKO}$) in the presence or absence of AAP2. In the absence of AAP2, AAV2mt1 yielded vectors at decent levels comparable to the yield obtained from the standard AAV2 vector production. In the presence of AAP2, AAV2 mt1 yielded vectors 2 times and almost a log higher than AAV2wt and $AAV2^{AAPKO}$, respectively. Based on these observations, we propose a hypothesis that ablation of heparin binding accompanied by a Y-to-F mutation at position 370 is sufficient to render AAV2 independent of the AAP2 requirement for assembly. Further work are currently underway to identify more AAPi mutants, including those derived from AAV9, another AAPd serotype. We have also introduced molecular dynamics simulations in our study to understand how changes in capsid flexibility and conformation play roles in determining AAP requirements.

73. Specific Di-Nucleotide Pattern in Adeno-Associated Virus Genomes

Conradin Baumgartl[1], Man Xu[1], Lena Naber[1], Dirk Grimm[2]

[1]Universitätsklinikum Heidelberg, Heidelberg, Germany,[2]Heidelberg University Hospital, Heidelberg, Germany

Viral genomic DNA is known to interact with the inside of the capsid in Adeno-associated virus (AAV) and other parvoviruses, but the extent of the interaction and the shape the single-stranded (ss)DNA genome has to assume are largely unknown. Intrinsic DNA shape has a well-known function in facilitating nucleosome formation in eukaryotes or supercoiling propensity in prokaryotes. Periodic appearances of different di-nucleotides about every 10-11 bp are known to shape double-stranded (ds)DNA into a bend and thus facilitate protein interaction or DNA coiling. The exact repeating di-nucleotide differs between species. Using spectral density estimators and non-linear regression, we have discovered a specific di-nucleotide periodicity in wild-type dependoparvoviral genomes in which the YY di-nucleotide repeats every ~15 bp. As genome-wide di-nucleotide periodicities become more obvious with sequence length and as viral genomes are considerably smaller than eukaryotic ones, we analyzed individual

taxonomic genera to gather comprehensive sequence information. Next to dependoparvovirus, we also investigated periodicities within members of the entire *Shotokuvirae* virus kingdom and found di-nucleotide periodicities to be relatively prevalent. Especially genera from *polyomaviridae* and *papillomaviridae* show a distinct 10-11 bp YY di-nucleotide repeat pattern. Another noteworthy genus is the turncurtovirus from the *geminiviridae* that shows a repeat of 9 bp in YY di-nucleotides. Notably, the 15-bp YY periodicity appears to be unique for the genus dependoparvovirus. While di-nucleotide periodicities have never been described in parvoviruses and their function in linear ssDNA remains enigmatic, their presence implies a biological significance. In fact, in libraries of genetically engineered AAV cap genes, we found that the periodicity of the packaged DNA sequences increased upon production compared to the parental sequences, which implies that these repeats provide an advantage during production. To investigate the function of this genomic pattern further, we introduced an inert AAV wild-type fragment containing the di-nucleotide pattern into a reporter construct flanked by AAV packaging signals and used it to produce recombinant AAV. Indeed, the rAAV containing the wild-type fragment yielded higher titers compared to controls and other viral genomes in a small-scale production format. Our surprising discovery of a genome-wide and periodic di-nucleotide pattern in AAV DNA raises the intriguing hypothesis that, next to traditional *cis*-acting packaging signals, AAV has evolved as-of-yet-unknown mechanisms involving molecular interactions of its genome e.g. with the viral capsid or host proteins that facilitate its replication and assembly, and that could be harnessed in the future to improve AAV vector manufacturing and application.

Next Generation Gene & Cell Therapies for Heart, Lung, and Kidney Diseases

74. Rescue of Lethal SP-B Deficiency in a Murine Model Using Lentiviral Vector-Mediated Gene Therapy

Kamran M. Miah, Aimee Ruffle, Mariana A. Viegas, Emily Castells, Hamid Dolatshad, Stephanie G. Sumner-Jones, Rebecca Dean, Yue Du, Alexandra McStea, Rosie J. Munday, Altar M. Munis, Stephen C. Hyde, Deborah R. Gill

Radcliffe Department of Medicine, Nuffield Division of Clinical and Laboratory Sciences, University of Oxford, Oxford, United Kingdom

There remains a clear unmet need for a curative gene therapy for the rare, but neonatally lethal, surfactant protein B (SP-B) deficiency. The autosomal recessive disease is caused by a lack of functional SP-B expression, which leads to an impairment in pulmonary surfactant homeostasis soon after birth in full-term infants. Surfactant is secreted from alveolar type II (ATII) cells in the lung as a complex mixture of lipids and surfactant proteins, including SP-A, -B, -C, and -D. It functions to prevent alveolar collapse and to allow efficient gas exchange at the air liquid interface. SP-B deficiency manifests clinically as a complex spectrum of lung pathologies, including pulmonary alveolar proteinosis, pulmonary inflammation, interstitial fibrosis, and severe respiratory distress. The respiratory distress presents shortly after birth and necessitates long-term mechanical ventilation to keep affected babies alive. Without lung transplantation SP-B deficient babies succumb to disease within the first year of life. To address this unmet clinical need we evaluated gene therapy for SP-B deficiency using a proprietary lentiviral vector platform based on recombinant Simian Immunodeficiency Virus (rSIV) pseudotyped with Sendai Virus Fusion (F) and Haemagglutinin-Neuraminidase (HN) glycoproteins (rSIV.F/HN) for efficient lung targeting. Here we report the use of rSIV.F/HN vector to deliver the human SP-B (hSP-B) transgene under the control of our hCEF promoter, to the lungs to protect a conditional SP-B knockout (KO) mouse model from lethal respiratory distress. We found that the rSIV.F/HN hSP-B vector, when administrated intranasally (2E8 TU daily over 4 consecutive days), prevented disease progression and restored SP-B levels and surfactant homeostasis to therapeutic levels. Lung histology, pulmonary neutrophil recruitment, and surfactant protein C (SP-C) processing were all restored to near wildtype levels. Importantly, the administration of rSIV.F/HN provided a substantial survival benefit after induction of the conditional SP-B deficiency, with >10 months ongoing survival, compared to ~5 days median survival of untreated control animals (Mantel-Cox test, Log-rank, $P = 0.0001$). At the time of abstract submission, our longest surviving treated animals were still alive at >300 days post disease induction. Following a single dose of rSIV.F/HN, the risk of respiratory decline in treated animals was substantially reduced (Hazard Ratio 9.3; 95% confidence interval 2.9-30.2), without the need for repeated administration of the vector to sustain therapeutic levels of SP-B expression over the course of these studies. This high degree of protection from respiratory decline means that treated animals have a survival indistinguishable from control (non-disease induced) littermates (69.2% versus 74.2% survival, respectively, measured up to 1-year from birth, $P = 0.9812$). We also aim to evaluate the efficacy of the rSIV.F/HN platform for SP-B gene therapy by expressing the hSP-B transgene under the control of several new ATII-specific promoters. In summary, our observations support the use of the rSIV.F/HN platform expressing hSP-B as a novel and potentially curative treatment for new-borns diagnosed with SP-B deficiency.

75. Aerosolized Gene and Oligonucleotide Therapy Targeting microRNA-224 Ameliorates Pulmonary Hypertension by Orchestrating the BMP Pathway

Olympia Bikou[1,2], Aymen Halouani[3], Catherine Swarts[2], Petros Avramopoulos[4], Malik Bisserier[2], Erik Kohlbrenner[5], Hajjar Roger[6], Stefan Engelhardt[4], Lahouaria Hadri[2], Sebastien Bonnet[7], Yassine Sassi[3]

[1]LMU University Hospital, Munich, Germany,[2]Icahn School of Medicine at Mount Sinai, New York, NY,[3]Fralin Biomedical Research Institute at Virginia Tech Carilion, Virginia, VA,[4]Institute of Pharmacology and Toxicology, Technical University, Munich, Germany,[5]Spark Therapeutics, Philadelphia, PA,[6]Gene and Cell Therapy Institute, Massachusetts General Brigham, Boston, MA,[7]Pulmonary Hypertension Research Group, Université Laval, Québec, QC, Canada

Pulmonary arterial hypertension (PAH) is a severe vascular disease that leads to right heart failure and death. PAH therapies primarily

target pulmonary vasoconstriction, but only modestly affect pulmonary vascular remodeling. Currently there is no cure for PAH and new therapeutic options targeting the roots of the disease are desperately needed. MicroRNAs (miRs) have emerged in the last decades as key regulators in health and disease. We identified miR-224 as a lung enriched miR and *in silico* approaches for PAH predicted miR-224 among the miRs that target PAH related genes. In this study we aimed to investigate the role of miR-224 in pulmonary vascular remodeling, to define the mechanism of miR-224 action in PAH and to test the therapeutic effect of miR-224 gene- and oligonucleotide therapeutic approaches in PAH. We found pulmonary miR-224 levels to be increased in PH-diseased lungs cross-species: in mice, rat, pig and humans. Furthermore, miR-224 was increased in human pulmonary artery smooth muscle cells (hPASMCs) isolated from PAH patients compared to healthy controls. *In vitro* studies revealed that miR-224 is enriched in hPASMCs and its expression is necessary and sufficient to induce hPASMCs proliferation. We next tested the therapeutic effect of miR-224 inhibition using three different PAH animal models in mice and rats. In a first approach, we intra-tracheally aerosolized an adeno-associated virus1-tough decoy-miR-224 (AAV1-TuD-224) to PH diseased mice (Sugen/hypoxia model). AAV-Control-treated mice displayed all the hallmarks of PAH (i.e., increased Fulton index, elevated pulmonary arterial- and right ventricular pressure, cardiomyocyte hypertrophy and pulmonary arterial medial thickness), whereas AAV1-TuD-224-treated mice displayed a marked and significant decrease in these parameters. We next intra-tracheally delivered a chemically modified antisense oligonucleotide specific for miR-224 (LNA-224) to PH diseased mice and rats (Sugen/hypoxia model). Consistent with our genetic approach, LNA-224 significantly protected mice and rats from cardiac hypertrophy and pulmonary vascular remodeling at the tissue and cellular levels. We finally delivered LNA-224 to monocrotaline-treated rats and found (by magnetic resonance imaging, hemodynamics, morphometric and histological measurements) that miR-224 inhibition improves survival and suppresses PAH. Mechanistically, we found that miR-224 represses BMP signaling by directly targeting four pathway factors. Our data suggest that miR-224 plays a pivotal role in pulmonary vascular remodeling by targeting the BMP pathway. Aerosolized gene and oligonucleotide therapy targeting miR-224 may have therapeutic value for PAH.

76. AAV2-GEC: Advancing Gene Delivery for Precision Treatment of Antibody-Mediated Kidney Diseases

Shuya Liu, Guochao Wu, Jakob Körbelin, Shun Lu, Tobias B. Huber

University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Background: The breakdown of glomerular filtration barrier (GFB) is relevant to the pathogenesis of most kidney diseases. Targeting the renal glomeruli by conventional drugs is challenging due to the lack of cell-targeting specificity, which significantly limits the development of novel kidney therapies. Adeno-associated virus (AAV) is a promising *in vivo* gene delivery platform showing the advantage in delivering therapeutic molecules to those difficult or non-druggable cells. However, natural AAV serotypes have insufficient targeting specificity and transduction efficiency in kidney cells. **Methods:** We developed an evolution-directed selection protocol specifically for kidneys and screened a random AAV2 display peptide library *in vivo*. Integrative experimental and bioinformatics workflows identified the most promising AAV vectors enriched in the renal glomeruli. The transduction specificity and efficiency of the selected AAV were evaluated *in vivo* under both physiological and pathological conditions, as well as in primary human glomerular endothelial cells (GEC). The potential for kidney-targeting therapies was evaluated in antibody-mediated kidney diseases. **Results**: A new AAV vector termed AAV2-GEC was identified, which specifically and efficiently transduced the GEC in the renal glomeruli after systemic administration. AAV2-GEC exhibited robust GEC tropism in C57BL/6J, Balb/c, BTBR mice and Sprague Dawley rats, as well as in disease models causing GFB breakdown. As a proof of concept, AAV2-GEC was used to deliver a bacterial cysteine proteinase named IdeS to GEC in the kidney. IdeS is an antibody-cleaving enzyme used to eliminate pathogenic IgG. AAV2-GEC mediated IdeS expression efficiently produced IdeS in the renal glomeruli, which provided sustained clearance of IgG bound to the glomerular basement membrane (GBM), thereby successfully prevented the onset or progression of anti-GBM glomerulonephritis. **Conclusion:** This study establishes an efficient selection protocol for renal glomeruli and identifies a novel GEC-targeting AAV vector with robust tropism maintained cross rodents in both physiological and pathological settings. The successful application of AAV2-GEC in treating antibody-mediated kidney diseases represents a significant step forward and opens up promising avenues for kidney medicine.

77. Adoptive Transfer of Allogeneic Murine Engineered T Regulatory Cells Ameliorates Disease in Models of Acute Kidney Injury in Mice

Maegan E. Hoover, Madison Milaszewski, Nicole R. Reed, Abigail Doherty, Dalia E. Gaddis, Marko Repic, Aravind Kancharla, Thomas Wickham, Tiffany F. Chen, Payam Zarin

GentiBio, Cambridge, MA

Foxp3$^+$ T regulatory cells (Tregs) are important mediators of tissue homeostasis and immune tolerance. The rarity and heterogeneity of Treg populations in circulation, as well as their instability in inflammatory environments, however, has limited the applications of Tregs in recent cellular therapy approaches. Our platform addresses these limitations, creating stable, tissue targeted, and selectively activated Tregs from more abundantly available T cell populations. Specifically, we utilize CRISPR-Cas9 mediated gene editing of PBMC isolated CD4$^+$ T cells to engineer Tregs for stable expression of Foxp3, and a rapamycin-activated signaling complex that provides tunable IL-2 signaling, divorcing Foxp3 expression from regulatory elements known to promote Treg instability under inflammatory conditions. A murine surrogate was developed to allow for *in vivo* evaluation of our cellular therapy in mouse models of inflammation and tissue injury. Polyclonal murine engineered Tregs (mEngTregs) were generated from bulk splenic CD4$^+$ T cells via CRISPR-Cas9 mediated knock-in of the MND promoter at the Foxp3 locus. To allow for allogeneic assessment, CB6F1 (C57BL/6 x BALB/c F1 cross) donor mice were used for cell generation. Acute Kidney Disease (AKI) is a life-threatening condition defined by a rapid decline in renal function. Common biomarkers of

AKI include gross tubular degeneration, increased local inflammatory cytokine response, shedding of kidney injury molecule-1 (kim-1) into both serum and urine, a severe reduction in glomerular filtration rate, and increased levels of blood urea nitrogen (BUN) and serum creatinine (sCR). AKI can be triggered by any condition in which renal blood flow is restricted, cardiac output is reduced, or direct tissue insult occurs via toxins, infection, medication, or local inflammation. Despite significant advancements in the field, treatment options for patients experiencing renal failure are limited and prognosis remains poor. In the current work, we assess the ability of mEngTregs to ameliorate disease in models of acute kidney injury in mice. Kidney injury was induced in recipient animals by either surgical or chemical intervention and mEngTregs were adoptively transferred by intravenous injection. mEngTregs were found to effectively engraft at the site of the kidney and significantly improve disease outcome in multiple models of AKI. Specifically, animals treated with mEngTregs showed a significant reduction in sCR, BUN, and Kim-1 levels compared to AKI controls. Key inflammatory cytokines such as IL-1α, TNF-α, and IL-6 were lower in the kidney of mEngTreg treated animals and reductions in infiltrating neutrophils were visible by flow cytometry. Animals treated with mEngTregs also showed normalization of the renal macrophage compartment by activation markers such as MHCII and CD64. Importantly, histopathology analysis of the kidneys of mEngTreg treated AKI animals showed significantly less tubular degeneration, cast formation, and loss of brush border as compared to AKI controls. Overall, the ability of mEngTregs to ameliorate AKI as measured by key, translatable, biomarkers of renal function, supports the potential for developing a potent off-the-shelf allogeneic engineered Treg cellular therapy for human AKI.

78. Addressing a Critical Limitation of First-Generation Dystrophin-Mimetics: AAV-Nanotrophins Provide Superior Cardioprotection and Circumvent Junctional Fragmentation *In Vivo*

Kyle A. Hubble, Coral M. Kasden, Maria P. Corzo, Tanvi Singh, Alaine I. Castillo, Rufranshell Reyes, Benjamin W. Kozyak, Hansell H. Stedman

University of Pennsylvania, Philadelphia, PA

Introduction. Duchenne muscular dystrophy (DMD) results from genetic, usually deletional, deficiency of the 427 kD isoforms of dystrophin. Despite groundbreaking progress, two enduring challenges have complicated the development of effective, durable, and safe gene therapy. 1) mRNAs encoding the native 427 kD isoforms of dystrophin are ~ 14 kb in length, vastly exceeding the < 5 kb cloning capacity of the AAV platform. Miniaturized variants of dystrophin encoded by internally deleted cDNAs have been advanced to the clinic by multiple groups, but data released to date suggest limited durability consistent with the pathobiology of Becker muscular dystrophy (BMD), including the instability of mutant dystrophin. 2) Reported findings in DMD patients receiving systemically administered adeno-associated virus (AAV)-dystrophin vectors include T cell mediated myositis/myocarditis and limited durability of therapeutic efficacy associated with progressive loss of transgene expression. Here we present the development and rigorous testing of a novel class of therapeutics providing compelling evidence for emerging solutions to address the limitations of current therapies. **Methods.** We have used complementary approaches to develop solutions to both problems. We addressed T cell immunity by substituting epitopes from the self protein utrophin for those of the non-self protein dystrophin. We addressed the problem of protein functionality by avoiding a pitfall of previously used approaches to dystrophin "downsizing" for AAV: non-native protein folding geometry at the deletional splice junction(s) with the capacity to locally destabilize the rod domain as in severe BMD. Our reconstruction of the evolutionary history of dystrophin (presented elsewhere) enabled insight into structural and functional constraint on proteins in this supergene family, most notably the conservation of junctions between adjacent triple helical repeats throughout animal phylogenesis. This allowed us to predict and subsequently address the instability of the rod with load bearing in the first-generation gene therapies. We harnessed structural biology tools such as Hidden Markov Modeling to develop a simple set of testable hypotheses about biomechanical load bearing and structure-function relationships. We used novel, clinically relevant pathobiological assays to investigate both physiological functionality and protein structural integrity under mechanical stress. **Results.** We constructed and tested in *mdx* mice a series of AAV vectors encoding dystrophin- and utrophin-based miniaturized proteins, including three nanotrophins in which the splice junctions in the rod domain share the unique capacity to preserve the three-dimensional folding geometry of adjacent triple helical repeats. To rigorously test our hypothesis, we performed randomized, blinded *in vivo* tests to provide quantitative indices of functionality conferred by each of the vectors. Following catecholamine-induced cardiac stress, multiple quantitative assays for acute myocardial injury revealed statistically significant differences between groups of *mdx* mice injected with microdystrophin or our best performing nanotrophin-5U (cardiac troponin I: 8.09 vs 1.07 ng/mL, p=0.0016 and Evans blue dye: 2.04 vs 0.25%, p=0.0100, respectively). Concordant with these functional results, by western blot analysis only the nanotrophins were resistant to load-dependent fragmentation in the rod domain. **Conclusions.** Rigorously designed and executed *in vivo* tests support the hypothesized correlation between load-dependent fragmentation and superior physiological performance of nanotrophin-5U, a novel candidate therapeutic for DMD based on the non-immunogenic utrophin sequence.

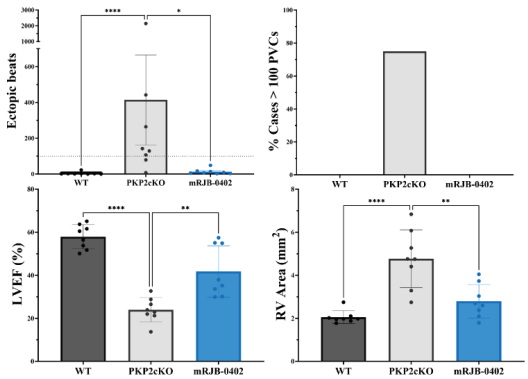
79. AAV FGF21 Gene Therapy as a Variant Agnostic Treatment for Arrhythmogenic Cardiomyopathy

Kristy Brown[1], Christopher De Solis[1], Mingliang Zhang[2], Sarah Boyce[2], Manuela Gencarelli[2], Xianming Lin[2], Maritza C. McIntyre[3], Daniel Oliver[1], Deborah D. Ascheim[4], Marina Cerrone[2], Mario Delmar[2], Noah Davidsohn[1]

[1]Rejuvenate Bio, San Diego, CA,[2]NYU, New York, NY,[3]Advanced Therapies Partners, Raleigh, NC,[4]Rejuvenate Bio, Los Angeles, CA

Arrhythmogenic cardiomyopathy (ACM) is a severe, debilitating, life threatening, and rare inherited heart disease. While more than 10 different genes have been implicated in the approximately 60% of patients with variant associated ACM, the majority involve one or more variants in desmosomal genes, which are key components of the cardiac

intercalated disc. The desmosome is a protein complex responsible for cell-to-cell adhesion, stability, and transmission of contractility and force in a coordinated fashion between cardiomyocytes. These gene variants result in premature ventricular contractions (PVCs) and life-threatening ventricular arrhythmias, risk of sudden cardiac death, and progressive fibrofatty replacement of ventricular myocardium resulting in cardiac dysfunction that can lead to heart failure. No disease modifying treatments have proven effective in humans for ACM, highlighting the profound unmet medical need. RJB-0402 is a novel systemically administered investigational adeno-associated virus (AAV) gene therapy that drives liver-specific expression of FGF21 protein, which targets multiple key pathological drivers of ACM in cardiomyocytes. FGF21 has known salutary effects on cardiac dysfunction as well as ventricular arrhythmias, adipogenesis, inflammation, and fibrosis, all of which are ACM hallmarks. Preliminary exploration suggests that FGF21's antiarrhythmic effect may be, at least in part, attributed to its role in restoring cardiomyocyte calcium handling. RJB-0402 efficacy was studied in a conditional PKP2 knockout (PKP2cKO) mouse model of ACM. FGF21 gene therapy significantly reduced arrhythmias following isoproterenol challenge, as evidenced by decreased incidence and severity of ventricular arrhythmias manifest by PVC burden in RJB-0402 treated PKP2cKO mice (n=8) relative to untreated PKP2cKO controls (n=8), with PVC frequency in treated PKP2cKO mice approaching those of the wild type controls (n=8). Partial preservation of cardiac function in PKP2cKO mice assessed by echocardiography was also observed in the FGF21 treated cohort compared to vehicle treated controls. Furthermore, a reduction in cardiac fibrosis was observed by histological assessments in FGF21 treated cKO mice relative to untreated controls. These findings demonstrate early proof of principle that FGF21 gene therapy is a promising variant-agnostic candidate for treatment of ACM that warrants further investigation and potential clinical application. This novel approach is particularly promising for treatment of ACM due to variants not amenable to gene replacement therapy; genes too large for traditional gene replacement such as the *desmoplakin* gene, or due to multiple variants.



In vivo evaluation of RJB-0402 in vehicle treated WT and PKP2cKO mice and PKP2cKO mice treated with 1.5 × 10^{13} vg/kg of mRJB-0402 (n=8 per group). Top left panel: total ectopic beats observed in 30 minutes following a challenge of ISO; Top right panel: PVC burden of mice with greater than 100 ectopic beats in 30 minutes following a challenge ISO. Bottom left panel: left ventricular ejection fraction. Bottom right panel: right ventricle area. *=p <0.05, **=p <0.01, ***=p <0.001, ****=p <0.0001

80. Increasing Saline Tonicity Enhances Airway Gene Transfer

Ashley L. Cooney[1], Kenan Najdawi[1], Christian Brommel[1], Laura I. Marquez Loza[2], Paul B. McCray[1], Patrick L. Sinn[1]

[1]Pediatrics, University of Iowa, Iowa City, IA,[2]Molecular Medicine, University of Iowa, Iowa City, IA

A fundamental challenge for cystic fibrosis (CF) gene therapy is ensuring sufficient transduction of airway epithelial cells to achieve therapeutic correction. Vehicles such as lysophosphatidylcholine (LPC), EGTA, perfluorocarbon, or methylcellulose have been used to enhance gene transfer of Ad, AAV or lentiviral vectors. However, advancing these formulations to a clinical setting adds an additional layer to translating a gene therapy therapeutic. To this end, we screened FDA approved reagents for their ability to enhance viral vector transduction of primary human airway epithelial cells. Hypertonic saline conferred a clear benefit. Hypertonic saline (3-7%) is frequently administered as an agent to enhance mucus clearance in people with CF. Hypertonic saline transiently disrupts epithelial cell tight junctions, but its ability to improve gene transfer has not been investigated. Here we asked if increasing NaCl tonicity in the vector formulation enhances the transduction efficiency of 3 gene therapy vectors: adenovirus, AAV, and lentiviral vectors. We observed that vectors formulated *in vitro* with 3-5% NaCl exhibited markedly increased transduction for all 3 platforms (**Figure 1A-D**), leading to phenotypic correction of the anion channel defect in primary cultures of human CF epithelial cells. The NaCl-enhanced transduction was enhanced by ionic strength but not osmolarity or pH. Ad entry remained receptor dependent and required low pH endosomal escape. Gene transfer *in vivo* was also enhanced in mouse and pig airways by formulating vectors in NaCl (5% or 7%). Increasing saline tonicity has the potential to increase the viral delivery efficacy with a reduced amount of vector. These studies open the door to many possibilities for other genetic lung diseases, far beyond CF.

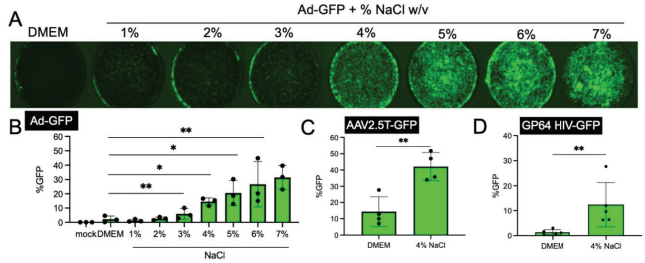


Figure 1: *Increasing saline tonicity enhances transduction efficiency of Ad, AAV, and GP64 HIV.* A) Ad CMVeGFP (MOI=250) was applied to the apical surface of well-differentiated human primary airway epithelia alone or with NaCl (1-7%) for 2 hours. B) GFP expression quantified by flow cytometry 5 day later from cultures in A). C) AAV2.5T CMVeGFP (MOI=100,000) was applied apically alone or in the presence of 4% NaCl for 2 hours. GFP expression was quantified by flow cytometry 2 weeks later. D) GP64 HIV CMVeGFP (MOI=50) was delivered with 4% NaCl for 2 hours. GFP expression was quantified by flow cytometry 2 weeks later. N=3-5 human donors, *p<0.05, **p<0.05.

Ophthalmic and Auditory: Delivery Innovations

81. Optimization of Viral Gene Therapy for Inherited Retinal Diseases Using Fibrin-Mediated Delivery

Aubrey Berger[1,2], Travis Knudsen[2], Raymond Iezzi[2], Francesca Zenti[2], Brittni Scruggs[2], Alan D. Marmorstein[2]

[1]Mayo Graduate School of Biomedical Sciences, Mayo Clinic, Rochester, MN,[2]Mayo Clinic Department of Ophthalmology, Mayo Clinic, Rochester, MN

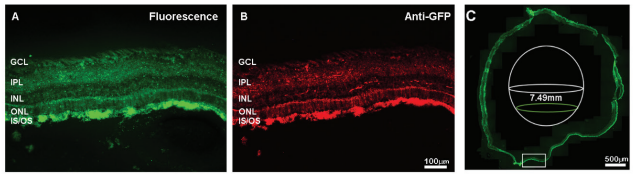
Purpose: Treatment of RPE65-mediated Leber Congenital Amaurosis involves an injection of AAV2 suspension between the RPE and retina. While this achieves RPE transduction, the procedure requires subretinal bleb formation. This increases the risk of macular holes, progressive chorioretinal atrophy, and choroidal neovascularization. These complications create a need for an alternative method of administration that avoids puncturing and detaching the retina. We hypothesize that a virus-embedded fibrin hydrogel will release virus to transduce RPE cells, and when placed on the epiretinal surface, the gel will result in locally transduced RPE. **Methods:** Fibrin hydrogels were cast with GFP-encoding AAV2/2 at 5.7e11 viral genomes (vg)/cm^3. These gels were characterized using electron microscopy, optical coherence tomography, immunofluorescence, and an ELISA-based diffusion assay. The gels were placed on the surface of confluent ARPE19 [BS2] cells to determine *in vitro* infectivity through detection of GFP expression. Domestic pigs underwent vitrectomy with implantation of virus-hydrogels placed directly the epiretinal surface (N=6) and in the subretinal space (N=3). AAV solution without hydrogel was injected using subretinal (N=3) and intravitreal injections (N=3) as controls at comparable viral titers. Eye exams were completed weekly, and after a month, eyes were enucleated. FITC imaging was used to observe expression of GFP in whole mount imaging and in histological sections. PCR was preformed to identify AAV genome presence in ocular tissues and the liver. **Results:** Immunofluorescence demonstrated that gels have a homogenous distribution of AAV. In the diffusion assay, gels achieved 50% viral diffusion after 48 hours with the remaining virus accounted for in the non-degraded gel. When placed in culture, observed cells displayed GFP signal after 2-4 days, confirming that released virus retained infectivity *in vitro*. In the pig, hydrogels degraded in the eye within five days. Observation of GFP signal in the RPE confirmed cell transduction from the epiretinal and subretinal implant groups with fewer complications (*e.g.*, chorioretinal atrophy, intraocular inflammation) than the groups without hydrogel. **Conclusion:** AAV2 transduces RPE and RPE-like cells *in vivo* and *in vitro*, respectively, when released from a fibrin hydrogel. Strong RPE transduction in pig eyes receiving epiretinal hydrogel highlights this novel surgical approach as an alternative delivery to subretinal bleb formation. These data hold promise for fibrin-mediated viral gene therapy delivery in patients with inherited retinal diseases. Future studies will test the effect of implant location, viral titer, and gel size on efficacy and safety with further characterization of viral diffusion, gene expression, and gel degradation across different conditions.

82. Suprachoroidal Injection of Poly(Beta-Amino Ester) Nanoparticles Enables Safe, Efficacious, and Durable Gene Delivery Across Multiple Species

Jordan J. Green[1,2], Jikui Shen[2], Raquel Lima e Silva[2], Mingliang Zhang[2], Kathryn Luly[1], Yeongseo Lim[1], Sean Hackett[2], Shirley Lowmaster[1], Sydney Shannon[1], David R. Wilson[1], Stephany Yi Tzeng[1], Peter A. Campochiaro[3]

[1]Biomedical Engineering, Johns Hopkins University, Baltimore, MD,[2]Ophthalmology, Johns Hopkins University, Baltimore, MD,[3]Ophthalmology, Johns Hopkins Hospital School of Medicine, Baltimore, MD

Introduction. Current challenges with ocular gene therapy to the retina include incomplete biodistribution and retinal penetration, limitations to DNA cargo carrying capacity, expensive manufacturing costs at scale, and potential safety risks. To overcome these challenges, we created hydrolytically degradable end-group modified poly(beta-amino ester) (PBAE) NPs that can safely and effectively deliver plasmids to photoreceptors in the retina. We combined this chemical innovation in gene delivery with innovation to the method of physical gene delivery through suprachoroidal injection. ***Methods.*** Cationic PBAEs were synthesized via Michael addition reactions and formed nanoparticles (NPs) with anionic plasmid DNA via self-assembly in acidic buffer. NPs were then lyophilized and stably stored until use. For NP administration, a needle was used to make a partial thickness circumferential opening in the sclera and a second needle was used to perform suprachoroidal injections into the scleral opening to reach the suprachoroidal space. Gene expression was quantified by immunohistochemistry and ELISAs. This NP delivery system was evaluated in multiple species including Brown Norway rats, Yucatan minipigs, and Göttingen minipigs. ***Results.*** PBAE NPs were found to encapsulate 100% of the plasmid DNA during particle self-assembly and hold on average 250 ± 20 kbp in each particle. The PBAE NPs are designed to be hydrolytically degradable in physiological conditions, and no signs of toxicity were observed following administration of the biodegradable PBAE NPs in any of the rats or minipigs evaluated, suggesting that this approach may be a broadly safe non-viral method for ocular gene transfer. In the human-sized eyes of minipigs, penetration of the NPs into the retina was found to be good, with photoreceptors non-virally transfected (**Fig 1A-B**). Biodistribution of the NPs and their corresponding gene transfer was also found to be broad, throughout the circumference of the minipigs eyes, from the anterior to the posterior (**Fig 1C**). Although the NPs break down quickly in water, the plasmids were observed to persist and exhibit durable gene expression for all time points evaluated, including lasting at least 8 months following a single suprachoroidal injection in rats. ***Conclusion.*** The PBAE NPs are easily scalable and avoid the challenges of viral manufacturing. They were found to have a large cargo capacity for nucleic acid delivery and to safely deliver those cargos to the retina without causing toxicity or retinal degeneration. The suprachoroidal approach was found to enable broad biodistribution throughout the circumference of human-sized eyes and could readily be administered in an outpatient setting. Together, this system led to durable non-viral ocular gene transfer and is promising for the treatment of inherited retinal degenerations as well as other causes of blindness.



Suprachoroidal injection of PBAE/DNA nanoparticles induces strong transgene expression in the retina of Yucatan minipigs. Robust expression throughout the retina is observed by **(A)** Direct GFP fluorescence as well as **(B)** Anti-GFP staining. Expression is observed throughout the retina including in **(C)** Posterior sections that demonstrate GFP fluorescence around the entire circumference of the eye.

83. Efficient Spiral Ganglion Neuron Transduction with a Novel Capsid-Engineered AAV Vector for Improved Cochlear Implant Outcomes in Hearing Loss

Jennifer Marx[1], Peixin Huang[2], Sereina Sutter[3], Moritz Ertelt[4], Odett Kaiser[5], Jennifer Harre[5], Juliane Schott[1], Josephine Macdonald[1], Axel Rossi[1], Athanasia Warnecke[5], Clara T. Schoeder[4], Axel Schambach[1], Hinrich Staecker[2], Hildegard Büning[1]

[1]Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany,[2]Department of Otolaryngology Head and Neck Surgery, University of Kansas School of Medicine, Kansas City, KS,[3]Institute of Virology, University of Zurich, Zurich, Switzerland,[4]Institute for Drug Discovery, University Leipzig Medical Faculty, Leipzig, Germany,[5]Department of Otolaryngology, Hannover Medical School, Hannover, Germany

Hearing loss (HL) affects over 460 million people worldwide, significantly affecting quality of life. Cochlear implants (CI) are used for severe to profound HL, but outcomes differ based on spiral ganglion neuron (SGN) survival. Recognizing the potential of neurotrophins to enhance SGN survival and improve CI outcomes, we developed a novel AAV vector by capsid-engineering displaying a heptamer peptide previously derived from a phage library screen. Our novel AAV vector, Var9, efficiently transduces SGN in adult mice already at low doses. Structure-focused modeling of Var9 revealed a distinct alteration in cell attachment receptor binding due to peptide insertion compared to its parental serotype AAV2. Predictions indicating reduced attachment receptor affinity were validated through affinity chromatography and competition assays. Interestingly, Var9 demonstrated faster transgene expression in HEI-OC1 cells, a murine auditory cell line, despite significantly lower entry efficiency, indicating enhanced intracellular processing. Subsequent IF-FISH analysis at single-cell level visualized Var9 uncoating to approximately threefold higher levels than AAV2 after 24h. In contrast to AAV2, Var9 uncoating appeared independent of cell cycle progression, potentially providing an advantage in post-mitotic otic cells. Furthermore, Var9 outperformed AAV2 with faster uncoating in vitro (HEI-OC1) in an indirect assay, correlating with corresponding transduction efficiencies. This advantage extended to in vivo settings, with Var9 demonstrating 6-, 4-, and 3-fold greater uncoating compared to AAV2 in isolated mouse cochleae at 1, 3, and 7 days post-injection, respectively. Finally, in a neurotrophic factor gene therapy approach, Var9 prevented SGN degeneration comparable to control levels by overexpressing BDNF in SGN of deafened mice. In conclusion, our novel AAV vector demonstrated superior properties crucial for efficient SGN transduction and will be further refined for clinical applications, aiming to enhance neural survival and improve outcomes for cochlear gene therapy in CI recipients.

84. Optimizing Ocular Gene Therapy: Harnessing the Potential of the Proteolipid Vehicle Nucleic Acid Delivery Platform

Douglas Brown[1], Maryam Hejazi[2], Ping Wee[1], Prakash Bhandari[3], Jitendra Kumar[4], Liliya Grin[1], Arun Raturi[1], John Lewis[3]

[1]Entos Pharmaceuticals, Edmonton, AB, Canada,[2]Entos, Edmonton, AB, Canada,[3]Entos Pharmaceuticals, San Diego, CA,[4]Entos Pharmaceuticals Inc, San Diego, CA

Age-related macular degeneration, retinitis pigmentosa, diabetic retinopathy, Stargardt disease, and glaucoma are prevalent eye conditions that lead to vison loss. Gene therapy has the potential to combat and prevent vision impairment; however, conventional viral and non-viral gene delivery platforms have limited their success due to challenges with delivery of nucleic acids, immunogenicity, and tolerability. We developed the Fusogenix™ proteolipid vehicle (PLV™) nucleic acid delivery platform to combat these limitations. The PLV platform leverages a proprietary fusion-associated small transmembrane (FAST) protein and a well-tolerated lipid formulation to achieve optimal delivery of nucleic acids to tissues throughout the body including the retina. Here, we investigated the ocular tropism of the FAST-PLV platform encapsulating short interfering RNA (siRNA), messenger RNA (mRNA), and plasmid DNA (pDNA). The FAST-PLV platform enables robust delivery and expression of mRNA and pDNA cargo in primary mouse and human retinal cell cultures without cytotoxicity. In mice, intravitreal injection of FAST-PLVs encapsulating siRNA targeting a functional protein resulted in potent gene knockdown throughout the eye. Intravitreal injection of FAST-PLVs encapsulating reporter pDNA and mRNA encoding firefly luciferase (FLuc) or eGFP resulted in localized and prominent FLuc expression in mice. Immunofluorescence analysis confirmed stable expression of pDNA and mRNA in retinal ganglion and photoreceptor cells. The efficacy demonstrated in rodents extended to rabbits and non-human primates, with pDNA-GFP and mRNA-GFP yielding substantial GFP expression in the retina. Optical coherence tomography confirmed minimal toxicity following these injections in both models. These collective findings underscore the promising potential of the FAST-PLV platform for ocular gene therapy.

85. Cochlear Gene Therapy Delivery Innovations, AAV Capsid Variants and Cell Type-Specific Regulatory Elements to Facilitate CSF-Mediated Administration

Paul T. Ranum[1], Ellie Carrell[2], Jenna Devare[3], Luis Tecedor[2], Yonghong Chen[2], Ryan Giovenco[2], Alejandro Mas Monteys[4], Stephen R. Chorney[5], Robert C. O'Reilly[5], Beverly L. Davidson[2]

[1]Latus Bio, Philadelphia, PA,[2]Children's Hospital of Philadelphia, Philadelphia, PA,[3]The Children's Hospital of Philadelphia, Philadelphia, PA,[4]University of Pennsylvania, Philadelphia, PA,[5]Division of Pediatric Otolaryngology, Children's Hospital of Philadelphia, Philadelphia, PA

The recent clinical success of AK-OTOF has demonstrated the feasibility and value of gene therapies for hearing and deafness. Current approaches to cochlear gene therapy use AAV intracochlear infusion via an inner ear surgical approach. This method necessitates general anesthesia, perforation(s) of the sealed inner ear space, and patients of sufficient age to facilitate the approach. However, the complexity of the auditory system and extreme diversity of genetic causes of hearing loss remain barriers to broader targeting of most hearing loss genes. Delivery continues to pose challenges at every level: the therapeutic window of opportunity, the vectors to target various hearing-relevant cochlear cell types, and payload design that limit expression to therapeutically relevant cells. Toward these goals we used our recently developed cerebrospinal fluid (CSF)-mediated route of gene therapy administration to the inner ear and round window membrane + canalostomy (RWM) infusions of WT and modified AAVs into NHPs to identify new hearing-relevant AAV capsids that target primate cochlear cells and developed a pipeline for splicing-based regulatory element discovery with validation in murine auditory inner hair cells (IHCs). CSF-mediated delivery of our top modified AAV variants yielded capsids capable of nearly complete IHC transduction across all cochlear turns, robust transduction of spiral ganglion neurons, and transduction of non-sensory supporting cells in the organ of Corti of the NHP cochlea. Additionally, using available single-cell RNA-Seq data, we tested a hair-cell-specific splice event with 100% sequence conservation between mouse and human for its ability to confer hair cell-specific payload expression. After ICV administration to mouse CSF there was auditory hair cell specific gene expression, and no expression throughout the non-cochlear central nervous system. In summary, we show an alternative method for gene delivery to the inner ear via the CSF, identified new capsid variants for cochlear gene delivery, and developed regulatory elements to limit gene expression to a cochlear cell-type of interest, all steps towards further development of gene therapies for hearing loss.

86. Identification of Novel Ligand-Conjugated AAV Vectors with Enhanced Properties for Suprachoroidal Gene Delivery

Gaelle M. Lefevre[1], Adrien Savy[1], Sigmund K. S. Tejada[2], Sungyeon Cho[2], Bezawit Ejigu[2], Delphine Compere[1], Andrew C. Mercer[2], April R. Giles[2]

[1]Coave Therapeutics, Paris, France,[2]REGENXBIO Inc., Rockville, MD

Adeno-associated virus (AAV) mediated gene therapy shows potential for treating prevalent retinal diseases like age-related macular degeneration and diabetic retinopathy, but it has not been widely applied yet. One of the main challenges is delivering the therapy effectively to the targeted areas in the eye, in particular the retinal pigment epithelium (RPE)/choroid and retina. The traditional method, subretinal administration, is considered effective but is an invasive surgery that must be performed in an operating room, which presents challenges for widespread implementation. As an alternative, administration to the suprachoroidal space (SCS) offers a less invasive way to deliver AAV gene therapies to the back of the eye, and developing capsids specifically tailored to delivery by this route of administration would further support its implementation, especially for delivering intracellular transgenes to the RPE and retina. To achieve this, we used the AAV-ligand conjugate platform (ALIGATER™) to create new AAV vectors optimized for SCS delivery. This platform relies on the chemical conjugation of functionalizing biomolecules to the surface of AAV capsids. As it does not require prior modification of the AAV capsid, this platform is compatible with virtually any natural or engineered AAV serotype. The conjugation process is performed in a single step, without the need for a catalyst, and is carried out post-AAV production, enabling platform manufacturing processes and a rapid translation of new candidates to the clinic. We built a barcoded library of conjugated AAV (coAAV) vectors and performed a parallel evaluation of these vectors with a benchmark capsid in non-human primates following SCS administration. Three weeks post injection, we evaluated the vector genome distribution and transgene mRNA expression in ocular and peripheral tissues. Several coAAV vectors showed significantly enhanced relative expression in the RPE/choroid and retina compared to the benchmark capsid, with lower transduction of off-target systemic tissues such as liver and kidney. These coAAVs represent the first chemically-improved capsids for SCS delivery, opening a potential path forward for the development of more efficient, more targeted, and well -tolerated gene therapies for the treatment of common retinal disorders.

87. Development of an *In Vivo* Non-Viral Ocular Editing Platform and Application to Potential Treatments for Glaucoma

Dominique L. Ouellet, Michael J. Lukason, Jack Philp, Marja Pimentel, Jimmy Breen, Izele Abure, Marian Themeles, Sushant Karnik, Jilong Li, Anass Agrud, Jordan Paine, Yen-Yu Liu, Anandan Paldurai, Daniel Pascual, Nicole Flanagan, Lauren Tso, Yin Tang, Kayla Urbaez, Nicolette Lee, Nassim Ajami, Katie Schum, Shashwat Nagar, Bethany Winans, Parin Sripakdeevong, Seshidhar Reddy Police, Alex Harding, Laura Serwer, Jonathan Terret, Lorenzo Aulisa, Mary-Lee Dequeant

CRISPR Therapeutics, Boston, MA

Open-angle glaucoma, one of the most common causes of blindness worldwide, is a condition for which the main risk factor is high intraocular pressure (IOP), which causes damage to the optic nerve and results in progressive vision loss. High IOP is a consequence of reduced aqueous humor outflow caused by blocked or altered trabecular meshwork (TM) tissue, a sieve-like structure located inside the eye

at the cornea-sclera junction. Lines of treatment involve mainly eye drops and trabeculectomy surgery aimed at reducing aqueous humor production or favoring the passage of aqueous humor. Unfortunately, patient non-adherence and surgical limitations often lead to disease progression. We developed a lipid nanoparticle (LNP) approach to deliver RNA molecules to the trabecular meshwork cells for potential treatments for glaucoma. LNP formulations were screened using primary TM cells *in vitro* and *in vivo* in the mouse eye. Selected LNP candidates carrying enhanced green fluorescent protein (eGFP) were confirmed in a human or non-human primate (NHP) *ex vivo* anterior segment organ culture (ASOC) model, as well as in NHPs following intracameral injection. Moreover, LNP candidates carrying Cas9/gRNA cargo targeting the mouse actin alpha 2 (ACTA2) gene successfully reduced the expression of the alpha smooth muscle actin (α-SMA), a resident protein of the trabecular meshwork, by more than 75% at 4 weeks post-injection. As a proof-of-concept of LNP delivery to the TM cells for glaucoma, we designed a gRNA targeting the myocilin (MYOC) gene. Mutations in the MYOC gene, and its altered expression in the trabecular meshwork cells, have been linked to severe glaucomatous conditions in which the manifestations often begin at a juvenile age. Myocilin mutations have been shown to be gain-of-function mutations, which makes the knockdown of this protein a potential target for a gene editing approach. The delivery of LNP candidates carrying Cas9 and a gRNA targeting MYOC in glaucomatous TM cells and in human and NHP ASOC models showed editing at the MYOC locus and reduction of the myocilin protein, including up to 98% editing and 85% protein knockdown in human primary TM cells. This novel approach aims to facilitate glaucoma treatment using transient expression of editing machinery in the trabecular meshwork cells to target genes, like MYOC, involved in glaucoma.

CAR T and Other Genetically Modified Immune Cells

88. Development of a FAP-CAR T Cell Protocol to Reduce Skeletal Muscle Fibrosis in a Murine Model of Duchenne Muscular Dystrophy

Maxime Ferrand[1], Celine Rocca[1], Sonia Albini[1], Valentina Buffa[1], Elodie Bôle-Richard[2], Francine Garnache-Ottou[2], Isabelle Richard[1], Anne Galy[1,3]

[1]UMR_S951 Integrare Research Unit, Genethon, Evry-Courcouronnes, France,[2]UMR1098 RIGHT, Université Franche-Comté, EFS Besançon, Besançon, France,[3]Art-tg, Inserm, Corbeil-Essonnes, France

Fibrosis is a feature of Duchenne muscular dystrophy (DMD) which progressively weakens skeletal and cardiac muscles. Immunotherapy with fibroblast activating protein (FAP)-specific CAR T cells has already been used to reduce cardiac fibrosis in mice during a drug-induced condition but this approach has not been tested in muscular dystrophy. The DBA2mdx mouse is a model of severe DMD in which rapid and strong fibrosis is observed, characterized by dense collagen deposits in skeletal muscles, with over-expression of the FAP and Col3 genes in the TA, GA, EDL muscles at 2, 3, and 4 months of age compared to their aged-matched DBA2 littermates. We developed a third-generation mFAP-CAR lentiviral vector (LV) to generate mFAP-specific CAR T cells from DBA2 spleen T cells. The transduction conditions were initially established with a LV co-expressing the mFAP-CAR and a CD19 tag, but subsequently, the CD19 tag was removed to increase titer and transduction efficacy, as measured by provirus digital PCR. The specificity and potency of the mFAP CAR T cells was demonstrated on FAP-expressing 3T3 cells using cytotoxicity and degranulation assays. To evaluate their *in vivo* efficacy, mFAP-CAR T cells were adoptively transferred into DBA2mdx mice. Dose finding tests showed that at least two injections of 1xE+06 FAP-CAR-LV-transduced cells were necessary to significantly reduce the histological and molecular biomarkers of fibrosis in muscles, as measured 2 weeks post-cell injection by reduced Col3 and FAP mRNA expression. Altogether, these results establish a basal protocol to reduce muscle fibrosis in DBA2mdx mice. Further studies are warranted to understand the effects of FAP CAR T cells in this model. *MF and CR contributed equally. IR and AG are co-senior authors of this study.*

89. Genetic Depletion of TIGIT Augments CAR-NK Cell Effector Function in the Solid Tumor Microenvironment

Ishwar Navin[1], Matthew Dysthe[2], Corrine Baumgartner[3], Robin Parihar[4]

[1]Immunology, Baylor College of Medicine, Houston, TX,[2]Translational Biology and Molecular Medicine, Baylor College of Medicine, Houston, TX,[3]Pediatrics, Baylor College of Medicine, Houston, TX,[4]Baylor College of Medicine, Houston, TX

Chimeric antigen receptor-expressing natural killer cells (CAR-NKs) have shown success against hematological malignancies. However, their efficacy has been limited in solid tumors which generate a tumor microenvironment (TME) that includes inhibitory myeloid derived suppressor cells (MDSCs) and M2 macrophages (M2s). We demonstrated that several methods of ex vivo manufacture of CAR-NKs significantly upregulated T cell immunoreceptor with Ig and ITIM domains (TIGIT), an inhibitory NK receptor. Neuroblastoma and sarcoma biopsy tissues showed TIGIT ligand expression on tumor cells and intratumoral MDSCs and M2s. *Our objective* was to determine the impact of TIGIT on CAR-NK function in the TME. Current TIGIT-targeting approaches using antibodies are handicapped by transient binding and poor bioavailability in the TME. *We hypothesized* that genetic deletion of TIGIT on CAR-NKs leads to a more robust and durable anti-tumor response within the TME. TIGIT knockout (KO) CAR-NKs expressing a 2nd generation GD2 CAR containing the 4-1BB endodomain were generated by sequential CRISPR/cas9 and retroviral transduction of expanded primary human NK cells. Degranulation (CD107a) and IFN-gamma by TIGIT^{KO}GD2.CAR-NK were assessed in short-term TME co-cultures containing LAN-1 neuroblastoma and human MDSCs. A novel long-term TME co-culture wherein CHLA255 neuroblastoma and human monocytes pre-established a suppressive TME for 72 hours prior to addition of TIGIT^{KO}GD2.CAR-NK was used to assess tumor growth and changes in caspase3/7, mitochondrial mass, and activated MTOR expression. In vivo efficacy and persistence were assessed in NSG mice containing a novel neuroblastoma TME xenograft.CRISPR/Cas9 and retroviral transduction resulted in TIGIT^{KO}CAR-NKs with >90% TIGIT deletion and >60% GD2.CAR

expression. TIGIT deletion did not alter surface expression of DNAM-1, NKG2D, NKG2A, TIM-3, PD-1 or LAG-3 on TIGIT^{KO}CAR-NKs. TIGIT^{KO} and GD2.CAR expression was stable in TIGIT^{KO}GD2.CAR-NKs over 7 days of secondary NK expansion. TIGIT^{KO} enhanced GD2.CAR-NK IFN-g secretion but not degranulation in short-term TME co-cultures. In long-term tumor co-cultures, TIGIT^{KO}GD2.CAR-NKs demonstrated greater anti-tumor activity vs TIGIT-expressing GD2.CAR-NKs (<10% viable tumor vs 35% viable tumor, p<0.01; n=4 donors). In TME co-cultures, TIGIT^{KO}GD2.CAR-NKs rapidly expanded and controlled tumor compared to control CAR-NKs (50% viable tumor vs 88% viable tumor, p<0.05; n=4 donors). Compared to control CAR-NKs, TIGIT^{KO}GD2.CAR-NKs continued to limit tumor growth in serial TME co-cultures but without changes in GD2.CAR expression, degranulation, nor differences in CAR-NK surface receptor profile. Furthermore, in long-term TME co-cultures, TIGIT^{KO}GD2.CAR-NKs compared to GD2.CAR-NKs expressed similar levels of caspase3/7, activated MTOR, and mitochondrial mass. Deletion of the activating receptor DNAM-1, the competitive binder of TIGIT ligands, did not abrogate the anti-tumor activity of TIGIT^{KO}GD2.CAR-NKs. Gene expression profiling was performed to define differentially expressed genes in TIGIT-expressing vs. TIGIT^{KO} GD2.CAR-NK prior to and after exposure to the TME. In vivo studies demonstrated improved NK cell persistence, stable GD2.CAR expression, and enhanced anti-tumor efficacy in TME xenograft-bearing mice treated with TIGIT^{KO}GD2.CAR-NKs compared to mice treated with TIGIT-expressing GD2.CAR-NKs.We defined a role for TIGIT in suppression of CAR-NK function and suggest TIGIT deletion as a novel CAR-NK therapeutic platform to evade immune suppression in the TME. This underscores the potential of gene-edited CAR-NKs to improve clinical outcomes in patients with solid tumors.

90. HLA-A2 CAR/IL-2-CISC Engineered Treg Exhibit Robust *In Vitro* and *In Vivo* Efficacy

Subhash Kumar Tripathi[1], Noelle P. Dahl[1], Annaiz Grimm[1], Yuchi C. Honaker[1], Peter J. Cook[1], David J. Rawlings[1,2]

[1]Center for Immunity and Immunotherapy, Seattle Children's Research Institute, Seattle, WA,[2]University of Washington, Seattle, WA

Chimeric antigen receptor expressing T regulatory cells (CAR-Treg) represent a promising potential cell therapy for autoimmunity, allograft rejection and graft-versus-host-disease (GvHD). Alloantigen-specific Treg are more potent than polyclonal Treg in suppressing GvHD in mouse models. Notably, HLA-A*02 antibody-based CAR (A2-CAR) expressing human natural (n)Treg exhibit significant clinical efficacy in animal models and are currently in use in Phase I/II clinical trials for renal allograft protection. However, nTreg based cell products face challenges associated with both production at therapeutic scale and *in vivo* engraftment and stability. We previously addressed these challenges, using homology-directed-repair (HDR)-based gene editing to generate antigen-specific and chemically induced IL-2 signaling complex (CISC) expressing CD4$^+$ EngTreg from CD4$^+$ T cells. This engineering approach results in high-level, stable FOXP3 expression, robust Treg phenotype and function and therapeutic benefit in Type-1-diabetes models[1-5]. Here, expanding on this engineering platform, we generated and tested the functional activities of CD4$^+$ A2-CAR EngTreg. First, we utilized lentiviral (LV)-mediated A2-CAR delivery in association with HDR editing to introduce the IL2-CISC (designed to mimic IL-2 signaling in response to an exogenous dimerizer) and the MND promoter into the *FOXP3* locus in human CD4$^+$ T cells. CD4$^+$ A2-CAR EngTreg expanded and enriched to high purity. Cell products exhibited stable expression of FOXP3 and Treg markers with minimal proinflammatory cytokine production and robust suppressive activity. *In vitro*, A2-CAR EngTreg responded in HLA-A*02 antigen-specific manner and exhibited minimal cytotoxicity. In comparison to polyclonal CD4$^+$ EngTreg, A2-CAR EngTreg exhibited superior control of GvHD mediated by HLA-mismatched allogeneic effector T cells in NSG recipient mice. Next, we assessed the efficacy of A2-CAR CISC EngTreg using sub-therapeutic doses in association with chemical dimerizer therapy to engage IL2-CISC signaling *in vivo*. Recipient mice were treated with the rapamycin analog, AP21967, that activates IL-2 CISC system without impacting the mTOR signaling in a xenogeneic allo-GvHD model. Strikingly, dimerizer therapy led to elimination of xenogeneic allo-GvHD at sub-therapeutic EngTreg doses correlating with improved engraftment of A2-CAR CISC EngTreg. Finally, to eliminate the requirement of LV-based CAR delivery, we demonstrate strategies that permit generation of A2-CAR CISC EngTreg using dual-HDR-based gene editing. Together, these findings illustrate the robust capabilities of CAR-CISC EngTreg products and suggest that this platform may provide broad therapeutic potential in transplantation, autoimmune and/or autoinflammatory diseases. ***References:*** **1.** Honaker, Y., et al. (2020). Sci Transl Med 12(546), eaay6422. **2.** Cook, P.J., et al. (2023). Mol Ther 31(8), 2472-2488. **3.** Hunt, M.S., et al. (2023). Mol Ther 31(10), 2872-2886. **4.** Yang, S.J., et al. (2022). Sci Transl Med 14(665), eabn1716. **5.** Uenishi, G.I., et al. (2024). JCI Insight, *in press*.

91. Anti-B7-H3 Chimeric Antigen Receptor NK Cells Suppress the Growth of Atypical Teratoid / Rhabdoid Tumor Orthotopic Xenografts

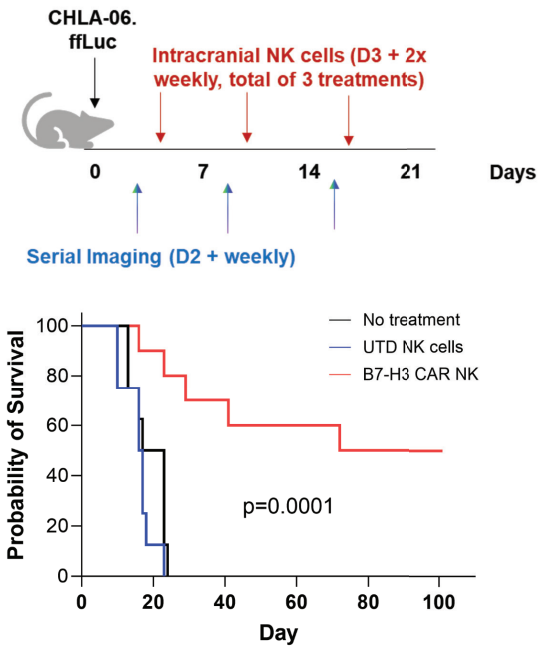
Jun Choe, Sachiv Chakravarti, Natalie Holl, Ruyan Rahnama, Megan Zinsky, Danielle Grace Jones, Stamatia Vorri, Arjun Modi, Eric H. Raabe, Challice L. Bonifant

Johns Hopkins University School of Medicine, Baltimore, MD

Background: Atypical teratoid/rhabdoid tumors (AT/RTs) are the most common malignant CNS tumor in infants. AT/RT patients have a 5-year overall survival rate of ~35% and high rates of relapse, emphasizing a dire need for new safe and effective therapies. These therapy-resistant tumors frequently overexpress the immune checkpoint cell surface molecule B7-H3 (CD276). CAR-T cell studies have an established safety profile in pediatric patients. We developed B7-H3 targeted chimeric antigen receptor (CAR) natural killer (NK) cells as a tumor-specific cell therapy for aggressive pediatric brain tumors. CAR-NK cells have several advantages over CAR-T cells. NK cells can be obtained from healthy donors and produced as an "off-the-shelf" product. NK cells also have a lower risk of inflammatory toxicity and graft-versus-host disease compared to T-cells when transferred across the HLA barrier from healthy donors to patients. **Methods:** We have designed a library of variable affinity B7-H3-targeted CARs, produced replication incompetent γ-retroviral vector, and used this for generation of B7-H3 CAR-NK cells. We verified B7-H3 expression in a panel of AT/RT

cell lines, and further engineered firefly luciferase expressing AT/RT (CHLA06.ffLuc, BT12.ffLuc, BT37.ffLuc) as well as a CHLA06-derived B7-H3 knockout. We developed an orthotopic CHLA06.ffLuc xenograft model. We tested CAR-NK cell functionality using *in vitro* co-culture and cytotoxicity assays. *In vivo* study was performed in our xenograft with intratumoral delivery of CAR-NK cells. Results: B7-H3 targeted CAR-NK cells demonstrate target-specific cytotoxicity when compared to untransduced NK cells (36.8±12.5% vs. 72.4±24.2% at effector:target ratio of 1:1, n=3). CRISPR/Cas9 mediated knockout of B7-H3 in target cells abolished the difference in CAR vs. no-CAR NK-mediated target killing. When delivered intracranially to CHLA-06 orthotopic xenograft bearing mice, B7-H3 CAR NK cells eliminate tumor cells and prolong survival, whereas unmodified NK cells did not (Figure 1. log-ranked median survival = 20 vs. 84 days, p<0.0001). We are currently testing intracranial delivery of our B7-H3 CAR NK against other AT/RT orthotopic xenografts. **Conclusions:** Targeting pediatric brain tumors with an anti-B7-H3 CAR-NK cell therapy may provide a safe and effective treatment for patients who have extremely limited therapeutic options. Direct intracranial injections of cell therapies into pediatric patients is currently being explored in clinical trials. As B7-H3 is a tumor associated antigen found to be overexpressed in a large variety of tumor types, this strategy may also be useful for treatment of other B7-H3 expressing cancers.

Figure 1.



92. An Immune Synapse-Stabilizing Receptor Enhances Sensitivity of CAR- and TCR-Mediated Tumor Targeting

Chiou-Tsun Tsai[1], Jorge Andres Ibanez[2], Norihiro Watanabe[1], Giedre Krenciute[2], Maksim Mamonkin[1]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX,[2]Bone Marrow Transplantation and Cellular Therapy, St. Jude Children's Research Hospital, Memphis, TN

Downregulation of the target antigen is a common mechanism of tumor escape in cellular immunotherapy. Multi-antigen targeting with cytotoxic receptors can reduce the risk of antigen escape but, in many indications, also increases off-tumor toxicity. We have developed an alternative strategy to improve targeting of tumor cells with suboptimal antigen expression without broadening the unwanted off-tumor toxicity. We designed an additional non-cytotoxic receptor that augments immune synapse and boosts signaling from the main antigen receptor (e.g., a CAR or a TCR), thus selectively improving cytolysis of antigen-low tumors. This synapse-stabilizing receptor (SSR) targets a secondary tumor antigen without inducing direct cytotoxicity, thus limiting the cytolysis only to cells expressing the CAR or TCR target. We evaluated this concept in preclinical models of acute myeloid leukemia (AML) where CAR T-cells targeting CLL1 antigen induce robust killing of CLL1-high targets but poor cytolysis of CLL1-intermediate/low leukemia. We designed an SSR targeting CD38, an antigen commonly expressed in acute leukemia but also in critical hematopoietic cells and primitive progenitors thus making direct cytotoxic targeting risky. To augment SSR function and further boost CAR signaling, we attached signaling endodomains derived from IgSF and TNFRSF genes as well as native and modified LAT. We found a modified LAT truncated at the amino acid 177 (modLAT177) produced highest amplification of CAR signaling and strongest cytotoxicity against antigen-low AML lines Molm13 and THP-1 compared to other SSR configurations. SSR co-expression did not affect cytolysis of antigen-high leukemia but augmented production of IFN-γ and IL-2 and enhanced lytic degranulation (measured by GzB and CD107a) and intracellular Ca++ flux in CLL1 CAR T cells upon coculture with antigen-low leukemia. Mechanistically, SSR engagement increased accumulation of F-actin and SLP76/WASP at the T-cell immune synapse and enhanced polarization of the microtubule organizing center (MTOC) and lytic granules demonstrating improved cytolytic immune synapse formation. In a mouse xenograft model of AML (MOLM13), SSR-armed CLL1 CAR T cells produced higher expansion and superior control of circulating leukemia significantly extending mouse survival compared to unarmed CAR T cells (p=0.0023). Of note, SSR-armed CLL1 CAR T cells did not induce cytolysis of normal CLL1-negative CD38+ NK cells or normal CD38+ hematopoietic progenitors in colony-forming assays with CD34+ cord blood cells thus illustrating desired tumor selectivity. To evaluate the benefit of SSR beyond CARs, we paired it with a TCR targeting a pan-cancer antigen survivin (SurTCR). SSR arming significantly enhanced targeting of HLA-low/intermediate AML cell line THP1 by SurTCR T cells while unarmed SurTCR T cells produced minimal cytotoxicity. Notably, SSR expression enhanced lytic degranulation of CD8+ SurTCR T cells and augmented production of IFNγ and TNFα in both CD8+ and CD4+ T cells, whereas unarmed SurTCR only activated CD8+ T cells, suggesting SSR can functionally replace a CD4 co-receptor. Finally, we showed

SSR expression markedly increased cytotoxicity of T cells redirected by a leukemia-targeting bispecific T-cell engager (BiTE) highlighting strong augmentation TCR-mediated tumor cytolysis. Collectively, these results demonstrate an effective strategy to boost sensitivity of CAR- and TCR-redirected T cells against tumors with suboptimal expression of the target antigen without broadening off-tumor toxicities with a non-cytotoxic synapse-stabilizing receptor.

93. Transduction with Bicistronic CD20 CAR with NIS Reporter Allows PET/CT Visualization of CAR-T Cell Trafficking *In Vivo*

Brynn B. Duncan[1], Justin Mirazee[2], Christopher Chien[2], Kevin Quiroz[1], Shiqin Judy Yu[1], Sogun Hong[1], Naomi Taylor[2], Noriko Sato[3], David Young[1], Cynthia Dunbar[1]

[1]TSCBB, NHLBI, NIH, Bethesda, MD,[2]Pediatric Oncology Branch, NCI, NIH, Bethesda, MD,[3]Molecular Imaging Branch, NCI, NIH, Bethesda, MD

Background: Tracking CAR-T cell expansion and persistence in patients with hematologic malignancies is currently limited to peripheral blood and marrow sampling, providing limited insights into how and where CAR-T cells traffic and persist beyond these compartments. Costimulatory endodomains that are known to influence CAR biology have unclear downstream effects on CAR-T cell trafficking. The sodium-iodide symporter (NIS) is a plasma membrane glycoprotein that actively uptakes iodide and iodide analogs that can be transduced into T cells along with a CAR, allowing real-time *in vivo* PET/SPECT imaging of CAR-T cells. **Methods**: CAR constructs with specificity for human and non-human primate (NHP) CD20 that contain various costimulatory endodomains (4-1BB, CD28, CD27) were generated via Golden Gate assembly. Rhesus NIS or truncated EGFR (for CAR identification) reporters were inserted downstream via a T2A skip sequence for expression under control of the same EF1a promoter driving CAR expression. *In vitro* cytotoxicity and functional iodide and fluorine-18 tetrafluoroborate (^{18}F-TFB) uptake was tested. NSG mice bearing CD20$^+$ Raji lymphoma xenografts were treated with CAR-T cells and imaged by PET/CT weekly. Syngeneic NHPs treated with autologous NIS-expressing CAR-T cells were monitored for cytokine release syndrome (CRS), neurotoxicity, and CAR-T cell expansion in response to endogenous and autologous reinfusion of B cells. NHP recipients of NIS-expressing CAR-T cells undergo weekly PET/CT imaging. **Results**: Successful bicistronic lentiviral transduction of both human and NHP T cells demonstrated equivalent *in vitro* CD20 antigen-specific cytotoxicity regardless of NIS expression and demonstrated NIS-specific iodide and ^{18}F-TFB uptake. NIS-expressing CAR-T cells homed to Raji lymphoma in NSG mice with variable trafficking kinetics and *in vivo* tumor control depending on the costimulatory domain. CD20/41BB/NIS most quickly and effectively induced remission (max SUV_{mean} day 12; day 45 survival 83%, n=6) followed by CD20/CD27/NIS (max SUV_{mean} day 18; day 45 survival 67%, n=6) and CD20/CD28/NIS (max SUV_{mean} day 33+; day 45 survival 33%, n=6). An NHP (NHP01) was given 2.72x10^6/kg CAR+ T cells consisting of both CD20/41BB/EGFRt and CD20/4-1BB/NIS CAR-T cells. NHP01 developed CRS, B cell aplasia, CAR-T cell expansion (ddPCR analyses of PBMCs for EGFRt and NIS), and CAR-T cell-associated cytokine production. B cell reinfusion at day +17 did not induce substantial CAR re-expansion or cytokine elevation. No neurotoxicity was observed. Another NHP (NHP02) received CD20/41BB/NIS CAR+ T cells (5.49x10^6/kg) and will undergo weekly PET/CT imaging as well as combined IV and subcutaneous autologous B cell reinfusion. **Conclusions**: CD20 CAR-T cells that express NIS demonstrate unhindered *in vitro* and *in vivo* anti-tumor activity, allowing sensitive and specific visualization of CAR-T cells *in vivo* via PET/CT. In a syngeneic NHP model, 4-1BB-costimulated CAR-T cells expressing NIS expanded as well as those with EGFRt and induced CRS and B cell aplasia. Currently, PET imaging of an NHP treated with CD20/41BB/NIS (NHP02) is underway with plans for another treated with CD20/CD28/NIS (NHP03) in the near future.

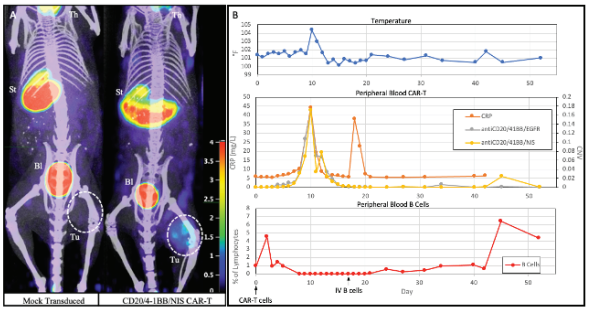


Figure 1. (A) Representative PET/CT image of tumor-bearing NSG mice 8 days after T cell administration. Day -4 subcutaneous challenge with 10^6 CD20+Raji lymphoma cells in the right thigh was followed by 2x10^6 T cells IV on day 0. Increased SUV is noted within the tumor (Tu) of mice treated with NIS-expressing CAR-T cells (right) but not mock CAR-T cells (left). Expected background uptake present in thyroid (Th), stomach (St), and bladder (Bl). (B) Evidence of CRS (top and middle), CAR expansion by ddPCR (middle), and B cell aplasia (lower) in NHP01 post-infusion of CD20/41BB/EGFRt and CD20/41BB/NIS CAR-T cells. Peak expansion and CRS occurred at day 10 with B cell aplasia noted from day 8 to 21.

94. Chimeric Cytokine Receptors Induce Antigen-Specific Activation in Natural Killer Cells Against Acute Myeloid Leukemia

Natalie J. Holl, Ruyan Rahnama, Jun Choe, Megan Zinsky, Danielle Grace Jones, Challice L. Bonifant

Pediatric Oncology, Johns Hopkins University School of Medicine, Baltimore, MD

Background: Chimeric antigen receptor (CAR) T cell therapies have revolutionized the standard of care treatment for B cell malignancies. However, CAR-T cell clinical trials for acute myeloid leukemia (AML) have had discouraging outcomes. As an alternative effector to T cells, natural killer (NK) cells are being investigated. NK cells are innately cytotoxic against cancer cells, are readily enhanced with CARs, and, unlike T cells, do not cause graft-versus-host disease. However, NK cells have exhibited a lack of persistence in multiple clinical trials, limiting their efficacy and the relapse-free survival of patients. In order to increase NK cell persistence, we have designed antigen-specific chimeric cytokine receptors (CCRs) based on the interleukin-15 receptor (IL-15R) to stimulate prolonged NK cell survival. **Methods:** Artificial receptors were designed with AlphaFold using sequences from either the native IL-15Rβ chain or the common gamma chain (γc) and single chain variable fragments to target CD123, an AML-associated antigen. Each CCR chain was subcloned into an expression vector with a fluorescent protein, GFP or mScarlet (mSc), to create various CCR-γc-GFP and CCR-IL-15Rβ-mSc constructs. Primary NK cells were expanded from peripheral blood mononuclear cells with irradiated K562.mbIL15.41BBL feeder cells following magnetic depletion of T cells. NK cells were then transduced with retroviral vectors. After testing constructs with promising predicted *in silico* folding, the optimal CCR pair was chosen for further testing based on

surface expression and antigen-binding. Antigen-specific signaling of CCRs was evaluated by stimulating CCR-NK cells with immobilized recombinant human CD123 (rhCD123) and detecting activation of STAT5 by phosphorylation (pSTAT5) using Western blot. Antigen-specific cytotoxicity was assessed in 18-hour bioluminescence assays with firefly luciferase-expressing target cell lines. Non-transduced NK cells were used as controls in all experiments. **Results:** CCR chains were expressed at similar levels individually (mean ± SD; CCR-γc-GFP, GFP+%: 61.7 ± 16.5%; CCR-IL-15Rβ-mSc, mSc+%: 63.3 ± 15.4%) and together (co-transduced, GFP+mSc+%: 53.2 ± 13.3%). CCR single chain pSTAT5 was not detectable with rhCD123 stimulation and while NK cells co-transduced with both receptors displayed low levels of tonic signaling, stimulation with rhCD123 resulted in a 2.1x fold increase in signal. Co-transduced NK cells displayed significantly higher cytotoxicity versus MOLM-13 (CD123+) at every effector-to-target ratio except the lowest compared to NKs expressing single CCRs and non-transduced cells (1:10 $p = 0.7935$, 1:5 $p = 0.0309$, 1:2 $p < 0.0001$, 1:1 < 0.0001 vs. non-transduced). NK cells did not show different cytotoxicity against Raji (CD123-) (all $p > 0.8145$ vs. non-transduced). **Conclusions and future directions:** NK cells expressing our IL-15R-based CCRs demonstrate antigen-specific signaling and cytotoxicity dependent on expression of both our chimeric IL-15Rβ and chimeric γc. We are actively evaluating the phenotype of NK cells expressing these receptors in long-term *in vitro* assays, specifically as relates to persistence and activation. These results will provide insights to the signaling biology of NK cells in the context of engineered cytokine-dependent activation. We next plan *in vivo* experiments in our established xenograft models of human AML to track NK cell persistence and disease clearance.

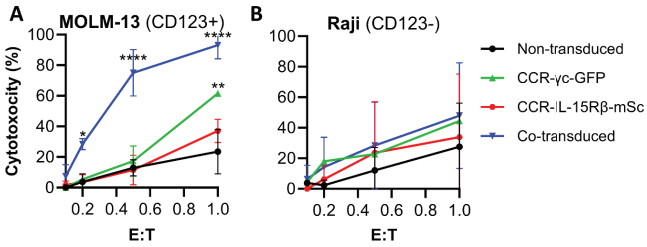


Figure 1. CCR-NK cells display antigen-specific cytotoxicity. **A)** NK cells were co-cultured with CD123+ and **B)** CD123- cell lines at various effector-to-target ratios (E:T). Cytotoxicity was quantified as the percentage of decreased bioluminescent signal after 18 hours of co-culture with firefly luciferase-expressing target cells compared to target cells not exposed to NK cells. * $p \leq 0.05$, ** $p \leq 0.01$, or **** $p \leq 0.0001$ vs. non-transduced. N = 2 unique NK cell donors, 3 technical replicates per donor.

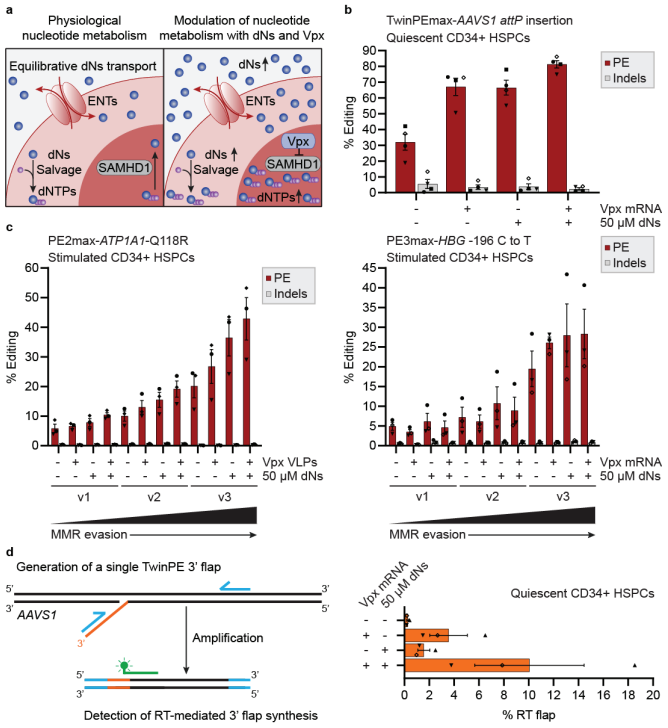
Base Editing and Prime Editing I

95. Nucleotide Metabolism Constrains Prime Editing in Hematopoietic Stem and Progenitor Cells

Sébastien Levesque[1,2,3,4,5], Andrea Cosentino[1,2,5,6], Archana Verma[1,2,3,4,5], Pietro Genovese[1,2,5], Daniel E. Bauer[1,2,3,4,5]

[1]Division of Hematology/Oncology, Boston Children's Hospital, Boston, MA,[2]Department of Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA,[3]Harvard Stem Cell Institute, Cambridge, MA,[4]Broad Institute, Cambridge, MA,[5]Harvard Medical School, Boston, MA,[6]Milano-Bicocca University, Milan, Italy

Therapeutic prime editing (PE) of hematopoietic stem and progenitor cells (HSPCs) holds great potential to correct pathogenic mutations causative of severe blood disorders. While recent advances have improved the technology, efficient prime editing remains challenging in primary hematopoietic cells. Since SAMHD1 has been identified as an antiviral factor restricting the nucleotide pool available for reverse transcription (RT) and virus replication, we hypothesized that modulating this metabolic pathway could enhance RT and thus prime editing in HSPCs. We devised strategies to modulate nucleotide metabolism by supplementing deoxynucleosides (dNs) and delivering the viral accessory protein Vpx using virus-like particles (VLPs) or mRNA. Exogenous dNs supplementation and Vpx-mediated proteasomal degradation of SAMHD1 markedly increased PE efficiency at seven loci in HSPCs from seven different healthy donors, achieving up to 68%, 77%, and 86% editing using the PE2, PE3, and TwinPE systems, respectively. Of importance, maximal enhancements were observed with epegRNAs designed to evade DNA mismatch repair. To substantiate our findings, we tested whether the limited level of dNTPs constrains RT in HSPCs. We installed the V223M mutation known to increase dNTP affinity to the M-MLV RT. This additional approach improved PE at three different loci, further expanding the toolkit available to overcome this metabolic constraint. We then developed a ddPCR assay to measure PE-mediated RT based on synthesis of a non-homologous 3' DNA flap that does not integrate to its target locus. Using this approach, we confirmed that modulating nucleotide metabolism increases PE flap synthesis by an average of 37-fold in quiescent HSPCs from three different donors. Altogether, modulation of nucleotide metabolism should enhance prime editing to facilitate the clinical translation of gene therapies for severe blood disorders through engineering quiescent cells. This work highlights a key metabolic restriction for therapeutic prime editing of primary cells and could help design future approaches to engineer HSCs in vivo.



Vpx and deoxynucleoside (dN) supplementation enhance prime editing in CD34⁺ HSPCs. (**a**) Schematic representation of the modulation of nucleotide metabolism. (**b**) PE and indel quantification as determined by CRISPResso2 analysis from amplicon sequencing. HSPCs were electroporated with Vpx and TwinPEmax RNAs targeting *AAVS1* and cultured in the presence or absence of 50 µM each dNs. Genomic DNA was harvested 3 days post-nucleofection. $n = 4$ independent biological replicates. (**c**) Same as in (**b**) with PE2max and PE3max RNAs targeting *ATP1A1* or the *HBG1/2* promoters using (e)pegRNAs designed to progressively evade DNA mismatch repair (MMR). $n = 3$ independent biological replicates. (**d**) Percentage of allele harboring the PE flap relative to a genomic reference as determined by ddPCR. $n = 3$ independent biological replicates.

96. Prime Editors Precisely Correct Pathogenic *RHO* Mutations and Preserve Photoreceptors *In Vivo*

Emily Brown, Abhishek Dass, Vanessa Yanez, Manuel N. Tapia, Bilva Burkule, Shreya Malhotra, Rafaela Mendes, Peter Jeffrey Chen, Billy Law, Jeff Jones, Yunchao Gai, Yi Pu, Jacob Stewart-Ornstein, Jia Tay, Vivian Choi, Deepak Reyon, Jeremy Duffield

Prime Medicine, Cambridge, MA

Mutations in the rhodopsin gene result in *RHO*-mediated Retinitis Pigmentosa (*RHO*-RP), characterized by progressive degeneration of rods and cones photoreceptors. Children or young adults develop loss of night and peripheral vision, ultimately losing central vision. Multiple gene therapy approaches have been investigated in treating patients with *RHO* p.P23H. However, due to the potential toxicity of overexpression of wildtype Rhodopsin (RHO) by gene transfer and limited options of gene correction by other gene editing (GE) mechanisms, there has only been incremental clinical success. Prime Editing is a next generation GE technology that can precisely correct more than 90% of all pathogenic human mutations without the need for DSB, with minimal byproducts at the edit site, minimal off-target activity, or risk of large chromosomal alterations or genotoxicity sometimes observed with CRISPR-Cas9. This approach potentially restores WT RHO expression in its endogenous locus and could address patients with mutations other than p.P23H. We developed a dual AAV system to deliver Prime Editor components (Dual AAV PEs) to photoreceptors via subretinal (SR) injection. Prime Editors (PEs) precisely correct prevalent disease-causing autosomal dominant (ad) mutations in *RHO* including p.P23H and a mutation hotspot located at the C-terminus of the protein that includes 18 pathogenic mutations, including *RHO* p.V345L and p.P347L, which could potentially benefit ~60% of *RHO*-mediated adRP patients. Comprehensive high throughput screening led to the identification of potent PEs to precisely correct either p.P23H or all 18 mutations in the mutational hotspot *in vitro*. Early PE candidates were tested in mutant or patient cell lines and human retinal explants using a Dual AAV PE, where we observed editing efficiencies as high as 40%. To test PE *in vivo*, we generated humanized mouse models with the mutant human *RHO* gene (with 5-point mutations) replacing the mouse *Rho* gene. Dual AAV PEs were injected subretinally to 6-8 weeks old humanized mice, resulted in >60% of photoreceptors with precise correction of p.P23H or p.V345L/p.P347L mutations within the transduced area. To assess if Prime Editors impacted disease progression, we generated humanized *RHO* p.P23H animals where photoreceptors degenerate rapidly after birth. Six- and 13-weeks post injection of Dual AAV PEs, animals were evaluated for mutational correction and for photoreceptor preservation. The transduced regions of the retina showed significant preservation by quantitative histological evaluation, without evidence of inflammation or toxicity while the untransduced area showed complete degeneration of the photoreceptor layer. Separately, Dual AAV PEs were tested in human retinal explants. Photoreceptor genomes were analyzed using Prime Medicine's comprehensive genome-wide off-target assays. Preliminary results have not identified any off-target edits or AAV genome integrations. The AAV Prime Editor platform effectively and safely delivers PE components to photoreceptors with high efficiency, precisely corrects pathogenic mutations that cause adRP and halts retinal degeneration in humanized mice. Genome-wide preliminary off-target analyses in human photoreceptors show no evidence of unintended edits. The AAV Prime Editor platform has the potential to be applied to additional retinal diseases by using other the Prime Editor guide RNA sequences.

97. In Utero and Postnatal Base Editing for a Metabolic Liver Disease in Mice and Nonhuman Primates

Ana Maria Dumitru[1], Marco Carpenter[1], Madelynn Whittaker[2], Cara Berkowitz[1], Valerie L. Luks[2], Nicole Kus[1], Rosa Choi[1], Nicholas F. Archer[1], William Johnston[3], Arjit Biswas[1], Julie Yeo Su Li[4], Yiping Fan[4], Xiao Wang[2], Mohamad Gabriel Alameh[1], Jerry Chan[5], Kiran Musunuru[2], William Peranteau[6]

[1]CHOP, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, PA,[3]Children's Hospital of Philadelphia, Philadelphia, PA,[4]NUS, Singapore, Singapore,[5]KK Women's and Children's Hospital / Duke NUS Medical School, Singapore, Singapore,[6]The Children's Hospital of Philadelphia, Philadelphia, PA

A number of genetic diseases amenable to therapeutic gene editing cause pathology before or shortly after birth. In utero gene editing has the potential to mitigate disease before the onset of this pathology. Moreover, normal developmental properties of the fetus including the small size, immature immune system, and abundant, accessible progenitor cell populations are hypothesized to facilitate efficient gene editing. Genetic diseases affecting the liver, including metabolic liver diseases, are some diseases that may benefit from in utero editing. As an example, we have chosen to evaluate a therapeutic base editing strategy for hereditary tyrosinemia type 1 (HT1) applied either before or after birth. HT1 is an autosomal recessive metabolic liver disease in which mutations in the *FAH* gene, the final step in the tyrosine catabolic pathway, result in accumulation of toxic metabolites and subsequent liver failure with an increased risk of hepatocellular carcinoma. The pathology can begin before birth. Current treatment for HT1 includes daily dosing of nitisinone which inhibits the HPD enzyme upstream in the tyrosine catabolic pathway and prevents the accumulation of toxic metabolites. We hypothesized that adenine base editing to disrupt a splice acceptor or donor site in the *HPD* gene could inactivate the gene as a therapeutic approach for HT1. To address this hypothesis, we screened a number of gRNAs together with ABE8.8 in HuH-7 cells, a human hepatoma cell line. A lead gRNA, HPD20, was chosen based on on-target editing efficiency and off-target editing profile. To assess the ability of this approach to rescue the disease phenotype, gestational day (E) 16 fetal HT1 mice or 8 week old HT1 mice were intravascularly dosed with an LNP containing ABE8.8 mRNA and a mouse gRNA orthologous to HPD20 gRNA. These studies demonstrated efficient on-target editing with survival, liver function and urine metabolites similar to that seen in HT1 mice maintained on nitisinone and significantly improved compared to HT1 mice in which nitisinone was removed, all of which died by 3 weeks off nitisinone. The target sequence of the human HPD20 gRNA is homologous to the sequence present in *Macaca fascicularis*. Thus, we next tested the feasibility of in utero base editing in the fetal nonhuman primate (NHP). LNP_ABE8.8_HPD20 was intravascularly delivered into midgestation *Macaca fascicularis* fetuses. Assessments at short time points after injection demonstrated efficient delivery of the LNP to the fetal liver and moderate editing throughout the liver. To assess a potential benefit of the fetal environment for in vivo editing, we dosed two 3 year old *Macaca fascicularis* NHPs with LNP_ABE8.8_HPD20 at a comparable dose to that given to the fetuses. On-target liver editing was noted although editing levels were approximately 2- to 4.5-fold lower compared to fetal recipients. This study supports a mutation-agnostic adenine base editing approach for HT1 and demonstrates the feasibility of *in utero* liver gene editing via LNP delivery in NHPs, highlighting potential advantages to editing during fetal development in this model.

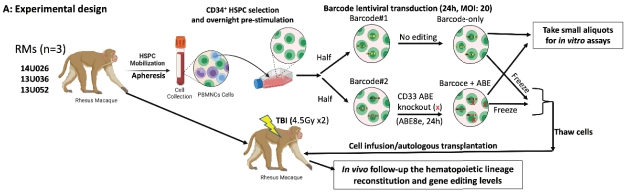
98. The Impact of Base Editing on Rhesus Macaque Hematopoietic Stem Cell Engraftment and *In Vivo* Clonal Behavior

Ashley Gin[1], Chuanfeng Wu[1], Diana Abraham[1], Yifan Zhou[1], Taha B. Hayal[1], Richard Lozano[1], Gregory A. Newby[2], David R. Liu[2], Cynthia Dunbar[1]

[1]National Heart, Lung, and Blood Institute, Bethesda, MD,[2]Broad Institute of MIT and Harvard, Cambridge, MA

CRISPR/Cas9 editing has transformed hematopoietic stem and progenitor cell (HSPC) gene therapies; however, Cas9 generates double-stranded breaks (DSBs), enhancing off-target risks, p53 activation, and HSPC genotoxicity. Base editors (BEs) mitigate these concerns by facilitating single-nucleotide conversions without DSBs. Investigation into BE impact on long-term HSPC engraftment and clonality is necessary to evaluate BEs for clinical HSPC gene therapies. Depleting CD33, a myeloid differentiation marker, protects HSPCs from CD33-targeted immunotherapies without effecting functionality. We evaluated the clonal impact of HSPC CD33-targeted adenine BE (ABE, ABE8e variant) in a competitive rhesus macaque (RM) autologous HSPC transplantation (HSCT) model (n=3). Following total body irradiation, RMs were infused with two genetically barcoded $CD34^+$ HSPC populations: Lib#1 was unelectroporated (Barcode-only arm) and Lib#2 was electroporated for CD33-targeted ABE (Barcode+ABE arm) (Fig. 1A). ABE efficiency in the Barcode+ABE arm varied from 11.7-88.2% in the 3 RMs (Fig. 1B). *In vitro*, no significant differences were observed in cell growth, viability, CFU plating efficiency, or lineage distribution between both arms. Following HSCT, blood cells were collected weekly to track engraftment via GFP expression and ABE alleles and to follow clonal diversity via quantitative barcode retrieval. *In vivo,* GFP marking peaked earlier than the editing level in granulocytes (Gr), suggesting slower engraftment of Barcode+ABE cells (Fig. 1C). CD33 ABE levels peaked at expected levels 2-3 weeks post-HSCT, but markedly decreased over time, indicating deficient long-term ABE cell engraftment. Unexpectedly, in the 2 RMs receiving ABE cells with the highest editing frequencies (13U036 & 13U052), the overall GFP^+ population, consisting of Barcode+ABE and Barcode-only cells, declined in tandem with the decrease of edited cells. In contrast, GFP^+ cells in the less efficiently edited RM (14U026) maintained consistently high GFP^+% for up to 14 months (m) despite a significant decrease in edited cells. These findings suggest high ABE efficiency may induce toxicities that impact infused HSPC engraftment via niche effects or paracrine signals, requiring further investigation. In RM 13U036, 1m $CD33^{+/-}$ Gr and 3 and 5m $GFP^+CD33^{+/-}$ Gr were sorted for barcode clonal analysis. The majority of $CD33^-$ Gr showed expected CD33 ABE (Fig. 1D), and the high-contributing clones revealed distinct clonal patterns between $CD33^+$ and $CD33^-$ Gr (Fig. 1E). Initially, short-term engraftment was polyclonal with high diversity in both $CD33^+$ lib#1 (Barcode-only) and $CD33^-$ lib#2 (Barcode+ABE) Gr. However, clonal diversity decreased over time, with $CD33^-$ lib#2 Gr's diversity declining the most, exhibiting oligoclonality (Fig. 1E-F). Despite Barcode+ABE cells maintaining normal characteristics *in*

vitro, our preliminary findings indicate reduced long-term engraftment potential of Barcode+ABE cells *in vivo*. These findings suggest high ABE levels may enhance HSPC toxicity, increasing risk for HSPC loss and oligoclonal hematopoiesis. While the combination of lentiviral transduction and ABE8e may have added to HSPC stress, these findings provide relevant information for the optimization and safe clinical development of ABE-based gene and cell therapies.



B: *In vitro* transduction, gene editing efficiency and monkey transplantation parameters

Monkey ID	Barcode-only Library ID	Barcode+ABE Library ID	Barcode-only transduction efficiency (GFP+%)	Barcode+ABE transduction efficiency (GFP+%)	Barcode+ABE CD33 ABE editing efficiency (%)	Barcode-only infused cell number (million)	Barcode+ABE infused cell number (million)	Infusion product total cell number (million)	Infusion product GFP+%	Infusion product overall editing level(%)	Cell dose million/kg
14U026	Barcode lib#1	Barcode lib#2	37.9%	49.5%	11.7%	26.2	18.8	45	n/a*	4.8%	5.3
13U036	Barcode lib#1	Barcode lib#2	31.1%	32.6%	79.2%	42	12	54	31.8%	23.9%	4.5
13U052	Barcode lib#1	Barcode lib#2	20.6%	22.4%	88.3%	17.5	7.5	25	21.0%	26.5%	2.2

*n/a: Infusion product GFP level was not measured.

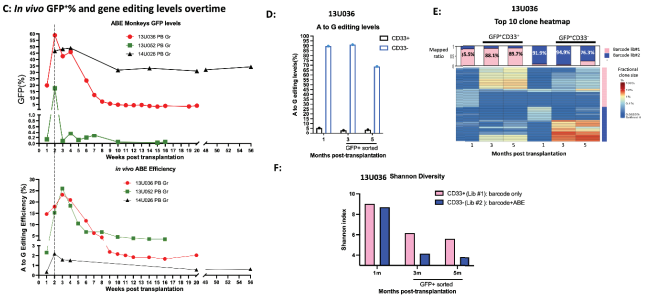


Figure 1: *In vivo* tracking of CD33 KO base edited and barcoded hematopoietic stem and progenitor cells (HSPCs) indicates poor engraftment and oligoclonality induced by adenine base editing (ABE) in rhesus macaque (RM) autologous transplantation model. (A) Experimental design of competitive Barcode-only and Barcode+ABE autologous RM transplantation model. **(B)** *In vitro* transduction, gene editing efficiency, and monkey transplantation parameters. **(C)** GFP+% levels (upper panel) and the ABE editing levels (lower panel) overtime post-transplantation. **(D)** Editing levels of CD33$^{+/-}$ Granulocytes (Gr) subsets overtime; the 1m Grs were sorted only based on CD33 expression, and the 3m and 5m GFP^{+} CD33$^{+/-}$ Grs were sorted. **(E)** Barcode clonal tracking of the CD33$^{+/-}$ Gr subsets overtime. The bar plots on top of the heatmap show the normalized proportion of the barcode libraries (lib) indicated by their percentage of mapping ratios. The pink bars show the lib#1 percent from the barcode sequencing reads in a sample; the blue bars show the lib#2 percent. In the CD33^{+} Gr samples, most of the barcode presented were from lib#1 (Barcode-only), and the majority barcodes in the CD33^{-} Gr samples are the lib#2 (Barcode+ABE). The lower barcode heatmap is the top 10 high contributing clones relative fractional clone size heatmap; each row is a barcoded clone, each column is a sample, the color scale shows the relative clonal size, redder indicates a bigger clone size. The pink/blue bar on the right of the heatmap indicated the barcodes in the heatmap belong to the corresponding lib. **(F)** Clonal diversity of Barcode+ABE and Barcode-only cells was calculated by the Shannon Diversity Index of the retrieved barcodes.

99. Base Editors Provoke Non-Predictable Chromosomal Translocations and Off-Target Editing

Manuel Rhiel[1], Kerstin Geiger[1], Masako Kaufmann[1], Julia Klermund[1], Melissa Whitehead[1], Viviane Dettmer-Monaco[1], Ala' Dibas[1], Carla Fuster-Garcia[1], Geoffroy Andrieux[1], Claudio Mussolino[1], Melanie Boerries[1], Tatjana Cornu[2], Toni Cathomen[1]

[1]Medical Center - University of Freiburg, Freiburg, Germany,[2]Medical Center – University of Freiburg, Freiburg, Germany

Base editing has emerged as a promising alternative to CRISPR-Cas nuclease-based genome editing, in part because of its promise of reduced genotoxicity. Here, we performed a comprehensive genome-wide analysis of base editing-induced off-target effects. Primary human T cells were edited at four genomic loci (CCR5, COL7A1, EMX1, FANCF) using side-by-side CRISPR-Cas9 nuclease, an adenine base editor (ABE8e), one of three cytosine base editors (CBEs), or Cas9 nickase. We used CAST-Seq to identify gross chromosomal aberrations and off-target sites (OTs), followed by rhAmp-Seq to evaluate on-target activity (44-84% modified alleles) and mutagenic effects at >100 nominated OTs. CAST-Seq revealed large (>200 bp) on-target aberrations ranging from 45% for Cas9 to 5% for ABE, 2-7% for CBEs, and 0% for nickase-edited cells. While ABE8e editing induced few translocations, more than 10% of the alleles were modified in 17 of the 40 identified OTs. In contrast, chromosomal translocation events in some CBE-treated cells exceeded that found in Cas9-edited T cells by 3-fold. Furthermore, extensive C-to-T conversion (≤99% of alleles) and mutagenesis (≤7% of alleles) were detected in T cells edited with a hyperactive CBE, whereas recently developed CBE variants (TadCBEd or CBE-T1.52) showed only marginal OT activity. In conclusion, our study revealed the genotoxic potential of ABE8e and the hyperactive evoCDA1-BE4max. These base editors induced mutagenic OT editing at sites that were not altered by CRISPR-Cas9 nucleases complexed with the same gRNA, suggesting that the mechanism of gRNA-dependent OT activity of base editors is different from that of CRISPR-Cas nucleases.

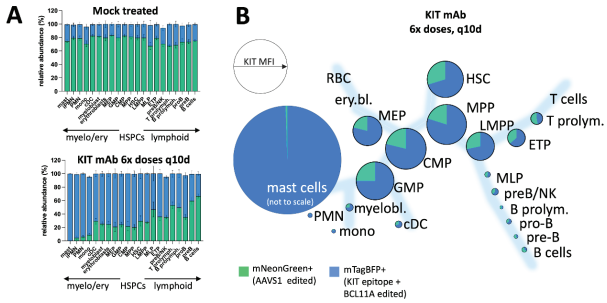
100. Epitope Editing Combined with Extended Schedule Anti-KIT Antibody Treatment Enhances Immune-Based *In Vivo* Selection of Multiplex Gene-Engineered Cells

Gabriele Casirati[1], Adele Mucci[2], Marta Freschi[1], Mohammed S. Mahmoud[1], Jan-Phillipp Gerhards[2], Andrea Cosentino[1], Jing Zeng[2], Christian Brendel[2], Daniel E. Bauer[3], Pietro Genovese[4]

[1]Hematology/Oncology, Boston Children's Hospital / Dana Farber Cancer Institute, Boston, MA,[2]Hematology/Oncology, Boston Children's Hospital, Boston, MA,[3]Boston Children's Hospital / Harvard Medical School, Boston, MA,[4]Hematology/Oncology, Boston Children's Hospital / Harvard Medical School, Boston, MA

Hematopoietic stem/progenitor cell (HSPC) transplantation (HSCT) offers curative options for conditions for which the substitution of endogenous hematopoiesis with gene-corrected cells can halt the pathogenic process. Yet, short and long-term effects of genotoxic conditioning remain a barrier to a wider application of HSCT and gene therapies. Whereas monoclonal antibodies (mAbs) targeting HSPCs have been proposed as alternative to chemo/radiotherapy, their pharmacokinetics hamper effective clinical use due to risk of on-target depletion of transplanted HSPCs, leading to increased risk of graft failure or incomplete myeloablation. We previously demonstrated that precise editing of the targeted epitope in HSPCs can endow hematopoietic cells with selective resistance to mAbs without affecting stem cell functionality. We identified amino-acid changes in the extracellular domain of KIT that abrogate the binding of a therapeutic mAb (Fab79D) without affecting surface expression, ligand affinity and downstream signaling. We exploited adenine base editing (BE) to efficiently (~80%) introduce these mutations in CD34+ HSPCs, which preserved long-term multilineage repopulation. As epitope-editing can be achieved through BE, it is well suited for combination with additional therapeutic genome modifications, such as the disruption of *BCL11A* erythroid enhancer to enforce fetal hemoglobin (HbF) expression, which can provide therapeutic benefit for patients affected by sickle cell disease. We multiplexed KIT and sgRNAs targeting the +55 and +58 BCL11A DHSs and obtained efficiencies comparable to single edits, while achieving upregulation of HbF proportional to the number of disrupted WGATAR motifs (up to 80%). Critically, we observed high degree of biallelic co-editing, with 60% HSPCs bearing biallelic KIT edits and at least 3 disrupted WGATAR motifs.

By performing a competitive transplant with co-injection of multiplex KIT+BCL11A BE and AAVS1 BE CD34+ cells in NBSGW mice. We observed progressive enrichment of both *KIT* and *BCL11A* edits (~2x) in mice treated with a standard dose-dense Ab regimen when compared to controls. We speculated that optimized Ab schedules with extended exposure to anti-KIT mAb may provide superior results. We performed a second competitive transplant with lentiviral fluorescent marking of the KIT+BCL11A BE (mTagBFP) and AAVS1 BE cells (mNeonGreen), to enable precise tracking by FACS. We treated mice with a dose-dense or extended Ab regimens (6 doses, either every 5 or 10 days). Extended regimens produced markedly improved in vivo selection with near complete replacement (>80%) within progenitor and myeloid/erythroid compartments (FIG.A), which translated to improved HbF increase. The selection was proportional to KIT surface levels (FIG.B) and lineage half-life. As epitope editing eliminates limitations due to Ab on-target toxicity, we can envision innovative, non-genotoxic gene therapy transplant regimens that will achieve therapeutic levels of corrected HSPCs with substantial advantages over alternatives in terms of safety and tolerability.



101. Development of a Prime Edited CD34+ Cell Drug Product for the Treatment of P47phox Chronic Granulomatous Disease

Jack M. Heath[1], Jacob Stewart-Ornstein[1], Christa E. Osuna[1], Ahmad S. Arabiyat[1], Justin G. Tedeschi[1], Maria Collier[1], Julia Kushakji[1], Allen C. Ng[1], Marina Lilieholm[1], Alan Wilhelm[1], Katie Green[1], Hari Prasanna Subramanian[1], Sarah Trusiak[1], Katya Kosheleva[1], Matthew J. Irby[1], Rowshon Alam[1], Joseph Elich[1], Terence Ta[1], Suk See De Ravin[2], Harry L. Malech[3], Barrett J. Nehilla[1], Hetal K. Patel[1], Brian C. Beard[1], Jeremy S. Duffiled[1], Jennifer L. Gori[1]

[1]Prime Medicine, Inc., Cambridge, MA,[2]National Institutes of Health, NIAID, Bethesda, MD,[3]NIH/NIAID Laboratory of Clinical Immunology and Microbiology, Bethesda, MD

Chronic Granulomatous Disease (CGD) is an immunodeficiency caused by mutations in genes encoding proteins of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex. A primary function of the NADPH oxidase is to produce reactive oxygen species (ROS) that kill pathogens to control infection. CGD patients lack a functional NADPH oxidase, causing infections and inflammatory complications. CGD patients' symptoms are managed with antimicrobials, immunosuppression for inflammation, and interferon to boost immune responses. Allogeneic hematopoietic stem cell transplantation (HSCT) is currently the only potentially-curative treatment but is not available to many patients. P47phox CGD is typically caused by a two-nucleotide deletion (delGT) in the *NCF1* gene which encodes the p47 protein of NADPH oxidase. The *NCF1* gene is flanked by two nonfunctional pseudogenes that are homologous to *NCF1* and contain the delGT mutation in *NCF1* of CGD patients. Toward the development of a Prime Edited (PE) CD34+ cell therapy for p47phox CGD, an *in vivo* study was conducted with PE CGD patient CD34+ cells transplanted into immunodeficient mice. At week 16 post HSCT, 80% of bone marrow (BM) engrafted colony forming cells (CFCs) from PE CGD CD34+ cells had ≥ 1 delGT allele corrected and there were no significant differences in human hematopoietic reconstitution between mice engrafted with mock treated and PE CGD CD34+ cells. To determine whether Prime Editing of CGD patient CD34+ cells restored NADPH oxidase activity *in vivo*, BM CD15+ neutrophils were evaluated in an *ex vivo* dihydrorhodamine (DHR) assay which detects ROS. The percentage of DHR+ BM neutrophils from mice transplanted with PE CGD CD34+ cells was significantly increased compared to neutrophils from mice transplanted with mock treated CGD CD34+ cells (Mean ± S. D. % DHR+ of PE vs. mock cells: 67 ± 6% vs. 0.03 ± 0.02%; $p<0.0001$) and was closer to mock treated healthy donor (HD) controls (82 ± 5%). The median DHR fluorescence intensity was not statistically different between PE CGD and HD control cells ($p=0.145$). To evaluate the safety of Prime Editing, chromosomal, off-target, and transcriptional analyses were performed on BM engrafted mock treated and PE CGD CD34+ cells. Chromosomal and genomic analyses revealed no significant differences between mock treated and PE CGD CD34+ cells and no editing at potential off-target sites. Transcriptome analysis showed that engrafted mock treated CGD patient cells had elevated interferon signaling compared to HD and that this was restored to HD levels in PE CGD patient cells. A clinical scale manufacturing process with optimized PE components produced under Good Laboratory Practice was conducted to generate CD34+ cell drug product (DP) for IND-enabling studies. HD CD34+ cells were used for cell process development because they are readily available and have pseudogenes that contain the delGT mutation identical to the target mutation in *NCF1* of CGD patient cells. Four lots of HD CD34+ cell DPs showed similar CD34+ cell purity (98±1%), PE efficiency (74 ± 8% of CFCs with ≥ 1 allele corrected), and viability after editing and a freeze/thaw of the final product (78 ± 3%). These results show that correction of delGT in CGD patient CD34+ cells may provide a safe and efficacious curative treatment for CGD and that the clinical scale manufacturing process supports consistent production of a PE CD34+ cell DP.

AAV Analytical Methods

102. Three-Dimensional Linkage Analysis with Digital PCR for Recombinant Adeno-Associated Virus Characterization Beyond Genomic Titer: Integrity and Identity

Tam Duong[1], Michele Firmo[2], Bingnan Gu[1], Peng Wang[1]

[1]R&D, Lonza Houston Inc., Houston, TX,[2]Global Biologics Technical Development, Lonza Basel Inc., Basel, Switzerland

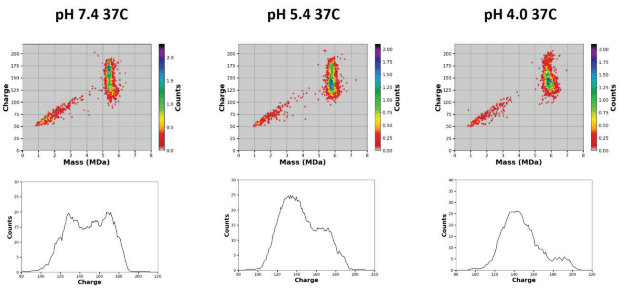
Recombinant adeno-associated virus (rAAV) has emerged as the vector of choice for *in vivo* gene delivery, with numerous clinical trials underway for the treatment of various human diseases. Utilizing rAAV in gene therapy requires a highly precise quantification method to determine the viral genome titer. This quantification process plays a pivotal role in establishing the optimal therapeutic dosage for a rAAV product, both in preclinical and clinical contexts. The conventional single-channel droplet digital PCR (ddPCR) method offers only partial information regarding the viral vector genome titer, lacking insights into its integrity, specifically whether it comprises a complete or partial genome. In our pursuit of further advancing rAAV analysis, we have developed a novel 3D ddPCR assay with advanced 3D linkage analysis. We have designed the three amplicon sites targeting both ends of the viral genome, as well as the center of key therapeutic gene of interest (GOI). This study aims to offer a more comprehensive and insightful assessment of rAAV products. Leveraging our 3D assay platform, we can determine not only the quantity of viral genome titer but also the quality, distinguishing between intact full-length viral genomes and partial ones, and even the identity of key GOI, therapeutic gene component. Importantly, we have developed algorithms to analyze 3D ddPCR linkage titer information. We rigorously tested our algorithms by conducting experiments involving the mixing of seven DNA fragments to represent various AAV viral genome populations, including 3 single partial, 3 double partial, and 1 full-length genomes. Across all 37 tested scenarios, we observed a significant correlation between the percentage of 3D linkage (representing full-length genomes) in the actual input and the model's output. Consequently, our comprehensive AAV analytical package not only offers insights into viral genome titer but also provides valuable information on its integrity and identity. This cost-effective approach, akin to the setup of traditional 1D or 2D dPCR, holds the potential to advance the application of rAAV in cell and gene therapy for the treatment of human diseases.

103. Quantification of pH Induced AAV9 Capsid Alterations and TLR9 Interactions by Charge Detection Mass Spectrometry

Li F. Lin[1], Zachary M. Miller[2], Evan R. Williams[2], David V. Schaffer[3]

[1]Chemical and Biomolecular Engineering, University of California Berkeley, Berkeley, CA,[2]Chemistry, University of California Berkeley, Berkeley, CA,[3]University of California Berkeley, Berkeley, CA

Vectors based on adeno-associated viruses (AAVs) are prevalently used in preclinical and clinical gene therapy, and their efficacy hinges on their delivery efficiency and subsequent transgene persistence following delivery. A recognized challenge for gene delivery is the activation of innate immune responses that trigger T-cell activation to target and eliminate cells expressing therapeutic proteins. In particular, the activation of toll-like receptor 9 (TLR9) is known to detect unmethylated CpG motifs and initiate T-cell activation signals. Acidification within endosomes, a necessary step for AAV transduction, could facilitate premature AAV genome release and subsequent exposure of AAV genomes to TLR9 recognition. Due to the heterogeneity of AAV capsids and the sensitivity of traditional assays, conventional methods fall short in elucidating individual AAV virion behaviors. In this study, we employed charge detection mass spectrometry (CDMS) to quantify the individual AAV9 virion's responses at selected pH points (7.4, 5.4 and 4.0), representing various endosomal stages. Through the analysis of over 1000 individual AAV9 virions per pH point, we observed a subpopulation of AAV9 capsids undergoing significant structural changes, indicated by the lower charge and increased mass, suggesting partial extrusion of viral genomes. Additional CDMS experimental results involving AAV9-TLR9 interactions that provide new insights into a vital aspect of AAV-mediated gene therapy and its immunological challenges will be presented.



104. Detection, Biophysical Characterization, and *In Vitro* Potency Impact of Distinct Empty/Light Capsid Populations in Recombinant AAV Preparations

Surya Simha Addepalli[1], Deepshika Gilbile[2], Wade Wang[2], Sergio Viedma Gonzalez[1], Daniel Barajas[1], John Muroski[2], Alexander Giaramita[1], Tu Nguyen[1], Kiernan Kringen[1], Laura Thacker[2], Ryan Liang[1], Leeann Alferness[1], Marissa Holmbeck[1], John Holtzinger[1], Abhigya Mookherjee[2], Vijay Raja[2], Wes Yonemoto[1], Vikas Bhat[2], Sreekanth Suravajjala[2], Terri Christianson[1], Pooja Agarwal[1], Bartlomiej Blus[1]

[1]Research and Early Development, BioMarin Pharmaceutical, San Rafael, CA,[2]Technical Development, BioMarin Pharmaceutical, Novato, CA

Recombinant Adeno-associated viruses (rAAVs) provide effective treatment for a variety of genetic diseases due to their broad tropism, favorable safety profile, and low immunogenicity. The commercial success of rAAV gene therapies has been, in part, driven by manufacturing advances, allowing for improved product yield, purity, and safety. The most common rAAV byproducts include extra-viral DNA and empty or light capsids that do not contain a copy

of a therapeutic transgene. The increased content of light capsids in rAAV preparations poses a manufacturing burden to meet often high dose requirements in the clinic and potentially elevates the risk of immunogenicity and toxicity. Therefore, pharmaceutical companies are actively optimizing manufacturing processes to reduce or eliminate light capsids from their products. In this study, we employed a combination of affinity and anion exchange chromatography (AEX) to purify light rAAV capsids from mammalian and insect cell harvests. During the AEX step, we observed distinct populations of light capsids for a subset of tested rAAV serotypes for both production systems. These species eluted at varying retention times and were collected separately for subsequent biophysical characterization. Our analyses on one such rAAV serotype produced from mammalian cells revealed significant differences in surface charge, viral protein composition, aggregation propensity, and impurity profiles. Notably, light capsid populations with longer AEX retention times had a higher content of VP1 and N-terminal, surface-externalized VP1 unique regions (VP1u), suggesting compromised capsid integrity. Finally, we evaluated the impact of the identified light capsid populations on the in vitro potency of purified full or heavy rAAVs using multiple cell lines. In summary, our results illustrate the need for in-depth characterization of rAAV light capsids to better assess their impact on product efficacy and safety as well as to inform product-specific manufacturing process development. As the viral gene therapy landscape continues to grow, such detailed impurity characterization may be particularly required for the successful expansion into next generation rAAV therapies.

105. Analytical Technologies in Development of Potency Assay for Gene Therapies

Shiqian Zhu, Jeff Patrick, Jessica Weaver, Andrew Taylor, Leigh Laundon, Bhoomi Jani, Bryan Thacker
BioAgilytix, Durham, NC

Purpose: Gene therapies - including AAVs, engineered viruses and polymer formulated oligonucleotides or plasmids - is among the highest growth area in biopharmaceuticals. They provide great promise as therapeutics but meanwhile present many development challenges due to their complex mechanisms of action (MOA). Potency assays play a pivotal role in the development of biologic therapeutics and face growing regulatory expectations from global regulatory agencies. The assay format depends on MOA of the product, and usually includes analysis of infectivity, expression, or function of the expressed protein. Therefore, it is critical to identify a suitable assay format and the associated analytical technology for potency assay development. Here case studies for representative gene therapies have been provided as examples of matrix approach and phase appropriate strategy for potency assay development, including the selection of analytical technologies, i.e. infectious titer, ELISA, MSD, Flow Cytometry, PCR, and LC-HRMS. We include discussion of phase appropriateness and the challenges and solutions for a variety of gene therapies. **Methods**: In all cases cells were cultured and maintained per vendor recommendation or established conditions. Flow cytometry was performed using a Cytoflex LX and plates were read using a BioTek Synergy Neo2 and Cytation. qPCR was performed on Quantstudio 7 and ddPCR was performed on QX200. LC-HRMS used to determine the expressed protein level was Orbitrap Exploris 240 coupled with Vanquich UHPLC system. Data was processed using Gen5, PLA, or Excel. Relative potency was determined by comparison to reference material. **Results**: Case 1 was an oncolytic Virus. One simple combined assay for infectivity and potency was developed in very early phase. The assay showed adaptable for formulation and dosing concentration changes. Continuous improvement of variation and sensitivity is required. Case 2 was about two AAV products. Each product contains a transgene for protein replacement treatment. However, due to the different MOAs, flow cytometry and In-cell ELISA were selected respectively. Western-Blot was used to screen knockout (KO) cell lines for flow cytometry assay. Upgraded software improved the data processing for In-Cell ELISA assay. Case 3 was a complicated nanoparticle containing plasmid DNA which encodes 3 transgenes. For early phase 3 methods (2 qPCR and 1 MSD) were developed to confirm the expression of 3 genes. When the product moved to Phase 2, two qPCR methods were upgraded to a duplex ddPCR method; MSD method was changed to ELISA method to improve the assay performance. Two additional cell-based assays were developed to test the function/activity of the transgene products. Drug substance (DS) transfection is procedurally different compared to DP transfection, which increased assay development workload, and the cytotoxicity at high dose levels limited the dose range so a linear curve fit was used for data analysis. Case 4 was a mRNA vaccine. This product was in early phase, no antibody available, so the potency assay was developed using LC-HRMS to test the expressed protein(s) as their peptide surrogates. The pilot study results showed promising does response curves. **Conclusions**: Our data and results demonstrated different approaches to the potency assay development for a variety of gene therapies at different phases. The selection of analytical technologies should base on the MOA of the product and should be phase appropriate. In addition to the traditional antibody-based technologies, e.g. MSD/ELISA and flow cytometry, molecular biology technologies, e.g. PCR, have been also playing an important role in potency assay for gene therapies. LC-HRMS provided an option for detection of expressed protein without the requirement of antibody, which could become a platform assay format for mRNA vaccines.

106. Quantification of Full and Empty Particles of Adeno-Associated Virus Vectors via a Novel Dual Fluorescence-Linked Immunosorbent Assay

Sereirath Soth, Mikako Takakura, Masahiro Suekawa, Takayuki Onishi, Takahiro Maruno, Mitsuko Fukuhara, Yasuo Tsunaka, Tetsuo Torisu, Susumu Uchiyama
Biotechnology, Osaka University, Suita, Japan

Background: The adeno-associated virus (AAV) vector is an attractive platform for gene therapy because of its low immunogenicity and non-pathogenicity. During the upstream process, both AAV vectors which do not contain DNA, empty particles (EPs), and AAV vector which contain DNA, full particles (FPs), are generated. It is difficult to completely remove EPs by purification because the physicochemical properties of EPs are only slightly different from those of FPs. Therefore, analysis and control of AAV vector purity are crucial for successful developments of AAV vector. Some analytical methods are available to assess the contents of FPs and EPs. Analytical ultracentrifugation (AUC) is the gold standard for characterizing AAV vectors and can quantify FPs, EPs with high precision. Mass photometry (MP) is a method that measures the mass of individual particles and provides

the percentage of each kind of particle against total counts (% counts). However, these analytical methods have limitations. For example, it is burdensome that prior purification is required before the analyses. A combination of enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR), which do not require purification before analysis, is a conventional method to quantify capsid and genome titers and to calculate EP/FP ratios. However, the combination of ELISA and PCR is subject to inherent drawbacks of error and variability because it relies on data from two independent quantitative analyses that use different mechanisms, and FPs and EPs must be quantified separately using non-identical samples. **Result:** In this study, we established a dual fluorescence-linked immunosorbent assay (dFLISA) that uses two different fluorescent dyes to quantify capsid and genome titers in a single analysis. After the addition of a secondary antibody conjugated to one fluorescent dye, the plate is subjected to heat treatment to release the genome from the capsid before addition of the second fluorescent dye. In dFLISA, capture of AAV particles, detection of capsid proteins, and release and detection of the viral genome are performed in the same well.Our study showed that the concentrations of EPs and FPs determined by dFLISA were comparable to those determined by AUC. The FP/EP ratios determined by dFLISA were consistent with the expected values. Furthermore, the concentrations of EPs and FPs in crude samples, which contain host cell DNA and proteins, were successfully obtained by dFLISA. Another advantage of dFLISA is that dFLISA can be readily adapted to measure other AAV serotypes and AAV vectors with different genome lengths. **Conclusion:** A novel analytical technique that allows the accurate and precise measurement of the abundance of both EPs and FPs, and the full/empty capsid ratio was developed in this study. The correlation between dFLISA and AUC indicated the reliability of the dFLISA results for both full and empty capsids. The dFLISA was also comparable or more accurate/precise compared with orthogonal techniques, including MP and a combination of dPCR and ELISA. In addition, dFLISA showed significant potential for evaluating the capsid and genome titers of unpurified samples and diverse AAV vector serotypes. These attributes render dFLISA an invaluable tool for developments of AAV-based gene therapies.

107. Analysis of Heterogeneous, Unpurified Gene Therapy Formulations by High Throughput Charge Detection Mass Spectrometry

Benjamin E. Draper, Lohra Mickelle Miller, Martin Jarrold

Megadalton Solutions, Inc., Bloomington, IN

Gene therapies produced in cells are expected to play an important role in emerging therapeutics. These biologics are generated using transfected cell lines with a major portion of the development cost associated with purifying the target drug from the host cell components. In the case of recombinant adeno associated virus (rAAV), typical purification strategies can involve a combination of ultracentrifugation, affinity capture and ion exchange chromatography to enrich and isolate the target drug. It is important to understand the quality of the product and the effects of different purification strategies at each stage. Charge detection mass spectrometry (CD-MS) has been shown to be a robust analytical method orthogonal to analytical ultracentrifugation for empty/partial/full quantification of AAV capsid heterogeneity for purified samples. In addition, intact mass measurements from CD-MS can identify the average viral protein ratio (VP1:VP2:VP3) and heterogeneity of the packaged DNA. Here, we show how advances in the throughput and dynamic range of CD-MS analysis have enabled the high resolution, multi-attribute analysis of purified rAAV to be applied to upstream, unpurified samples. The single particle analysis of mass and charge by CD-MS can be leveraged to characterize excess low molecular weight material, free DNA, and excess surfactant through the entire purification life cycle of rAAV.

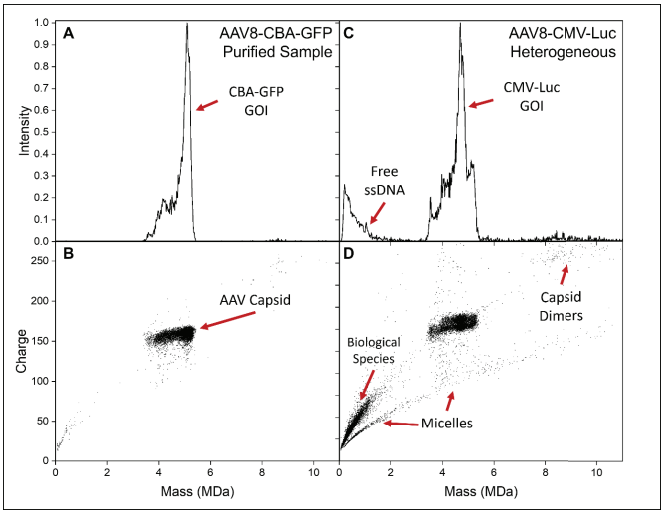


Figure 1 shows the CD-MS analysis of two samples, a well purified AAV8-CBA-GFP sample (a-b) and a representative in-process sample from AAV8-CMV-Luc (c-d). The mass histogram of the AAV8-CBA-GFP (Figure 1a) shows mostly full peak with some partial genomes and a small peak at residual empty capsid. The charge vs mass spectrum (Figure 1b) shows a well-defined charge population with small amounts of free protein aggregates at low molecular weight. The AAV8-CMV-Luc mass histogram (Figure 1c) has a much greater abundance of molecular weight smaller than the capsid with a resolved peak at the mass of the gene of interest (GOI). Even more information can be gleaned from the charge vs mass scatter plot (Figure 1d) where two separate charging bands can be seen with one attributed to biological species and one attributed to micelle species from non-ionic surfactant.

108. Rapid Optimization of AAV Production: Integrating Custom Design of Experiment, Cutting-Edge Production and Analytical Methods in Advancement of Gene Therapies

Tricia S. Jennings, Austin E. Smith, Gretchen Smith

Advanced Medicine Partners (AMP), Durham, NC

In the past decade adeno-associated viral vectors (AAV) have become a widely accepted therapeutic modality and a cornerstone of gene therapy. With continuous process improvements, optimized cell lines, and improved raw materials such as plasmids and transfection reagents, AAV production is evolving at a relentless pace. Amidst these impressive advancements, the pursuit of a repeatable process that yields high productivity and purity at scale remains a challenge. Transient transfection of HEK293 cells with three plasmids; including

the transgene flanked by ITRs, the packaging plasmid with the rep and cap genes, and helper plasmid for AAV lifecycle genes, not only offers a straightforward production method but also grants the flexibility to substitute plasmids. This production model provides significant advantages for rapidly assessing new genes of interest (GOI), AAV serotypes, and engineered capsid manufacturability. Nevertheless, differences in plasmid construct design and AAV serotype can result in diverse outcomes impacting productivity, product purity (such as capsid distribution), and unexpected losses in downstream purification steps. Given the unmet patient needs and the ongoing improvements in plasmid design, rapid and efficient screening and early optimization methods are critical at the current inflection point of gene therapy. Leveraging extensive expertise with a wide set of GOIs and serotypes, and utilizing the capabilities of our advanced production platform, we have developed a comprehensive Design of Experiment (DOE). For this customization, JMP®, Version 17 was utilized to design a mixture DOE with three mixture variables and two process variables. AAV production occurred in 24 single-use 250mL bioreactors using the automated Ambr® 250 High-Throughput platform bioreactor system. The implementation of the DOE and Ambr® approach ensures a high-quality experiment with a notable level of consistency and confidence, as indicated by statistical significance ($p < 0.0001$) and a high coefficient of determination ($RSq = 0.96$) for genomic titer by ddPCR (gTiter) at lysate. Additionally, the use of minibioreactors with analogous geometry and process controls to large-scale production bioreactors establishes a robust foundation for future scale-up. To further evaluate the DoE and Ambr® approach, selected bioreactors were processed through clarification and capture chromatography to assess productivity and de-risk downstream operations. Within a single DOE, plasmid screening and initial optimization for a GOI were performed, assessing feasibility and scalability, while notably enhancing both the product quantity and quality. The design, coupled with advanced analytical methods, allowed for analysis of lysate samples for genomic titer by ddPCR, total AAV by ELISA (tAAV), residual plasmid DNA by ddPCR, and capsid distribution. Rather than relying solely on the estimated full-to-total capsid ratio derived from the gTiter/tAAV ratio, our cutting-edge analytical methods enabled the incorporation of more robust capsid speciation data into our model. This experiment delivered rapid and meaningful data transfer to large-scale production within months. This approach can reduce drug product development timelines, costs of goods, and risk throughout the process, which are essential considerations for meeting current patient needs.

Neurologic Diseases II

109. Gene Replacement of Human EPM2A and EPM2B as a Potential Therapy for Fatal Childhood Neurodegenrative Disease, Lafora

Esther Alao

Pediatric Neurology, University of Texas Southwestern Medical Center, Dallas, TX

Background: Lafora Disease (LD) is a fatal progressive myoclonus epilepsy affecting otherwise healthy adolescents and leading to death within 10 years of onset, usually in a massive convulsion. LD is inherited in an autosomal recessive manner and caused by loss of function mutations in genes *EPM2A* encoding the glycogen phosphatase laforin or *EPM2B* encoding laforin's interacting ubiquitin E3 ligase partner malin. The absence of either protein results in a glycogen storage disorder driven by the accumulations of cytotoxic insoluble glycogen molecules called Lafora bodies (LBs), the driver of pathology in LD. LBs are present in all tissues with the brain being the severely affected organ. LBs form in both neurons and astrocytes and drive neurodegeneration and neuroinflammation respectively. Since LD is associated with mutations in either of two genes, it makes it an ideal candidate for gene replacement therapy whereby a functional copy of the mutated genes (*EPM2A* or *EPM2B*) can be delivered to make up for deficient proteins. **Hypothesis**: We **hypothesize** that delivering a functional copy of both *EPM2A* and *EPM2B* genes in brain tissue of respective LD Knockout mouse models would stop further formation of lafora bodies and reduce neuroinflammation. **Methods**: Non-pathogenic and neuronal trophic Adeno-associated virus serotype 9 (AAV9) was used as the vector of choice for delivering human *EPM2A* and *EPM2B* genes into the respective LD mouse model. To circumvent the blood-brain barrier (BBB), mice were treated with intrathecal injections of vectors at post-natal day 10. To evaluate the safety of gene therapies, a high titer of AAV9 delivering transgenes were injected into wild type mice and tissues were evaluated for any toxicity via H&E staining one month post treatment. The efficacy of both gene therapies was measured by injecting low and high titers of AAV9 in LD Knockout mice and evaluating outcome measures such as insoluble glycogen, lafora bodies, neuro-inflammatory markers three months post treatment. **Results**: The brain and other peripheral tissues revealed the expression of both laforin and malin following IHC and RNAscope respectively. Safety studies revealed that overexpressed human laforin (*EPM2A*) in wild type mice causes a dose dependent toxicity whereby lafora bodies form in tissues (DRG and Spinal Column) proximal to injection site and are absent in other tissues. Meanwhile, no toxicity was observed in safety study of human malin (*EPM2B*) expression. While both gene therapies presented with some efficacy, *EPM2B* gene replacement had a statistically significant effect wherein there was approximately 25% reduction in insoluble glycogen amounts, lafora bodies and about 50% reduction in the expression of *CXCL10*, a neuro-inflammatory marker upregulated at 3 months old (**Figure 1**). **Conclusions**: Preliminary results reveal a therapeutic potential for human *EPM2B* gene replacement in treating LD. However, to improve efficacy for potential clinical translation, focused ultrasound (FUS) technology would be used to increase the transduction efficiency of AAV9- delivered human *EPM2B*. Furthermore, to determine the mechanism underlying lafora body formation when human laforin is overexpressed in wild type mice, we would be investigating the role of excess laforin phosphatase activity in altering glycogen structure.

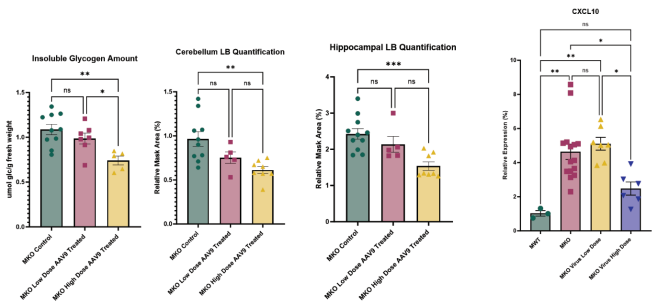


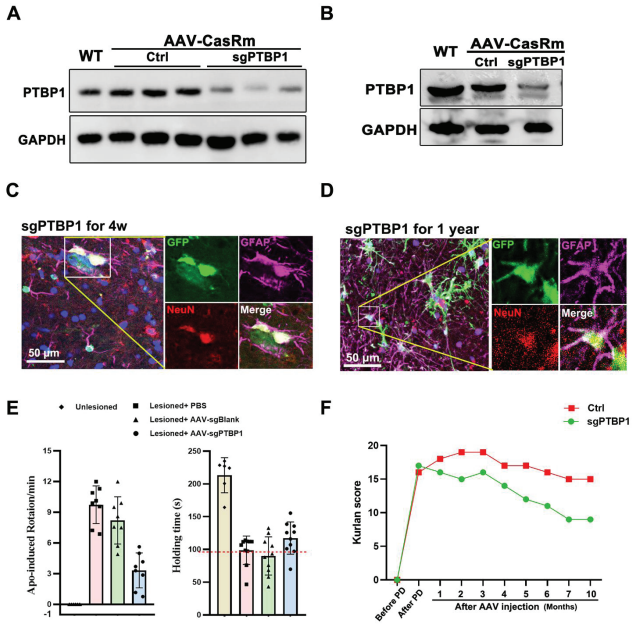
Figure 1: a) Insoluble Glycogen Measurement in EPM2B Knockout mice (MKO Control) and AAV9 treated mice at low and high viral titers b,c) LB quantification in control and AAV9 treated, and d) QPCR data showing MRNA levels of CXCL10 in control and AAV9 treated mice at 3 month of age.

110. CasRm-Mediated PTBP1 Knockdown Improves Motor Functions in Parkinson' Disease Mouse and Non-Human Primate Models

Shimin Wang[1], Piao Zhang[1], Huabao Liao[1], Xin Li[1], Hui Xu[1], Zhendong Xiao[2], Junjiu Huang[3], Junbin Liang[1]

[1]Reforgene Medicine, Guangzhou, China,[2]Biotherapy Center, The Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China,[3]School of Life Sciences, Sun Yat-sen University, Guangzhou, China

Background: Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopamine neurons in the midbrain and reduction of striatal dopamine level, leading to motor dysfunction. In situ induction of glial cells transdifferentiate into neurons has emerged as a promising treatment for PD. Polypyrimidine tract-binding protein 1 (PTBP1) is an RNA-binding protein that plays a key role in regulating nerve-specific splicing, however, its role in regulating the transdifferentiation of astrocytes into neurons remains to be studied. **Methods:** We developed an AAV-mediated PTBP1 RNA targeting therapy (RM-201) based on CasRm, a highly specific novel CRSIRP/Cas13 RNA targeting tool. The potential of RM-201 in treating PD was evaluated both in 6-OHDA-induced PD mouse model and MPTP-induced non-human primate (NHP) model. RM-201 was dosed by intra-nigra injection along with a GFP-expressing AAV that carries the same GFAP promoter as RM-201 (RM-201: AAV-GFP = 15:1). As negative controls, an AAV expressing CasRm and a control guide RNA was also given to mice and the monkey (control: AAV-GFP = 15:1). The performance of movement behaviors of animals were evaluated monthly following treatment. At the end of experiments, we detected the expression of PTBP1 in the midbrain of animals and the existence of GFP/GFAP/NeuN triple-positive intermediate neurons that transdifferentiated from AAV-infected astrocytes. **Results:** PTBP1 expression was significantly down-regulated in the midbrain of both PD mice (Fig. A) and the PD monkey (Fig. B) after a single dose of RM-201 treatment. GFP/GFAP/NeuN triple-positive cells can be captured in the midbrain of RM-201-treated mice (Fig.C) and treated side (right) of the monkey (Fig.D), but cannot be observed in control mice or control side (left). Both apomorphine (APO) induced rotation behavior and rotarod test showed that RM-201 treatment could significantly improve the motor function of PD mice (Fig. E). In PD monkey, Kurlan score of RM-201-treat side decreased significantly compared to the control side and the efficacy is durable (Fig. F), indicating that the motor function of PD monkey can be continuously improved by RM-201 treatment. **Conclusions:** Our results demonstrated that RM-201 can effectively suppress the expression of PTBP1 and induce astrocyte-to-neuron transdifferentiation, which could significantly improve the motor function of PD animals. This study provides direct evidence supporting RM-201 as a potential therapeutic gene editing for the treatment of patients with PD.



111. Unveiling the Therapeutic Potential of Targeted AAV-Mediated Glycogen Clearance in the Central Nervous System in a Glycogen Storage Disease IV Mouse Model

Su Jin Choi, Fiona E. Weaver, Rebecca A. Gibson, Rebecca L. Koch, Priya S. Kishnani, Jeong-A Lim

Duke University Medical Center, Durham, NC

Glycogen serves as the central nervous system (CNS)'s primary energy source required for proper brain functioning and is primarily stored in astrocytes under normal conditions, with dysregulation of this pathway causing a group of rare, inherited, disorders known as glycogen storage diseases. Specifically, glycogen storage disease IV (GSD IV) results from a deficiency of the glycogen-branching enzyme (GBE) which leads to widespread accumulation of poorly branched glycogen, known as polyglucosan. The adult-onset form of GSD IV is known as Adult Polyglucosan Body Disease (APBD) and is characterized by progressive neuromuscular dysfunction, neurogenic bladder, gait abnormalities, and poor motor coordination. The GSD IV mouse model (*Gbe1*$^{ys/ys}$), is a knock-in mouse model of the most common GBE1 mutation, p.Y329S, found in APBD patients of Ashkenazi Jewish ancestry and recapitulates many phenotypes observed in APBD patients. In this study, we utilized the *Gbe1*$^{ys/ys}$ mouse model to reveal the intricacies of CNS involvement during GSD IV progression. We report significant polyglucosan accumulations throughout the brain and spinal cord, initiating early in the disease progression. Furthermore, *Gbe1*$^{ys/ys}$ mice presented with severe, widespread astrogliosis in comparison to wildtype (WT) mice. At 3 months of age, *Gbe1*$^{ys/ys}$ mice performed

similarly to that of WT mice, however by 6 months, they showed a significant decline in the behavioral tests, exhibiting decreased muscle endurance and poor motor coordination. These data highlighted the significance of the CNS in GSD IV pathogenesis, and therefore, we targeted the CNS, using a neuron-specific promoter (Synapsin1; Syn1), for the treatment of GSD IV in the *Gbe1*$^{ys/ys}$ mouse model. At 6 weeks of age, two cohorts of mice received an intravenous injection of AAV9-Synapsin1-humanGBE (AAV9-Syn1-hGBE) and were subsequently sacrificed 2 weeks and 9 months post-injection. In the short-term (2 weeks) cohort, we observed an increase in GBE protein expression in the liver and brain of *Gbe1*$^{ys/ys}$ mice, and promisingly, the treatment led to preventing polyglucosan bodies accumulation in both tissues. Notably, the AAV treatment successfully rescued the behavioral defects in 6-month-old *Gbe1*$^{ys/ys}$ mice, who exhibited a significant improvement in motor coordination and neuromuscular function and performed comparably to age-matched WT mice. Interestingly, these effects persisted for approximately 6 months post-injection before we noted a dwindling of the performance improvements in AAV-treated *Gbe1*$^{ys/ys}$ mice, despite detectable GBE protein expression at that time. These data suggest that using the synapsin1 promoter to target the CNS for expressing glycogen branching enzyme in the APBD mouse model successfully prevented the early accumulation of polyglucosan. Furthermore, our vector restored GBE protein expression and rescued the neuromuscular phenotype in 6-month-old *Gbe1*$^{ys/ys}$ mice, revealing the therapeutic potential of neuron-directed gene therapy. Interestingly, our findings also alluded to non-neuronal CNS involvement in GSD IV pathology, thereby highlighting additional therapeutic avenues, which we are currently exploring for the treatment of GSD IV.

112. hfCas12Max-Mediated Gene Editing Therapy for Amyotrophic Lateral Sclerosis

Dong Yang[1], Aiguo Guo[1], Mengsi Duan[1], Meixian Wu[1], Shaoran Wang[1], Tong Li[1], Xuan Yao[1], Alvin Luk[1], Hui Yang[1,2], Linyu Shi[1]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: 97% of amyotrophic lateral sclerosis (ALS) patients exhibit cytoplasmic aggregation of mutated TDP-43 protein, contributing to neuron death. Ataxin-2 is implicated in the formation of stress granules that contain TDP-43. Knockdown of Ataxin-2 has been shown to potentially prevent the aggregation of TDP-43 stress granules. Here, we developed a potential therapeutic strategy using a high-fidelity Cas12Max (hfCas12Max) system, an engineered Cas12i with high activity and specificity, to target *ATXN2* as a novel treatment for ALS. **Methods**: We generated a single AAV vector packaging hfCas12Max and gRNA targeting *ATXN2* (g*ATXN2*) capable of accessing neurons in the brain and spinal cord when administered through intracerebroventricular (ICV) injections in neonatal humanized *TDP-43*$^{Tg/Tg}$ mice, a mouse model of ALS displaying abnormal gait, severe tremors, and motor function decline and develop increasingly severe kyphosis, which typically begins manifesting at P14. We, therefore, monitored these phenotypes tri-daily between P15 and P21, as well as weight, which is also adversely affected in *TDP-43*$^{Tg/Tg}$ mice. The lifespan of mice injected with AAV-hfCas12Max-g*ATXN2* or PBS was observed daily. The editing efficiency of *Atxn2* DNA in the cortex and spinal cord of *TDP-43*$^{Tg/Tg}$ mice was detected by NGS analysis 21 days after the treatment. The Ataxin-2 and p-TDP43 protein levels within the cortex and spinal cord of these mice were also detected by western blot or immunofluorescence (IF). **Results**: Compared to *TDP-43*$^{Tg/Tg}$ mice with PBS injection, *TDP-43*$^{Tg/Tg}$ mice treated with different doses of AAV-hfCas12Max-g*ATXN2* had improved gait ($P<0.05$), decreased kyphosis ($P<0.05$), reduced tremors ($P<0.05$), and increased weight ($P<0.05$) in a dose-dependent manner (Fig.1a-d), suggesting that the *TDP-43*$^{Tg/Tg}$ mice treated with AAV-hfCas12Max-g*ATXN2* possessed improved motor coordination and neuromuscular function. Additionally, high-dose AAV-hfCas12Max-g*ATXN2* treatment dramatically extended the lifespan in *TDP-43*$^{Tg/Tg}$ mice. Median survival soared by ~77 days (101.5 vs. 24 days; $p < 0.05$)(Fig. 1e), with mean survival also extending by ~50 days (73.83 ± 10.80 vs. 23 ± 0.67 days; $p < 0.01$) (Fig. 1f). Remarkably, 50% (6 out of 12) of high-dose-treated mice surpassed 100 days of survival. NGS results at 21 days after dosing showed that the editing efficiency of *Atxn2* in the cortex and spinal cord of *TDP-43*$^{Tg/Tg}$ mice treated with low-, middle-, and high-dose was increased, with 12.73±2.01%, 22.13±3.97%, 35.60±6.93% in cortex and 3.13±0.19%, 8.50±1.86%, 9.60±1.35% in spinal cord, respectively. After treating *TDP-43*$^{Tg/Tg}$ mice with high-dose, the ATXN2 protein level in the cortex decreased to 59.37±17.32% compared to the control mice. IF results showed a significant knockdown of ATXN2 protein level in the cortex and spinal cord p-TDP43 level in the motor cortex was almost rescued to WT mice level. **Conclusions**: Our findings suggest a promising therapeutic strategy using AAV-mediated delivery of hfCas12Max-g*ATXN2* for precision genome editing of *ATXN2*, which has the potential to extend lifespan and improve motor function and neuromuscular performance in ALS.

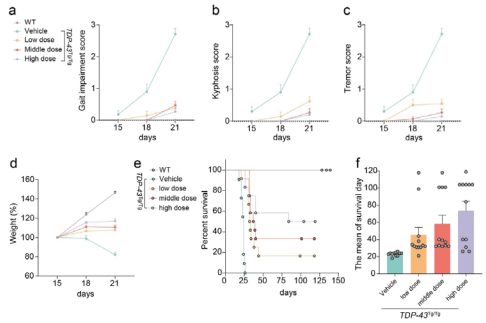


Figure 1. AAV-hfCas12Max-gATXN2 dosing improved the motor coordination, neuromuscular function, weight, and lifespan of *TDP-43*$^{Tg/Tg}$ mice.

113. All-in-One Adeno-Associated Virus Delivery for Therapeutic miR-21 Editing by saCas9 *In Vivo*

Lisa Nieland[1], Anne B. Vrijmoet[1], Isabelle W. Jetten[1], David Rufino-Ramos[1], Alexandra J. E. M. de Reus[1], Koen Breyne[2], Benjamin P. Kleinstiver[3], Casey A. Maguire[4], Marike L. D. Broekman[1], Xandra O. Breakefield[5], Erik R. Abels[6]

[1]Massachusetts General Hospital, Charlestown, MA,[2]Massachusetts General Hospital, Charleston, MA,[3]Massachusetts General Hospital, Boston, MA,[4]The Massachusetts General Hospital, Charlestown, MA,[5]Massachusetts General Hospital / HMS, Charlestown, MA,[6]Massachusetts General Hospital, Amsterdam, Netherlands

Glioma, the most aggressive tumor of the CNS has poor patient outcome with limited effective treatments available. CRISPR-Cas9 technology has opened a new avenue for gene therapy and has been previously used to target non-coding RNAs, including microRNA-21 (miR-21), a microRNA highly expressed in glioma cells responsible for tumor progression. In our previous research we showed that miR-21 is crucial for tumor growth and a complete knock out *in vitro* reduced its tumorigenesis potential. Here, we designed a single all-in-one recombinant adeno-associated vector (rAAV) containing *Staphylococcus aureus* Cas9 (saCas9) guided by a single-guide RNA (sgRNA) to target miR-21 coding sequences in the genome *in vivo*. We utilized a CRISPR delivery system using AAV-based gene therapy in a glioma mouse model. We showed that the AAV-8 serotype is capable in reaching the tumor cells in an orthotopic glioma model where it can deliver saCas9. We demonstrated that saCas9 is functionally expressed in glioma cells upon AAV-mediated delivery. This resulted in miR-21 downregulation in tumor cells leading to reduced tumor growth and improved overall survival. This all-in-one AAV-sgRNA-saCas9 approach specifically targeting microRNA coding sequences can be applied not only in the context of glioma, but for a range of genome editing purposes *in vivo*. Besides specifically targeting miR-21, this strategy could be used to target any microRNA by designing a sgRNA targeting the microRNA of interest. Applying CRISPR to microRNAs has the advantage that microRNA becomes non-functional based on a single cut, whereas the effect of CRISPR in genes could still result in a truncated protein to still be expressed with potential negative effects. Together, we show that engineering a single AAV can achieve therapeutic genome editing outcomes. We anticipate that successful *in vivo* delivery of this accurate and effective saCas9 can also advance therapeutic gene-editing in humans.

114. Chemogenetics for the Treatment of Spasticity

Nicholas Boulis, Kelly M. Poth, Michelle Zhang, Jareer Imran, Olivia Shi, Anthony Donsante

Neurosurgery, Emory University School of Medicine, Atlanta, GA

A consequence of many insults to the central nervous system, such as spinal cord injury and stroke, is the development of spasticity. The underpinnings of spasticity are not completely understood, but it involves the loss of descending innervation from the brain, increases in motor neuron sensitivity, and signaling through type Ia fibers. Ultimately, this results in the unrestrained activation of motor neurons, leading to unwanted muscle contractions. This can cause additional injuries, pain, and decreased quality of life for patients. Pharmacologic treatment with baclofen, alpha-2 agonists, and anticonvulsants can provide relief for some patients. However, these drugs can have significant side effects that lead patients to discontinue therapy, and tolerance can develop over time, requiring greater and greater doses. Therefore, there is a compelling need for better therapeutic approaches. Since the ultimate problem in spasticity is the aberrant activation of motor neurons, strategies that directly reduce the activity of these cells may be therapeutic. Chemogenetics is one such option. This approach involves two components: 1) an engineered receptor that suppresses neuronal activity, and 2) a biologically inert drug (ligand) to activate the receptor. For spasticity, the strategy would be to express the receptor in the motor neurons driving spasticity. Oral administration of the ligand would then reduce spasticity in a dose-dependent manner. However, the ability of chemogenetics approaches to regulate the activity of spinal motor neurons has not previously been evaluated. We hypothesized that an engineered chloride channel that opens upon binding of the ligand, bradanicline, could hyperpolarize motor neurons and inhibit their activity, resulting in changes in ambulation and grip strength. To test this hypothesis, we used an adeno-associated virus serotype 9 vector to deliver the cDNA for this channel to the lumbar spinal cord. Expression of the cDNA was driven by a human synapsin promoter to limit expression to neurons. In order to identify a maximum tolerated dose of the vector, three doses (10^9, 10^{10}, and 10^{11} vg) were injected unilaterally, spread over five injection sites. For eight weeks, motor function was quantified using hindlimb grip strength measurements and BBB scores. By two weeks post-delivery, the highest dose produced significant deficits on the treat side that worsened over time. These deficits were accompanied by a substantial increase in Iba1 staining, suggesting toxicity caused by the receptor. The lower two doses were better tolerated, and the 10^{10} vg dose was carried forward for further testing. To demonstrate ligand-dependent inhibition of motor neurons, a second cohort was again injected unilaterally with the vector. Animals receiving an empty vector served as a control. Five doses of ligand were tested (0, 1, 10, 30, and 100 mg/kg body weight), delivered by oral gavage. Animals receiving the control vector experience no change in motor function at any dose of the ligand. Animals receiving the receptor exhibited dose-dependent reduction in grip strength and gait on the ipsilateral side, demonstrating that the motor system can be modulated using chemogenetics. Further experiments in a rat spasticity model will demonstrate the utility of this approach to treat this disorder.

115. Delivery of scAAV9.co*GM2A* for Phenotypic Correction of GM2 Gangliosidosis AB-Variant, in the Newly Characterized *Gm2a/Neu3* Double Knockout Mouse Model

Camilyn S. Cheng[1], Natalie M. Deschenes[1], Melissa Mitchell[2], Jagdeep S. Walia[1,2,3]

[1]Centre for Neuroscience Studies, Queen's University, Kingston, ON, Canada,[2]Pediatrics, Queen's University School of Medicine, Kingston, ON, Canada,[3]Kingston Health Sciences Centre, Kingston, ON, Canada

GM2 Gangliosidoses AB-Variant (ABGM2) is an autosomal recessive lysosomal lipid storage disorder characterized by an overaccumulation of GM2 gangliosides in neuronal lysosomes, ultimately resulting in neuronal cell death. This disorder arises due to a mutation in the *GM2A* gene, which encodes for GM2 Activator Protein (GM2AP), an essential cofactor in the hydrolysis of GM2 gangliosides. Overaccumulation of GM2 gangliosides and eventual neuronal cell death present as phenotypic symptoms of sensory, motor, and cognitive decline and ultimately result in a shortened lifespan. In its most severe and common 'infantile' form, death can occur as early as 4 years of age. Currently, there are no curative treatments available for ABGM2; however, due to the monogenic nature of this disease, the delivery of a gene therapy using an Adeno-Associated Virus expressing a codon-optimized *GM2A* transgene holds great promise to treat ABGM2. Previous *GM2A* transgene administration in *Gm2a*$^{-/-}$ murine models showed reduction in GM2 ganglioside accumulation as well as widespread vector biodistribution to the central nervous system; however, that

model does not accurately mirror the severe human form due to its normal lifespan. This has led to the creation and characterization of a *Gm2a*$^{-/-}$/*Neu3*$^{-/-}$ murine model in the Walia lab, which more closely parallels the symptoms and lifespan of the severe human form of ABGM2 as these mice die at approximately 27 weeks of age. This study aims to investigate the dose-response of intrathecal delivery of scAAV9.co*GM2A* in the newly characterized *Gm2a*$^{-/-}$/*Neu3*$^{-/-}$ murine model for the first time. 5 cohorts each containing 6 mice received intrathecal administration at 6 weeks of age of either vehicle or three different doses of vector per mouse - low (0.5e^{11}vg), medium (1.0e^{11}vg) or high (2.0e^{11}vg). Monthly behavioural testing and blood collections were performed starting at 8-weeks of age and were continued until either short-term endpoint of 27-weeks of age, or humane endpoint. At each respective endpoint, serum, gross organs and tissues from the central nervous system were collected for biochemical analysis of GM2 ganglioside accumulation, GM2AP activity, vector biodistribution, immune response and histology. Data will be shown to support the hypothesis that there will be a dose-dependent response in GM2 ganglioside accumulation, GM2AP activity, vector biodistribution to the central nervous system and improvement in behavioural outcomes in short-term and added survival benefits in long-term. Furthermore, immune responses will be tested through immunological assays. These results will provide more clinically translatable results on the efficacy and safety of scAAV9.co*GM2A* in the treatment of ABGM2.

Breaking Barriers to the CNS via AAV Capsid Engineering

116. Human Blood-Brain Barrier Receptor-Guided Rapid Evolution of AAVs for Brain Gene Therapy

Changfan Lin[1], Xinhong Chen[1], Yujie Fan[1], Erin E. Sullivan[1], Tomasz Gawda[1], Alex Jin Hyung Chung[1], Xiaozhe Ding[1], Bill Kavvathas[1], Lillian J. Campos[2], Cameron R. Jackson[1], Jonathan Hoang[1], Sripriya Ravindra Kumar[1], Andrew S. Fox[2], Timothy Shay[3], Viviana Gradinaru[1]

[1]Caltech, Pasadena, CA,[2]University of California Davis, Davis, CA,[3]CLOVER Center, Caltech, Pasadena, CA

Despite substantial advancements in technologies to genetically modify cells, the systemic delivery of such gene therapies to cells in the brain continues to present significant challenges. Our research focuses on developing brain delivery tools, specifically harnessing adeno-associated viral vectors (AAVs). These non-pathogenic vectors show some ability to cross the blood-brain barrier (BBB), yet there is considerable room for improvement in their efficiency, specificity, and performance across mammalian species. In recent years, many engineered AAVs identified through directed evolution have shown greatly enhanced brain efficiency and specificity in mice and non-human primates (NHPs) with systemic delivery. The ultimate clinical utility of these vectors in humans, however, remains unproven. In vivo directed evolution of AAV capsids involves transduction of an animal with a library of AAV variants, followed by selective recovery of those variants that effectively reach, for example, the rodent or NHP brain. Notably, this method is resource-intensive and achieves only limited success in translating performance to other species, due to receptor differences across species, including between NHPs and humans. In response, we have developed a fast and efficient in vitro capsid engineering strategy that focuses on directed evolution of AAVs to target human BBB receptors such as carbonic anhydrase IV (CA4), recently identified by our group (Shay et al. Science Advances, 2023). Notably, CA4 expression is primarily concentrated in the primate brain, rather than other organs, unlike previously-characterized BBB transporters. CA4's expression profile offers the potential for AAVs targeting it to have fewer off-target effects. Employing a hybrid approach integrating both cell-free (resin-based) and cell-based selection methods, we successfully isolated AAVs exhibiting specific affinity for human CA4 (huCA4). We validated our approach using known receptor-binder pairs targeting the rodent BBB receptors LY6A (Huang et al., PLOS ONE, 2019) and CA4 (msCar4). Three rounds of selection yielded a set of AAVs with enhanced binding to huCA4 via a specific N-terminal surface loop of huCA4. These AAVs exhibit increased infectivity in cell culture, improved BBB penetration in an in-vitro human BBB model, and broad transduction of neurons in human brain organoids. Finally, testing in huCA4-expressing mice confirmed enhanced receptor-mediated delivery to mouse brain cells in vivo. Overall, our work presents an animal-free pipeline to engineer human receptor-targeting AAVs in a shortened development timeline. The AAVs identified by this mechanism-driven strategy show promise for enhancing gene delivery efficiency across species, including to the human brain, an improvement over current mechanism-agnostic AAV development methods.

117. Identification and Characterization of STAC-BBB, an Engineered AAV Capsid That Exhibits Widespread Transduction of the Central Nervous System in Cynomolgus Macaques

Matthew R. Tiffany, Stephanye Frias, Lori Andrews, Ankitha Nanjaraj, Russell Darst, Stephen Wist, Satria Sajuthi, Yuri Bendaña, Hung Tran, Sarah Mueller, Bryan Zeitler, Amy Pooler, David Ojala

Sangamo Therapeutics, Inc., Richmond, CA

The blood brain barrier (BBB) is a stringent biological barrier that severely restricts central nervous system (CNS) delivery with naturally occurring AAV serotypes. Previously we described our SIFTER (Selecting In vivo for Transduction and Expression of RNA) platform for in vivo selection of engineered AAV capsids that mediate improved CNS delivery. Here we applied this platform to identify STAC-BBB, a novel AAV capsid that exhibits robust and widespread CNS transduction in cynomolgus macaques after intravenous delivery. Three rounds of in vivo library screening were conducted in cynomolgus macaques using the SIFTER method. In the third round of screening, three macaques were dosed intravenously with a multiplexed library of 1,260 capsids, each linked to at least four unique barcodes driven by either a neuron-specific human synapsin 1 or ubiquitous CMV promoter. Nucleic acids were isolated from CNS and peripheral tissues and the level of mRNA expression and DNA delivery mediated by

each capsid was assessed. STAC-BBB was identified as the highest performing capsid for CNS delivery across all performance metrics, mediating a 700-fold improvement in neuronal expression compared to AAV9. Moreover, enrichment in both neuronal and ubiquitous transcript expression was highly consistent in all CNS tissues and across all animals on study. CNS delivery was robust in 150 brain punches collected from key brain regions, across six coronal brain slices spanning the rostro-caudal axis, and along the entire length of the spinal column. STAC-BBB also demonstrated de-targeting of dorsal root ganglia and peripheral tissues. We additionally analyzed capsid performance across barcode replicates and counted the number of unique molecular identifiers linked to each barcode. All four STAC-BBB barcode replicates performed equally well, and thousands of unique molecular identifiers were recovered from CNS tissues. An individual evaluation of STAC-BBB in cynomolgus macaques is underway with results expected shortly. Study readouts include vector genome biodistribution, transgene expression levels, and single cell analysis of transduction efficiency.

118. Antibody-Based AAV Retargeting to Transferrin Receptor Mediates Efficient Blood Brain Barrier Crossing and *In Vivo* Gene Delivery to the CNS in Mice and Non-Human Primates

Kalyani Nambiar, Kyle Plasterer, Ammar Tareen, Pascaline Aime-Wilson, Nicole Keating, Caryn Galligan, John Dugan, Tazeen Ishmam, Anthony LoMastro, Min Gao, Aishwarya Shevade, Zhe Zhang, Sheldon Mink, Andrew David Tustian, Lynn Macdonald, Christos Kyratsous, Leah Sabin

Regeneron Pharmaceuticals, Inc., Tarrytown, NY

Adeno-associated virus (AAV) is a leading viral vector used in gene therapy to treat human diseases. Although AAV has many advantages as a gene therapy vector, some of the limitations to its use for disorders of the central nervous system (CNS) are the inability of wild-type AAV serotypes to efficiently cross the blood-brain-barrier (BBB) when delivered systemically, as well as their tendency to preferentially transduce the liver. The high AAV doses required to effectively transduce extrahepatic tissues have led to severe adverse events in patients, including liver toxicity and death. While directed evolution-based selection approaches are a powerful method to identify novel capsids with desired properties, the translatability of such capsids for human treatment remains challenging. Thus, we sought to develop a species-agnostic, modular AAV retargeting platform that utilizes rational capsid engineering and monoclonal antibodies to redirect AAV particles to cross the BBB, while minimizing off-target tissue transduction. We are using a protein tagging system to covalently attach antibodies to surface-exposed variable loops on the viral capsid. The tropism of the conjugated capsid can be further modified through the introduction of mutations in the amino acid residues that serve as primary determinants of AAV tropism. This allows detargeting from the liver while enhancing on-target specificity. We have successfully used this approach to retarget multiple AAV serotypes to specific cell types *in vitro* and *in vivo,* using the cellular target of the retargeting antibodies to drive the specificity of transduction. Transferrin Receptor 1 (TfR) is a conserved receptor expressed on the apical surface of brain endothelial cells that is known to facilitate transcytosis of cargo across the BBB. We demonstrated that multiple AAV serotypes can efficiently cross the BBB following systemic administration when conjugated to anti-TfR antibodies, achieving comparable CNS transduction to wild-type AAV9 at lower doses. One of the key features of our antibody-mediated AAV retargeting platform is the cross-species translatability of the approach. By targeting surface-exposed cellular proteins with conserved expression patterns between mice, non-human primates (NHPs), and humans, we aim to develop AAV vectors with predictable tropism and potency across species. We screened a panel of anti-human TfR fabs for their ability to mediate BBB-crossing and transduction of the CNS following systemic delivery in mice expressing human TfR and identified multiple fabs with a range of binding properties that facilitated potent AAV delivery to the brain. To demonstrate translation of TfR-mediated BBB crossing in a large animal model, we evaluated the retargeted AAVs in cynomolgus macaques. Importantly, after intravenous delivery, TfR-retargeted AAVs were significantly enriched in multiple brain regions, including in cortical, subcortical, and cerebellar tissues. TfR-retargeted AAVs were also markedly detargeted from the liver, heart, and dorsal root ganglion. Thus, our novel TfR-targeted AAV vectors dramatically outperformed AAV9 in both efficiency and specificity of CNS transduction. This modular, antibody-based AAV retargeting system represents a robust method to enhance delivery of AAV to the CNS by targeting a conserved receptor, while simultaneously reducing off-target tissue transduction to mitigate safety risks. We aim to apply this technology to the development of therapies for diseases of the CNS.

119. Continued Directed Evolution of VCAP-101 and VCAP-102 Identifies Second Generation Capsid Variants with Increased Brain Tropism and in Non-Human Primates and Mice

Tyler Moyer, Kristin Graham, Jiachen Liu, Xiaoqin Ren, Joseph Clement, Alexis Bloedel, Rebecca Spellman, Camila Arce, Hamza Khalid, Anupriya Kulkarni, Alexandra Tsolias, Rong Zhao, Wenya Yang, Amy Bruce, Chiso Egbuchulam, Tatianna Knox, Brett Hoffman, Ishan Shah, Michael Grannan, Charlotte Chung, Alexander Kutchin, Johnson Amy, Kyle Grant, Nilesh Pande, Jeff Thompson, Johnny Yao, Chanchal Sadhu, Todd A. Carter, Mathieu E. Nonnenmacher

Voyager Therapeutics, Cambridge, MA

The treatment of central nervous system (CNS) diseases by Adeno-Associated Virus (AAV)-mediated gene therapy has been hindered by the availability of AAV capsid variants capable of efficiently crossing the blood-brain barrier (BBB). We recently applied our RNA-driven TRACER™ capsid evolution platform to identify VCAP-101 and VCAP-102, members of a family of capsids with peptide insertions in Variable Region IV (VR-IV) of AAV9. These capsid variants demonstrate significantly increased CNS-tropism and liver de-targeting relative to AAV9 in non-human primates (NHPs) and mice. Here, we perform two further rounds of directed evolution to VCAP-101 and VCAP-102 to generate second generation (Gen2) capsid variants.

In a pooled, next generation sequencing (NGS) based screen, these Gen2 capsid variants showed up to 7-fold improved BBB-penetrance across broad CNS regions relative to the parental VCAP-101 or VCAP-102 capsids in NHPs. Upon intravenous dosing of cynomolgus macaques (*Macaca fascicularis*) with these Gen2 capsids expressing a self-complementary histone transgene at 3E13 vector genomes per kilogram (VG/kg), we observed upwards of 9 vector genomes per cell across broad CNS regions and upwards of 60% of cells being targeted, including both neurons and astrocytes. Gen2 capsids demonstrated further liver de-targeting relative to both the parental VCAP-101 and VCAP-102 capsids. Additional studies in mice reinforce the dual cell-type targeting of these Gen2 capsids. Furthermore, we identify the receptor for this family of capsid variants and demonstrate that the capsid-receptor binding is both specific and direct. Given the high conservation of this receptor across mammals, our data collectively suggest a novel, highly CNS-specific capsid for use in the clinic.

120. Second Generation AAV Capsids Reprogrammed to Bind Human Transferrin Receptor are Targeted to the Brain and De-Targeted from the Liver in Human TFRC Knock-In Mice

Ken Y. Chan, Qin Huang, Shan Lou, Casey Keyes, Jason Wu, Nuria R. Botticello-Romero, Qingxia Zheng, Chin-Yen Lin, Jencilin Johnston, Allan Mills, Pamela Brauer, Gabrielle Clouse, Simon Pacouret, John W. Harvey, Thomas Beddow, Jenna K. Hurley, Isabelle G. Tobey, Megan Powell, Catherine P. Pirtle, Albert Chen, Andrew J. Barry, Fatma-Elzahraa Eid, Yujia A. Chan, Benjamin E. Deverman

Broad Institute of MIT and Harvard, Cambridge, MA

Developing vehicles that efficiently deliver genes throughout the human central nervous system (CNS) will broaden the range of treatable genetic diseases. We developed a high-throughput *in vitro* screening approach to reprogram AAV9 to bind the human Transferrin Receptor (TfR1), a protein expressed on the blood-brain barrier (BBB). We showed that one TfR1-targeted capsid, BI-hTFR1, was actively transported across a human brain endothelial cell layer and achieved 40-50 times greater reporter expression in the CNS of human *TFRC* knock-in mice compared to AAV9 in wild type mice after intravenous delivery. The enhanced tropism of BI-hTFR1 was CNS-specific and absent in wild type mice (Fig. 1A). Through sequence diversification, we identified a second generation capsid, BI-hTFR1.2, that was capable of transducing more than 60 percent of NeuN-positive neurons in the cortex of human *TFRC* knock-in mice when dosed at 5E10 vg/mouse, while also being de-targeted from the liver (Fig. 1B). Similar to BI-hTFR1, BI-hTFR1.2 exclusively binds to human but not macaque or mouse TfR1. Using amino acid swaps between species, we pinpointed human-specific residues of TfR1 that are critical for the species-specific binding by BI-hTFR1 and BI-hTFR1.2. Importantly, there are no common human polymorphisms that would be predicted to directly impact the ability of these capsids to bind TfR1 in patients. Our work establishes these TfR1-targeting capsids as promising vectors for human CNS gene therapy.

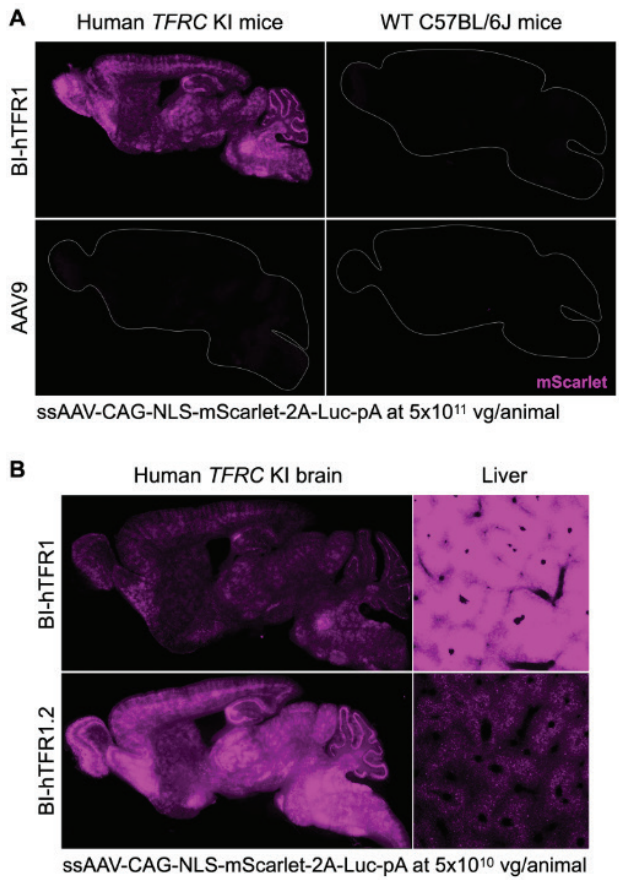


Figure 1. TfR1-targeted AAV capsids achieve efficient CNS-wide transgene expression in human *TFRC* knock-in mice. (A) BI-hTFR1 or AAV9 were intravenously injected into adult wild type C57BL/6J or *TFRC* knock-in mice at 5E11 vg/animal. Representative sagittal brain section images from three weeks post-injection are shown. **(B)** BI-hTFR1 or BI-hTFR1.2 were intravenously injected into adult *TFRC* knock-in mice at 5E10 vg/animal (~2.5E12 vg/kg). Representative sagittal brain and liver section images from three weeks post-injection are shown.

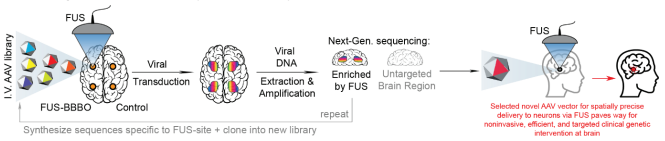
121. Engineering Viral Vectors for Acoustically Targeted Gene Delivery to the Brain across Species

Hongyi Richard Li[1], Jerzy Szablowski[2], Mikhail Shapiro[1]

[1]California Institute of Technology, Pasadena, CA,[2]Rice University, Houston, TX

Spatially targeted gene delivery to the brain has the potential to treat prevalent neurological and psychiatric diseases. However, the site-specific delivery of vectors such as adeno-associated viruses (AAVs) is typically performed via invasive injections, limiting scope of clinical applications. Over recent decades, focused ultrasound blood-brain-barrier opening (FUS-BBBO) has emerged as a noninvasive procedure enabling the site-specific entry of small viral vectors into the brain from systemic circulation. However, when used in conjunction with natural AAV serotypes, this approach has limited transduction efficiency, and results in undesirable transduction of peripheral

organs. In this project we employ in vivo evolution to develop new AAV serotypes with enhanced transduction at sites of FUS-BBBO and decreased transduction peripherally in rodents and nonhuman primates (NHPs). In our screening strategy, a library of AAVs with mutated capsids based on AAV9 is injected intravenously into hSyn1-Cre mouse and delivered via FUS-BBBO to one hemisphere. When a particular AAV variant transduces Cre-expressing neurons, its viral genome is modified, becoming detectable by a Cre-dependent PCR and next-generation sequencing (NGS). Repeated rounds of selection for vectors that uniquely appear in the targeted hemisphere led to desired novel AAV vectors (AAV.FUS) with 12.1-fold improvement in overall tissue specificity and above 56% improved neuronal tropism in brain. AAV.FUS's capability of improved FUS-mediated delivery to brain is validated across multiple murine strains. As a bonus, AAV.FUS has 2.5-fold improved transduction efficiency over AAV9 when delivered via intracranial injection. To develop improved viral vectors for FUS-BBBO in nonhuman primates (NHPs), we performed viral screening in macaques with a strategy similar to that of rodent model but introduced neuronal Cre expression through AAV9-hSyn1-Cre transduction due to lack of transgenic NHP. After two rounds of evolutions, 145 viral variants are selected for further screening based on their unique enrichment in acoustically targeted brain regions of NHP. Overall, this study shows that the molecular engineering of AAV capsids can lead to improved noninvasive, site-specific ultrasound-mediated gene delivery to the brain across different species, paving the way for large-scale disease model studies and potential future human clinical translation. Potential applications of noninvasive spatially targeted gene therapy include epilepsy, Parkinson's disease, chronic depression, obsessive compulsive disorder (OCD), and any other condition that is currently being treated with local ablation or DBS. By developing viral serotypes that more efficiently enter the brain at the site of FUS-BBBO, reducing the transduction of off-target organs, and reducing the required systemic dose, this work will greatly improve the safety profile and reduce the manufacturing cost of this class of therapies, making it more likely that they will benefit patients.



122. Directed Evolution of AAV9 Libraries in Non-Human Primates Identifies a Capsid Family with Enhanced Central Nervous System Tropism and Liver De-Targeting Following Systemic Delivery

Xiaojing Shi, Sean Gross, Michelle Ling, Reem Elteriefi, Zach Mason, Caden Holloway, Yixi Wang, Mark Chin, Kimberly McDowell, Brandon Wheeler, Nicholas Flytzanis, Nick Goeden, Susan Catalano

Capsida Biotherapeutics, Inc., Thousand Oaks, CA

Gene therapy for central nervous system (CNS) disorders has seen significant advancements, but achieving efficient and widespread CNS transduction by viral or non-viral vectors remains challenging. Local delivery of Adeno-Associated Virus (AAV) vectors to the CNS typically results in limited transduction, challenges in achieving therapeutic efficacy for diseases requiring systemic treatment and is associated with potential risks related to invasive delivery. Meanwhile, intravenous delivery of wild-type AAVs has limited brain tropism and toxicity risk due to off-target transduction in peripheral organs like the liver, presenting a significant opportunity for the development of engineered AAV capsids that can effectively cross the blood-brain barrier (BBB) while minimizing off-target transduction. We applied our high-throughput, non-human primate (NHP)-based AAV engineering platform for the directed evolution of an AAV9 capsid library and have generated capsids that overcome the limitations of these early gene therapy approaches. Following two rounds of intravenous dosing and screening, we identified several families of capsids with improved vector biodistribution across CNS regions compared to AAV9. Further evolution of these capsids, focusing on high CNS enrichment and liver de-targeting, led to the emergence of a capsid family with superior CNS tropism and liver de-targeting capabilities. One capsid was subsequently tested individually in cynomolgus monkeys by intravenous dosing. Compared to AAV9, this novel capsid showed a remarkable 137-fold improvement in transgene expression in the brain and a 16-fold decrease in vector biodistribution in the liver. Immunohistochemistry data confirmed that the novel capsid achieved extensive and neuron-specific transduction throughout CNS regions, including a 30% transduction in the frontal cortex, over 60% in the thalamus, and over 50% in the substantia nigra. Our work has identified a new family of CNS-tropic and neuron-specific AAV capsids with significantly decreased liver transduction, offering a promising avenue for effective and safe CNS-targeted gene therapies.

Strategies and Technologies for Advanced CMC

123. FDA and NIST Collaboration to Evaluate Assays and Control Materials for Characterizing Animal Biotechnology Products Generated by Genome Editing

Patricia Kiesler[1], Natalia Kolmakova[1], Sierra Miller[1], Sofiya Shevchenko[1], Stella Lee[2], Alexis Norris[2], Mayumi Miller[3], Adam Moyer[2], Samantha Maragh[1]

[1]Biosystems and Biomaterials Division, National Institute of Standards and Technology, Gaithersburg, MD,[2]Office of New Animal Drug Evaluation, US Food and Drug Administration, Center for Veterinary Medicine, Rockville, MD,[3]Office of Applied Science, US Food and Drug Administration, Center for Veterinary Medicine, Laurel, MD

Genome editing technology has revolutionized the ability to make targeted changes to an animal's genome (intentional genomic alterations or IGAs), offering exciting promise for the development of animal biotechnology products that address animal and public health needs. The U.S. Food and Drug Administration Center for Veterinary Medicine (FDA CVM) oversees regulation of such IGAs in animals that have been created using genome editing technologies. Characterization of these IGAs is an important part of the regulatory process to ensure that the edit to the animal is what it is intended to be and to

identify any unintended changes. However, there are currently no validated measurements and standards for characterizing unintended genomic alterations in animals. Most of the work to evaluate genome editing methods has been focused on human therapeutic use, and there is limited information available regarding their reliability and reproducibility for characterization of animal biotechnology products. To address these needs, FDA CVM has established a collaboration with the U.S. National Institute of Standards and Technology (NIST) that will generate resources including standardized measurements for characterizing both intended and unintended alterations in animal biotechnology products resulting from genome editing. These resources will provide animal biotechnology product developers and FDA regulators with example characterization approaches that they could use as part of the development and regulatory process for IGAs in animals as well as for validating methods, materials and/or data. Here, we present preliminary outcomes of this NIST-FDA CVM collaboration. NIST qualified commercially available swine and bovine cell lines and their DNA as potential control materials. The cell lines were characterized for genomic stability prior to editing, as well as for sequence by Sanger and NGS before and after genome editing. Multiple CRISPR/Cas9 editing assays, including two newly developed by NIST, were evaluated using purified animal DNA and the animal cell lines. Off-target sites identified from three in silico predictors and the biochemical assay CHANGE-seq were also compared. A subset of the off-target sites predicted by in silico tools were identified by CHANGE-seq and analyzed via targeted NGS for evidence of off-target editing in edited animal cells. Additionally, NIST onboarded the SITE-Seq off-target assay and did assay qualification and replicate analysis on human genomic DNA. SITE-Seq was found to perform similarly on animal genomic DNA as compared to human genomic DNA. Experimental design, protocols, datasets, and measurements that NIST generated will be published and made accessible to animal biotechnology product developers and the general public. Future work will focus on generating prototype DNA spike-in control materials to be used as controls for qualifying DNA sequencing methods that may be used for the detection of both intended and unintended genomic alterations in animal biotechnology products.

124. Purification Process Optimization of mRNA Lipid Nanoparticles via Microfluidic Platform to Improve Payload Distribution and Transfection

Xiang Liu, Fangchi Shao, Sixuan Li, Jinghan Lin, Yining Zhu, Tza-Huei Wang, Hai-Quan Mao

Johns Hopkins University, Baltimore, MD

Introduction Despite the extensive progress in lipid nanoparticle (LNP) composition optimization for tissue/organ targeting and therapeutic applications,[1] post-synthesis processing and purification have received limited attention. Commonly used LNP purification technologies include dialysis and tangential flow filtration (TFF); however, the composition stability during the purification process remains elusive and difficult to explore. Microfluidic technology has been successfully employed for the production of LNPs; the laminar flow within microfluidic channels facilitates integrated separation modules, enabling in-line purification, thus providing the unique opportunity to investigate the mechanism of LNP purification process in detail. By finetuning the flow rate and buffer conditions, and using the payload distribution detection method we previously developed,[2] we systematically studied LNP composition change during different purification programs, and aimed to ultimately optimize LNP purification process with improved payload distribution and transfection efficiency. **Materials and Methods** A widely used LNP formulation containing ionizable lipid SM-102, helper lipid 18:0 PC (DSPC), cholesterol, PEGylated lipid DMG-PEG2000, and fluorescently-labeled lipid TMR-PC (50:10:38.5:1.5:0.5, molar ratio) was formulated with Cy5-labeled mRNA using a T-junction at controlled flow rates according to our previously reported method.[2] The purification condition used in the microfluidic device was 1:24 volume ratio of LNP mixture to PBS buffer. Size and zeta-potential were determined by dynamic light scattering. Multi-color cylindrical illumination confocal spectroscopy (CICS) analyzed single-particle fluorescence burst quantification,[2] enabling particle classification and mRNA payload characterization. B16F10 cell line was used to assess the transfection efficiency of purified LNPs *in vitro*. **Results and Discussion** We utilized a microfluidic-based purification of LNPs via on-chip dialysis as illustrated in Figure 1A. We examined the effect of microfluidic dialysis flow rate from 0.5 to 50 mL/h on the quality of LNPs. As the flow rate increased, the polydispersity index (PDI) gradually decreased. The transfection efficiency peaks at 5 mL/h (Fig. 1B). Data from CICS analysis demonstrated that at 5 mL/h, the purified LNPs contained fewer empty particles and a higher average payload compared to those purified at 0.5 mL/h (Fig. 1E-G). These results suggest that a faster buffer exchange occurring at a higher flow rate may substantially reduce the composition drift and improve the uniformity of the payload distribution and transfection efficiency of LNPs.[2] This new microfluidic-based LNP purification platform and our results pave the way for further optimization of the LNP purification process and understanding of LNP structural features and their effect on transfection efficiency. **References** [1] X Hou, *et al.*, Lipid nanoparticles for mRNA delivery, *Nat. Rev. Mater.* 6: 1078 (2021). [2] S Li, *et al.*, Payload distribution and capacity of mRNA lipid nanoparticles, *Nat. Comm.* 13: 5561 (2022).

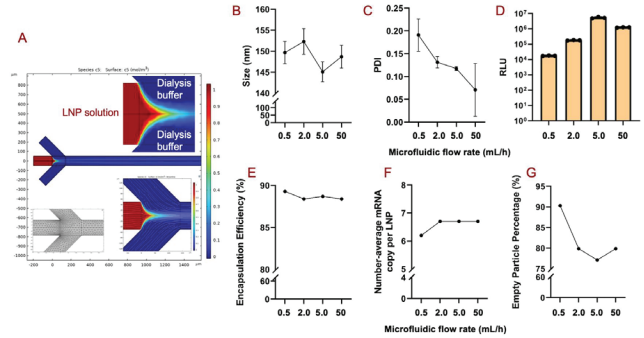


Figure 1. (A) Microfluidic-enabled dialysis design. Two side channels are the dialysis buffers, and the middle channel is LNP suspension in acetate buffer with 25% ethanol. Comparison of different microfluidic flow rate dialysis methods for LNPs in terms of (B) size, (C) PDI, (D) transfection efficiency in B16F10 melonoma cells, (E) encapsulation efficiency, (F) effective payload in terms of average copy number of mRNA per particle, and (G) percentage of empty LNP particles.

125. Recombinant Nuclease Cas9 for Therapeutic Genome-Editing - The GMP Manufacturer´s Point of View

Hans Huber
Biomay, Vienna, Austria

Genome editing with the CRISPR/Cas method has revolutionized molecular-biologic methods and the resulting therapeutic approaches. Nuclease Cas9 is an essential component of a recently market-approved therapy against sickle cell disease (SCD) and transfusion dependent beta-thalassemia (TDT). In this case study, we present an overview on the corresponding manufacturing campaign to supply those therapeutic programs with recombinant nuclease spCas9 under the requirements and conditions of current Good Manufacturing Practice (cGMP). Cas9 was expressed as a recombinant protein by using IPTG-induced *E. coli* culture as production system (pET/T7 promoter). A manufacturing process was developed and optimized, essentially consisting of fed-batch cultivation, high-pressure cell disruption, a three-step chromatography regime, formulation by ultra/diafiltration and final aseptic filling. Therapeutic nucleases for genome editing have to fulfill high and defined quality standards with respect of functionality/activity, homogeneity and purity. A set of analytical assays for routine in-process control and final quality control was developed and validated, and a respective list of specifications was defined. In addition, extended biochemical characterization of Cas9 was performed. Particular emphasis was put on the determination of the activity of Cas9 by application of an in-vitro potency assay. With respect to homogeneity, Cas9 aggregates and truncated variants thereof were characterized by high-performance size exclusion chromatography (HP-SEC). Long-term storage stability was shown to be sufficiently given by conducting multi-year stability studies. By performing forced degradation studies, the impact of extreme physico-chemical conditions on Cas9 potency was evaluated. For market submission, the performance of a manufacturing process is required to be validated. Exemplary data will be shown on critical quality attributes (CQA), down-scaled process characterization studies and their evaluation by statistical design of experiments (DOE). Finally, process validation was performed by conducting consecutive, full-scale and cGMP conform runs for process performance qualification (PPQ). In this comprehensive overview, the concepts, difficulties and results of late-stage clinical / pre-marketing manufacturing of GMP-grade recombinant Cas9 will be discussed.

126. Addressing the Impact of RM Critical Quality Attributes and Variability on Complex Biologic Manufacturing

Nandu Deorkar
Avantor, Radnor, PA

The complex biologics such as gene therapy, cell therapy, multi-specific antibodies, oncolytic viruses, and fusion proteins are being pursued in clinical development to address various hard to treat diseases and conditions. The pipeline of these molecules is increasing significantly due to rapid understanding of underlying biology and new discovery tools. These therapies require efficient and cost-effective manufacturing processes to realize its benefits. There are many challenges that appear when scaling-up complex biologic products. The two most critical challenge includes (1) managing yield and quality and (2) addressing new impurities that are not seen during previous studies. The root cause of these includes limited understanding of the process, limitations of process steps due to properties of complex biologics and limited understanding of impact of raw material attributes on the process performance and product quality. Beyond attributes impacting the process, other impurities such nitrosamine, ethylene glycol, genotoxic impurities, and bioburden/adventitious agents, if not addressed in time, can delay the potential product entering into clinical/commercial phase. This presentation will discuss the critical quality attributes of various raw materials, impact of variability on the process and approaches to control variability and thereby improve complex biologic manufacturing processes. Specific case studies and lesson learned will be discussed by comparing and contrasting with traditional monoclonal antibody or recombinant protein manufacturing processes.

127. Validation of Rapid Microbial Testing in an Academic GMP Cell Therapy Facility

Kavya Hemmanur, Janaina Machado, Daniel J. Kota
Houston Methodist Research Institute, Houston, TX

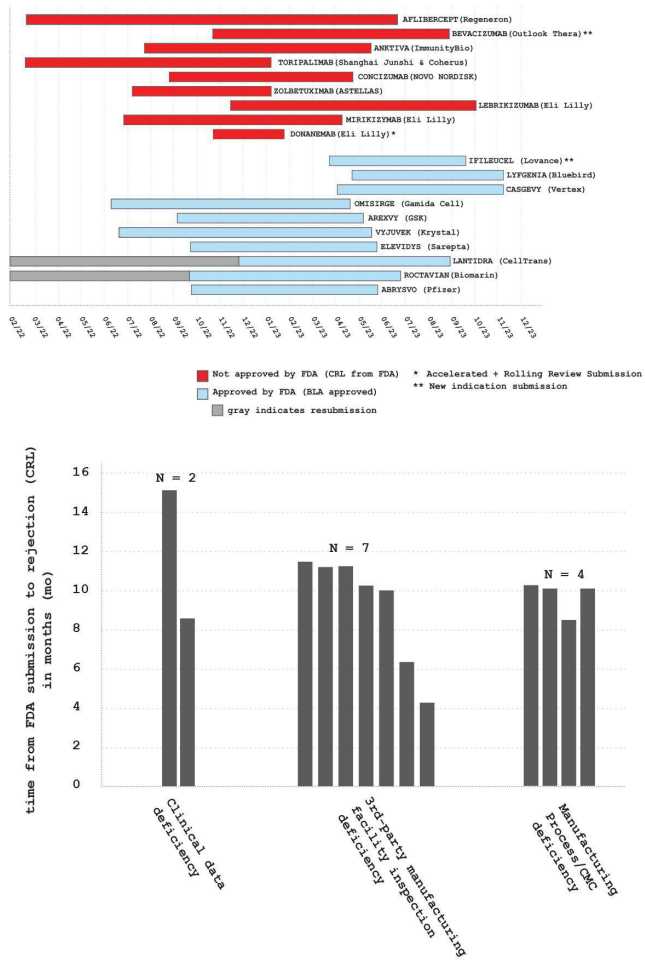
The Ann Kimball and John Johnson Center for Cellular Therapeutics (KJCCT) is an academic GMP facility composed of ISO7 cleanrooms and a dedicated quality control laboratory. KJCCT focuses on the translation of cell therapy products from research to first in human trials. Overall, cell therapy products must undergo a series of testing protocols to ensure their safety, identity, quality, strength and purity before release for patient use. Rapid microbial methods are alternative methods that can detect microbial contamination faster than traditional testing methods and are easier to implement in-house and can provide quick turnaround time, lower sample volume requirements, and ability to be automated. Here we demonstrate the feasibility to validate 2 alternative rapid microbial methods for sterility and mycoplasma using a BioMerieux BACT/ALERT 3D Dual T - an automated microbial detection system, and the Biofire® Mycoplasma (NAT) system - a multiplex PCR-based system. Methods, Results & Conclusion: We used an Installation, Operation and Performance Qualification process to validate our rapid microbial methods. Briefly, we use microbial strains listed in USP <71> using the BACT/ALERT® 3D Microbial Detection System, and seven mycoplasma strains listed under USP <63>and performed the testing according to manufacturer's instructions. Results show that our validation efforts meet all the acceptance criteria related to the requirements for instrument qualification and growth promotion of all microbial and mycoplasma strains down to 10 and 2 CFUs respectively with detection times below 5 days and 2 hours respectively. In addition, we have successfully performed two product-specific matrix inhibitory interference testing for suitability. Therefore, our center is confident in only adopting the suitability qualification to confirm the Limit of Detection and Specificity for each product before using the alternative methods for cellular products, reducing testing time and significantly reducing cost.

128. Systematic Analysis of Primary Field Data from Biologics Therapy Regulatory Non-Approvals: Why Aren't We Talking About FDA Rejections?

Limin Wang[1], Andreia Domingues[2], Angela Johnson[3,4]

[1]Cytiva Global Regulatory Strategy, Marlborough, MA,[2]Cytiva Global Regulatory Strategy, Eysins, Switzerland,[3]Cytiva Global Regulatory and Compliance, Marlborough, MA,[4]Department of Professional Studies, Regulatory Science, Northeastern University, Boston, MA

Background While drug approvals are transparent and widely analyzed, comprehensive analysis of non-approval in complex biologics and cell and gene therapy (CGT) is lacking, in part, because unlike approvals no readily accessible public databases exist for non-approval actions. Non-approval trends, however, can inform future CGT development, offering a unique perspective to both drug developers and regulators in how to accelerate these, often complex, therapeutics to market. **Methods** Data were extracted by a single researcher and confirmed by a second independent researcher from primary sources for 2023 biologic drug approvals vs non-approvals, defined as Food and Drug Administration (FDA) Biologics License Application (BLA) 'Complete Response Letter' (CRL). Sources included public drug sponsor and regulatory websites (FDA.gov), and nonpublic data (i.e. news aggregators) were excluded for reliability. Resulting non-approvals (CRL) dates, manufacturer, candidate info, and codified (NVivo, QRS) CRL content were analyzed with SAS/GRAPH Proc GPLOT. **Results** Of 19 drug submissions (Fig.1) for approval (FDA BLA submissions), 10/19 (53%) received regulatory non-approval (FDA CRL). Heuristic coding on primary source data revealed that the basis for non-approval (Fig.2) were clinical (N = 2), 3rd party facility issues (N = 7), and manufacturing process (CMC) deficiencies (N = 4), with 1 non-approval reporting both CMC and clinical deficiencies, and 2 non-approvals reporting both CMC and facility inspection issues; with most facility and CMC non-approvals received as 10-11 months after filing, and clinical non-approval issues significantly later (up to 15 months after filing). Mean approval time to receive approval from FDA was 8.6±1.6 months vs non-approval (CRL) mean time was 9±2.5 months, excluding accelerated approval/rolling review cases which received non-approval approximately 6 months sooner than non-approval in conventional review. **Conclusions** Manufacturing site inspection issues and deficiencies in documentation (CMC) regarding manufacturing processes remain the leading cause of non-approval of novel biologics and CGT products, received 10 to 12 months after filing. To accelerate drug development in this space, earlier manufacturing review processed are needed, through collaboration between regulatory agencies, drug developers, and manufacturing equipment suppliers for novel biologic and CGT products. It remains to be seen if new FDA programs recently announced (Advanced Therapy manufacturing (AMT) and rare disease and gene therapy pilot programs modeled on Covid Operation Warp Speed and oncology Project Orbis successes) can accelerate these timelines and move the needle on time-consuming roadblocks for novel therapeutics.



Lipid Nanoparticles

129. PET Imaging of *In Situ*-Engineered CAR-T Cells in B Cell Lymphoma with ^{68}Ga-PSMA-11

Nisi Zhang, Jai Woong Seo, Elise Robinson, Angelie Rivera-Rodriguez, James Wang, Yutong Guo, Bo Wu, Spencer K. Tumbale, Marina N. Raie, Mohammed Inayathullah, Debra K. Czerwinski, Ronald Levy, Katherine W. Ferrara

Stanford University, Palo Alto, CA

Chimeric antigen receptor (CAR)-T cell therapy offers promising outcomes in the management of hematological malignancies. Current CAR-T cell *in vitro* manufacturing routes are complex, and the process duration and cost present significant barriers. While *in situ* engineering of T cells is being explored to include viral and non-viral vectors to modify ligands for T cell targeting, lipid nanoparticles (LNPs) offer a safe alternative with tunable particle size, surface chemistry, and ease of cargo loading. However, the effectiveness of LNP *in situ* CAR T cell engineering has not been extensively explored *in vivo*. Positron emission tomography (PET) is a molecular imaging

technique to depict biochemical processes in patients. Combined with a radiotracer specifically for CAR-T cells, PET monitors the activity and spatial distribution in the body. Herein, we use prostate-specific membrane antigen (PSMA) as the PET reporter transgene, with restricted expression in normal tissues, and the PET reporter gene-probe pair of PSMA and ^{68}Ga-PSMA-11. We developed aCD5 mPSMA/mCAR19 LNPs that encapsulate PSMA mRNA and anti-CD19 CAR-mRNA encoded with a flag tag. To evaluate the feasibility of CAR-T cell radiolabeling, we first treated an immortalized mouse T cell line (EL4) with LNPs, followed by ^{68}Ga-PSMA-11 incubation and measured radioactivity. The cells treated with aCD5 mPSMA/mCAR19 LNPs had significantly higher ^{68}Ga counts per minute (CPM) (p=0.016), compared to cells without treatment or treated with IgG mPSMA/mCAR19 LNPs. In *ex vivo* studies with mouse T cells, we found with flow cytometry that PSMA and CAR19 were co-expressed on T cells (5.6%), while PSMA and CAR19 single positive populations were 8.9% and 12.1%, respectively. For initial *in vivo* imaging studies, we encapsulated firefly luciferase mRNA (mFluc) in place of PSMA mRNA along with CAR19 mRNA into LNPs (aCD5 mFLuc/mCAR19 LNPs) and tracked the bioluminescence signals using *in vivo* and *ex vivo* imaging. We observed positive FLuc expression in lymphoid organs, particularly in the spleen. Additionally, we treated the mice with aCD5 mCAR19 LNPs and performed immunophenotyping analysis of the organs for CAR19 using spectral flow cytometry. We showed enhanced LNP binding (65.2%) and CAR19 expression (11.64%) in the CD3$^+$ T cell population in the spleen, as compared with non-treated controls or IgG mCAR19 LNPs (0%) at 24 h post treatment. Meanwhile, CD11c$^+$ dendritic cells and F4/80$^+$ macrophages had low levels of LNP binding (<5%) and CAR19 expression (<2%), and other immune cells remained unaffected. Furthermore, in CD19$^+$ A20 tumor-bearing mice (a B cell lymphoma mouse model), treatment with aCD5 mCAR19 LNPs resulted in a significant increase in chemoattractant and pro-inflammatory cytokines in blood, and infiltration of T cells and other immune cells in tumors, compared to the non-treated group, demonstrating potential efficacy of *in situ*-engineered CAR-T cells. In conclusion, our studies have shown an LNP transfection system that can load mRNA for imaging with different modalities and CAR19 mRNA for co-expression in T cells. While we have demonstrated in vivo imaging to track T cells based on the expression of Fluc, our initial in vitro studies showed the potential to quantitatively track T cells with 68Ga-PSMA-11 paired with PSMA mRNA. This strategy allows for active surveillance of the level of CAR-T cells (Fig. 1).

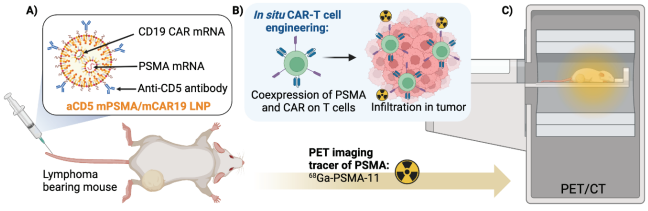


Fig 1. Schematic graph illustrates PET imaging of *in situ* engineered CAR-T cells. A) LNP vehicle is composed of DSPC, DSPE-PEG2000, Lipid 8, cholesterol and DSPE-PEG5000 conjugated with anti-CD5 antibody. The aCD5 mPSMA/mCAR19 LNP encapsulates PSMA mRNA and CAR19 mRNA encoded with a flag tag. We also prepare IgG mPSMA/mCAR19 LNP as the control with no T cell targeting. **B)** In a B cell lymphoma mouse model, we administer aCD5 mPSMA/mCAR19 LNPs to *in situ* engineer T cells expressing CAR19 and PSMA. **C)** Combined with CT scanning, PET imaging with ^{68}Ga-PSMA-11 monitors CAR-T cell spatial distribution in the body.

130. COATSOME® SS Series: Highly Versatile Ionizable Lipid as Vital Component of Lipid Nanoparticle for Gene Therapy and Vaccine Application

Ryosuke Fukuda[1], Shinya Tamagawa[1], Yuta Nakai[1], Hiroki Tanaka[2], Hidetaka Akita[2]

[1]NOF Corporation, Kasawaki, Japan,[2]Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan

BACKGROUND: Nucleic acid vaccines, DNA and messenger RNA (mRNA), have emerged as promising modalities for infectious disease and for cancer immunotherapy due, in part, to shortened manufacturing cycles and high potency. Lipid nanoparticles (LNP) composed of ionizable lipids are important components of such vaccines as they can convey and present the nucleic acid effectively to the immune system. However, toxicity of ionizable lipids and the challenges of cold chain logistics of LNP have limited their clinical use. Further development of LNP requires 1) a reduction in the systemic toxicity, 2) improved endosomal escape, 3) potent and cytotoxic T-cell specific adjuvating function, 4) improved stability at room temperature. *RESULTS:* We have previously demonstrated that LNPs composed of our biodegradable ionizable lipids (COATSOME® SS series), can deliver pDNA or mRNA in mice to the liver or the solid tumor via intravenous injection and achieve high levels of expression with less toxicity. Here, we describe their potential applications for tissue-specific gene therapy and mRNA-based vaccines. First, we finely tuned the LNPs formulation to achieve liver or spleen delivery and tissue-specific mRNA expression via intravenous injection. In addition, subcutaneous administration of ovalbumin (OVA)-encoding mRNA encapsulating LNPs composed of ssPalm-O-Phe (COATSOME® SS-OP) induced higher anti-OVA antibody production than a counterpart of globally-marketed ionizable lipid. This result implies high vaccination effects for infectious disease. Furthermore, addition of ssPalm E-P4C2 (COATSOME® SS-EC), another type of our ionizable lipids, significantly improved cytotoxic T-cell activity as a cancer vaccine. Next, we demonstrated that our SS-lipid formulation clearly suppressed the tumor growth in a mouse model of disseminated malignant diseases. In addition, we successfully delivered LNPs containing SS-EC to the spleen efficiently, and they induced high levels of cytotoxic T-cell activity. Thus, SS-EC would be used as a promising material for spleen-targeted cancer vaccines. Importantly, our LNPs with attractive functions were found to be stable as a freeze-dried formulation at room temperature for at least 3 months. *CONCLUSION:* We here demonstrate that SS-lipid bearing LNPs stimulate humoral or cellular immune responses, in particular, remarkably high cytotoxic T-cell responses and the spleen-selective cytotoxic T-cell responses. These responses were shown to be effective in limiting growth of tumor cells in a disseminated disease model. Furthermore, we achieved the development of freeze-dried LNPs which were stable for at least 3 months at room temperature, potentially leading to avoiding the cold-chain limitation of conventional mRNA vaccines.

131. Map the Formulation Space of Lipid Nanoparticles (LNPs) Using Microfluidic Assembly in the Intraperitoneal (IP) Administration of RNA Therapeutics

Farhana Islam[1], Ling Ding[2], David Oupicky[2]

[1]Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE,[2]Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, NE

Purpose: Pancreatic cancer (PC) is a challenging disease with limited treatment options and a poor prognosis. Despite progress in chemotherapy and targeted therapies, treatment remains difficult. A promising approach is utilizing lipid nanoparticle (LNP) technology for delivering therapeutic mRNA to pancreatic cancer cells. This technology enables specific mRNA delivery to cancer cells, enhancing effectiveness with fewer side effects than traditional therapies. Our goal is to synthesize and characterize LNPs optimized for mRNA delivery to pancreatic cancer cells, aiming to advance the development of mRNA-LNP as a potential therapeutic approach for PC. *Methods:* A microfluidic system was utilized in the development of the library of mRNA-LNP formulations, where various parameters were modified to optimize the formulations. To characterize the formulations, their size, polydispersity index (PDI), encapsulation efficiency (EE%), and stability were assessed using DLS and ribogreen assay. Additionally, the cytotoxicity of the formulations was evaluated by CTB assay on KPC8060 cell lines. Cellular uptake was investigated by luciferase assay and *in vitro* tumor spheroid penetration was observed under confocal microscopy by delivering GFP mRNA. *In vivo* biodistribution of mRNA-LNP formulation was examined in both healthy and tumor-bearing c57BL/6 mice and tracking performed using in vivo imaging system (IVIS). Luciferase mRNA was employed to assess mRNA delivery and expression in various organs. In the tumor mice model, orthotopic pancreatic tumor implantation was performed, and LNP formulations were administered upon reaching a specific tumor size. We also conjugated a peptide as a targeting moiety to one of the lipids used in the LNP synthesis. *Results:* The modification of process parameters had a notable impact on the size, PDI, and EE%. The resulting LNP formulations have demonstrated stability and reproducibility in terms of size, PDI, and EE%. LNP formulations exhibited significant cellular uptake when compared to free mRNA uptake. Both formulations showed efficient penetration of tumor spheroids, ensuring successful delivery of the mRNA payload *in vitro* (Fig 1). *In vivo* experiments conducted on healthy and tumor-bearing mice reveal that both LNP3 and LNP4 formulations exhibited distribution to the pancreas. Additionally, LNP4 demonstrated a significant presence in the liver, while LNP3 showed distribution to the tumor (Fig 2). The peptide-lipid conjugation was initially confirmed by thin layer chromatography. *Conclusion:* The utilization of a microfluidic system for the mRNA-LNP formulation development, coupled with the optimization of process parameters, has yielded promising outcomes concerning their physicochemical attributes, cellular uptake, tumor spheroid penetration and *in vivo* biodistribution to pancreatic tumors. As part of our future work, we aim to incorporate the peptide conjugated lipid into the LNPs to enhance their specificity for pancreatic tumor delivery.

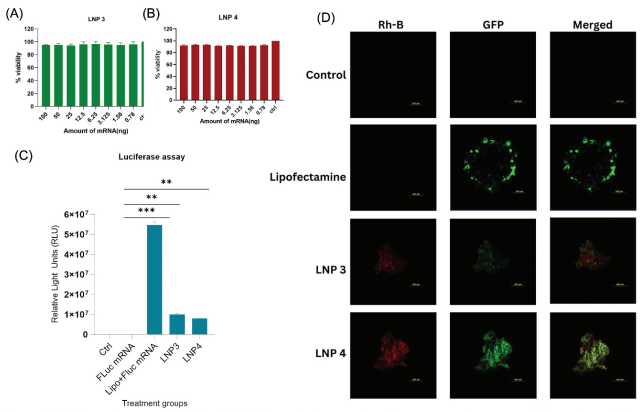


Figure 1: Cell-based assays of LNP3 and LNP4 formulations conducted on pancreatic cancer cell line. Cytotoxicity assessment, as determined by the CTB assay on KPC8060 cell line, indicates the impact of LNP3 (Fig. A) and LNP4 (Fig. B) formulations compared to untreated (control) cells. The study involves a range of mRNA concentrations from 0.78 ng to 100 ng (C). Furthermore, the cellular uptake of LNP formulations is explored through luciferase assay, comparing it with lipofectamine-treated mRNA uptake and free mRNA uptake. (D) The *in vitro* tumor spheroid penetration capacity of LNP formulations is also evaluated, and the level of penetration is compared with lipofectamine-treated mRNA using post-treatment confocal imaging and z-stacking. N=3 biological replicates, scale: 100 μm.

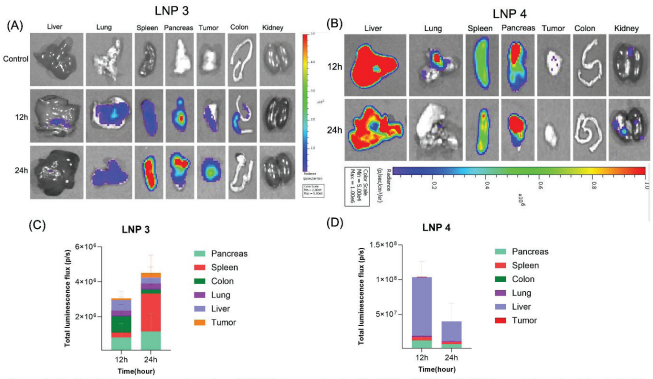


Figure 2: Biodistribution assay conducted on C57BL/6 tumor mice (n=3) utilizing LNP3 and LNP4 formulations, both loaded with fLuc mRNA. Orthotopic tumor implantation in pancreas was done and ultrasound was used to detect tumor size. When the tumor reached to a specific size, the mice treated with fLuc mRNA LNP formulations. Following post-treatment incubation, the animals were euthanized, and their organs such as pancreas, spleen, colon, lung, liver and tumor were collected for imaging. The expression of mRNA in various organs was assessed using an in vivo imaging system to track the biodistribution of LNP3 (Fig. A) and LNP4 (Fig. B) formulations. The total luminescence flux in different organs was quantified and is presented in Fig. C and Fig. D for LNP3 and LNP4 formulations respectively, with analysis conducted using living image software.

132. *In Vivo* RNA Delivery to T Cells and Hematopoietic Stem Cells in Humanized Mice and Non-Human Primates Using Targeting Lipid Nanoparticles

Rahul Palchaudhuri[1], Mohit Gupta[1], Michael T. Monte[1], Lorenzo Tozzi[1], Yu Cao[1], Han Gu[1], Rebecca Levy[1], Apiwat Wangweerawong[1], Lei Liu[1], Laura Saunders[1], Rebecca L. Goldman[1], Thomas V. Colace[1], Aaron Zannini[1], Travis Shute[1], Christina Ferren[1], Giulia Schiroli[1], Alyssa Sanfilippo[1], Tiffany Morales[1], William E. Salomon[1], James B. Rottman[1], Ulrike Gerdemann[2], Francesca Alvarez Calderon[2], Lev Gorfinkel[2], David Didio[1], Pinky Htun[1], Jack Chu[1], Zhijie Ma[1], Gilbert Ndzeidze[1], Chelsea Habif[1], Michael S. Magee[1], Jason Rodriguez[1], Madhusudan V. Peshwa[1], Rachit Jain[1], Bill Querbes[1], Leslie S. Kean[3], Hari Pujar[1], Michael C. Holmes[1], Jane Wang[1]

[1]Tessera Therapeutics, Somerville, MA,[2]DFCI, Boston, MA,[3]Boston Children's Hospital, Boston, MA

Non-viral *in vivo* delivery of genetic medicines could unlock new therapeutic areas and provide broader patient access. However, the success of *in vivo* delivery is dependent on the development of safe delivery vehicles capable of transporting the genetic medicines to their intended target tissues and cells. In recent years, lipid nanoparticles (LNPs) have emerged as a powerful *in vivo* delivery platform for RNA therapeutics to reach the liver, but efficient delivery to extrahepatic tissues remains a challenge. Tessera Therapeutics is developing novel LNPs designed to deliver RNA-based gene-modifying payloads to both T cells and HSCs *in vivo.* We have taken a multi-pronged approach to design our LNPs to target a desired tissue type. First, we optimized lipid chemistry and composition to skew biodistribution and facilitate endosomal escape. Second, we added specific cell targeting moieties to enable cell-specific delivery. By combining passive and active targeting strategies, we have observed efficient delivery to T cells and HSCs in preclinical mouse and non-human primate (NHP) models. *In vivo* delivery was assessed with LNPs containing Green Fluorescent Protein (GFP) reporter mRNA. Using our novel T cell-targeting LNPs, we demonstrated successful single-dose *in vivo* delivery of GFP in humanized mouse models generated from immunodeficient NSG mice. In NSG mice pre-engrafted with human HSCs for 12-weeks, GFP expression was observed in 34% of splenic T cells with minimal expression in both monocytes and B cells. In NSG mice pre-infused with human T cells for 14-days, GFP expression was observed in ~80% of splenic T cells. We also demonstrated successful GFP delivery in rhesus macaque, with a single dose leading to ~40% expression in peripheral T cells with negligible GFP expression in B cells. We also evaluated our Gene Writer in combination with a beta-2 microglobulin (*B2M)* template in a single dose of LNP and observed a ~30% knockout of *B2M* in T cells through a specific rewrite. Separately, we are developing HSC-targeting LNPs. In humanized NBSGW mice, HSC-targeting LNPs with a single dose led to dose-dependent delivery with GFP expression observed in over 90% of long-term HSCs (LT-HSCs defined as Lin-CD34+CD38-CD90+CD45RA- cells). With further improvements to the LNP, we also achieved over 90% GFP expression in long-term HSCs in NHPs. We then evaluated our Gene Writer in combination with B2M template in a single dose of LNP, where we observed ~15% *B2M* knockout in LT-HSCs of humanized mice after a single dose. **Together**, we believe these *in vivo* delivery results demonstrate our LNP delivery platform may be capable of efficiently delivering RNA payloads to T cells and HSCs, providing a potential delivery platform for *in vivo* genome engineering.

133. Machine Learning-Guided Optimization of Lipid Nanoparticle Composition for B Cell Transfection

Wu Han Toh, Leonardo Cheng, Gene Weng, Charles Shin, Ataes Aggarwal, Hai-Quan Mao
Johns Hopkins University, Baltimore, MD

Statement of Purpose: B lymphocytes (B cells) play a critical role in adaptive immunity, making them an important target for gene therapies. Despite this, efficient delivery systems for B cell transfection have yet to be developed. Lipid nanoparticles (LNPs) have emerged as promising non-viral vectors for immune cell delivery; however, they require extensive optimization across a complex design space - a task where manually directed approaches have proven insufficient[1]. Here, we build and deploy an iterative machine learning (ML)-guided screening platform to rapidly and exhaustively optimize LNP composition for B cell transfection *in vitro*. From a base library screening, we select, train, and refine high performing ML algorithms that predict transfection outcomes as a function of LNP design features[2]. Then, based on these predictions, we leverage Bayesian optimization to search for new test formulations, focusing on areas with the greatest transfection potential. We evaluate whether an *in silico* guided approach can uncover LNP formulations with improved experimental transfection. **Methods:** To generate a diverse LNP library for ML training, we used DLin-MC3-DMA as the ionizable lipid, cholesterol, DMG-PEG2000, and one of six helper phospholipids with varying charge. We varied four parameters, as previously described[1]. In total, 486 LNP formulations were screened for *in vitro* transfection efficiency in Ramos B cells using luciferase reporter mRNA. The sci-kit learn package (1.2.2) was used to conduct model training and prediction on a panel of tree-based, linear, and least squares regression models. Helper lipids were parameterized using select chemical features, and the data underwent 5-fold cross-validation with an 80-20 train-test split, optimized through randomized grid search to minimize mean absolute error. Gaussian process-based Bayesian Optimization via Scikit-Optimize (0.9.0) was used to suggest 15 *de novo* LNP formulations to test per helper lipid, using expected improvement (EI) to trade-off exploration and exploitation. **Results:** A total of 486 LNP formulations were screened for *in vitro* transfection in Ramos B cells, yielding a diverse library for robust ML model training (Fig. 1A, B). Of models tested, XGBoost (XGB) demonstrated the best predictive ability, achieving impressive Pearson's correlations (r = 0.91), and Spearman's rank (r = 0.89) on hold-out datasets (Fig. 1C). Feature importance analysis, using Shapley Additive Explanations, reveals structure-function relationships successfully learned by our model (Fig. 1D). Side-by-side experimental validation of ML-optimized test formulations yielded higher overall transfection efficiency compared to grid-screened formulations (Fig. 1E), with the top ML-optimized formulation achieving a 1.36-fold increase in RLU, 500% of the transfection signal benchmarked against the top-grid-searched formulation (Fig. 1F). Our platform efficiently tunes LNP formulation composition (Fig. 1G), achieving significant improvement over conventional screening.

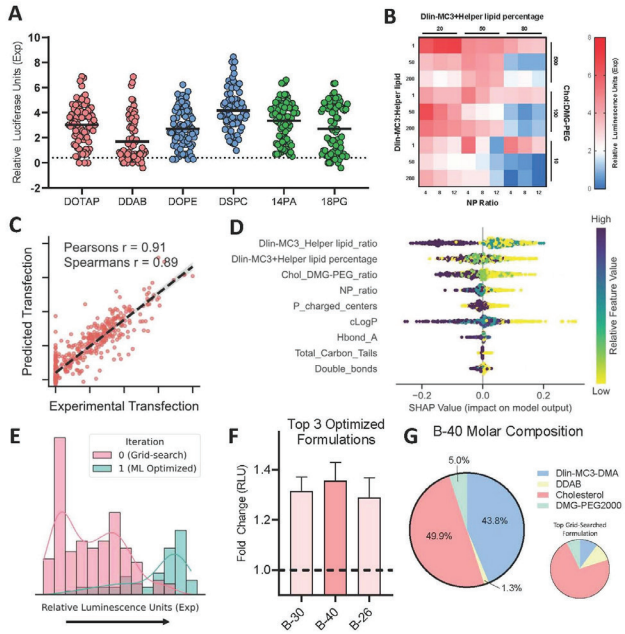


Figure 1. (A–B) Initial grid-based library screen of *in-vitro* Ramos transfection. (C–D) Model training and evaluation. (C) Experimental ground-truth vs. optimized XGB model-predictions on hold out set. (D) SHAPley feature importance beeswarm plot. (E–G) Experimental validation of ML-optimized test formulations. (E) Overall RLU distribution of optimized vs. grid-screened formulations. (F) Fold change in RLU of top 3 optimized LNPs over top grid-screened formulation. (G) Molar composition of top optimized LNP.

Conclusion: This platform is the first to use an ML-guided approach for screening LNP compositional ratios. This platform can be iterated to further increase transfection and can be adapted to optimize delivery to other historically hard-to-transfect cell-types. **References:** 1. Zhu Y, *et al.* **Nature Commun**. 2022;13:4282. 2. Cheng L, *et al.* **bioRxiv**. 2023. [Preprint]. Available from: https://doi.org/2023.12.07.570602

134. Formulation Optimized Circular RNA-LNP Vaccines Elicit Enhanced Immune Responses Against SARS-CoV-2

Edo Kon[1], Niv Cohen[1], Maya Shlosberg[1], Sofiya Pashko[1], Roi Mashiach[1], Bo Hu[2], Xiaowen Zou[2], Mengyao Zheng[2], Siqi Li[2], Emma Pan[2], Weiyi Zhang[2]

[1]RiboX Therapeutics (Israel) Ltd, Ness Ziona, Israel,[2]RiboX Therapeutics, Shanghai, China

mRNA Lipid Nanoparticles (LNPs) have emerged as an essential vaccine platform due to their success in the recent COVID-19 pandemic. The RNA-LNP platform is effective and, among other advantages, is modular regarding the ability to encapsulate different antigen encoding RNA sequences in the same LNP formulation. LNPs comprise a lipid formulation typically containing a phospholipid, cholesterol, PEG-lipid and an ionizable lipid, in varying molar ratios. Beyond mRNA, the covalently-closed structure of circular RNA (circRNA) prevents the degradation by exoribonucleases, therefore, leading to an intrinsic stability. This stability results in a more robust protein expression which, in a vaccine context, can correlate with improved immune responses. Furthermore, the circRNA structure may require formulation optimization that differs from optimal formulations for linear mRNA payloads. To optimize a circRNA-LNP vaccine formulation, a Design of Experiment (DoE) approach was used to maximize vaccine immunogenicity. The optimization was performed by altering the LNPs' lipid molar ratios without altering the lipid component identities. To determine the prophylactic vaccine efficacy, circRNA-LNPs coding for the SARS-CoV-2 receptor-binding domain fused to an Fc sequence (RBD-Fc) were utilized. Animals were intramuscularly administered with the circRNA-LNP vaccines and probed for serum IgG and antigen-specific cellular responses against the SARS-CoV-2 Spike glycoprotein as well as live-virus neutralizing titers. We demonstrate increased anti-Spike IgG titers in circRBD-Fc-LNP vaccinated animals compared to linear modified mRNA RBD-Fc-LNPs (Fig B). Furthermore, we show that by optimizing the LNP formulation we substantially enhanced both the virus neutralizing titers and antigen-specific cellular responses (Fig D, E). In conclusion, we have optimized a vaccine formulation capable of substantially enhancing the immunogenicity of circRNA-LNP vaccines. The optimized formulation enables a significant reduction in the administered dose while maintaining the circRNA-LNP vaccine efficacy. This advantage can mitigate vaccine related-reactogenicity and toxicities and may enable the development of vaccines against antigens that require a more profound expression profile.

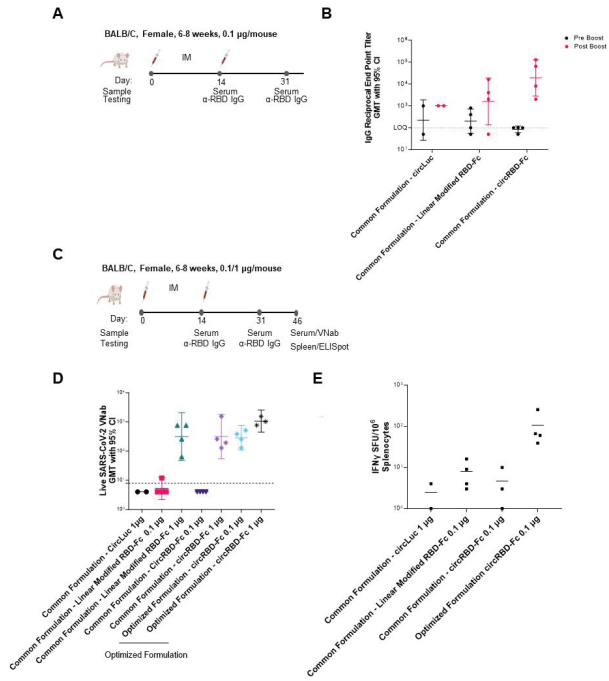


Figure: Formulation Optimized Circular RNA-LNP Vaccines Elicit Enhanced Immune Responses Against SARS-CoV-2. (A) Scheme depicting the vaccination regimen in Fig B. (B) Pre and post boost anti-Spike IgG titers recovered from sera of immunized mice. (C) Scheme depicting the vaccination regimen in Fig D,E. (D) SARS-CoV-2 virus neutralizing titers recovered from sera of immunized mice. (E) Spike-specific cellular responses quantified by IFNγ spot-forming units from splenocytes of immunized mice. Determined by ELISpot assay.

135. Development of a Novel "CLAMP" Platform for Scalable Preparation of Antibody-Conjugated Lipid Nanoparticles for T Cell-Targeted mRNA Delivery

Xi Zhu, Hong Chen, Jiaming Ren, Dalang Li, Chenxi Hou, Shin-Shay Tian, Xiaoping Zhao
Shanghai Vitalgen Biopharma, Shanghai, China

The advent of antibody-conjugated lipid nanoparticles (LNPs) for targeted delivery of nucleic acids has emerged as a compelling strategy for developing immuno and genomic therapies. This approach combines the robustness of LNPs as nucleic acid carriers with the specificity afforded by the conjugated antibodies, enabling precise and targeted delivery to the extrahepatic tissues. This approach has in particular, been validated by the encouraging proof-of-concept results of the T cell targeted LNPs (tLNPs) encapsulating CAR encoding mRNAs, resulting in the in-situ generation of functional CAR-T cells. However, the technical challenges associated with the reported tLNPs preparation methods, such as maleimide conjugation and post-insertion, have hindered the scale-up of LNP production and translational development. In response to these challenges, we developed a Controllable Ligand Attachment Modification and Purification (CLAMP) platform, an innovative technology tailored for the manufacturing of tLNPs. CLAMP integrates the processes for surface conjugation and purification of tLNPs, by incorporating systematically optimized components such as chemical linkers, surface anchors, site-specific residues engineered onto antibodies, coupling reactions, and purification processes. This platform simplifies complex conjugation procedures, ensuring compatibility with the standard LNP manufacturing processes. Applying the CLAMP technique, we successfully prepared anti-human CD5 antibody-conjugated LNPs (CD5-tLNPs) for T cell-targeted mRNA delivery. In the *in vitro* transfection studies, CD5-tLNPs exhibited high transduction efficiency in CD5-expressing Jurkat cells, with positivity exceeding 95%, while positivity for the human hepatic cell line HepG2 remained below 7%. Studies with unstimulated human peripheral blood mononuclear cells (PBMCs) demonstrated remarkable T cell specificity, with approximately 30% positivity in T cells and less than 1% in other evaluated immune cell types. To assess the robustness of the CLAMP technique, multiple independent batches of CD5-tLNPs were produced and tested. The high quality and consistencies of the different batches were confirmed by the physicochemical parameters evaluated and the consistent results from the *in vitro* transfection studies. In a stark contrast, the CD5-tLNPs prepared using the conventional maleimide conjugation method exhibited significantly greater batch-to-batch variations. To further demonstrate the T cell targeting efficiency of the CD5-tLNPS prepared by CLAMP, *in vivo* studies were conducted in hPBMC-engrafted NSG mice and CD5 humanized transgenic (hCD5) mice, employing tdTomato mRNA as a reporter. Expressions of tdTomato in subsets of cells in the peripheral blood and various tissues were assessed with flow cytometry 24 hours post-intravenous administration. In the hPBMC mouse study, 27.5% of the peripheral blood T cells expressed the tdTomato, contrasting with 0.23% in the CD5 antibody-negative LNP control group. In the hCD5 mouse study, tdTomato expression were observed in 32.9%, 10.3%, and 7.0% of the T cells isolated from the peripheral blood, spleen, and liver, respectively. In contrast, only 2.1%, 0.4%, and 0.9% of the T cells from the corresponding tissues did so. Notably, in the liver of the CD5-tLNP treated group, we observed a significant decrease in CD3 off-target cell positivity (7.7%) compared to the CD5 Ab negative LNP group (36.1%). In summary, we have shown that the novel CLAMP platform consistently produced fully functional and scalable antibody-conjugated tLNP preparations that could be adapted to clinical applications.

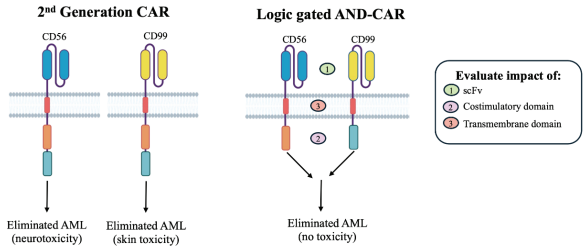
Genetically Modified Immune Cells for AML and Solid Tumors

136. Logic Gated CAR T Cells Targeting Acute Myeloid Leukemia Effectively Clear Leukemia While Mitigating Skin and Neuronal Toxicity

Phoebe Duong, Mark E. Kohler
University of Colorado, Anschutz Medical Campus, Aurora, CO

Chimeric antigen receptor (CAR) expressing T-cells have demonstrated remarkable efficacy in inducing minimal residual disease (MRD)-negative remissions in pediatric patients with relapsed and/or chemotherapy refractory acute lymphocytic leukemia (ALL). CAR T cell therapy for myeloid leukemias, however, has not demonstrated the same level of clinical success. There are multiple factors that may limit the effectiveness of CAR T cells in AML, such as clonal heterogeneity, a more suppressive cytokine milieu, and increased toxicity when targeting lineage restricted antigens. Furthermore, there is mounting evidence that leukemia stem cells (LSC) are drivers of relapse and chemotherapy refractoriness in AML patients, making this an essential population to target. However, many of the putative antigens that define LSCs are expressed on non-malignant cells of vital tissues, making them unsuitable for current CAR T cell strategies. Logic gated CAR T cells have the potential to overcome the lack of tumor-specific antigens through the targeting of unique combinations of antigens that are restricted to malignant cells and absent from healthy tissues. To this end, we evaluated an AND-CAR design to target AML antigens while mitigating on-target/off-tumor toxicity. Preclinical testing of logic gating strategies is limited by a paucity of syngeneic CAR T cell models where targeted antigens are present on normal tissues. Furthermore, testing of logic gating in xenograft models frequently evaluates responses to tumors engineered to express a single antigen, however, the continuous growth of tumors makes it unlikely for these models to fully capture the impact of off-target leakiness of logic gated CAR on normal tissues. To interrogate the efficacy of our AND-CAR strategy, we took advantage of two scFv's that targeted human AML antigens and cross-react with murine antigens expressed in normal tissues. We first generated a 2nd generation CAR using a cross-reactive scFv targeting CD56, which eliminated human AML from xenograft models, but led to lethal neurotoxicity in mice. Similarly, a 2nd generation CD99 CAR with a cross-reactive scFv was effective at eradicating AML but caused an inflammatory infiltrate in the skin of the paws of mice. Using an AND-CAR targeting CD56 and CD99, in which costimulatory domain and CD3z domain were expressed on two separate CAR molecules, we were able to clear human AML from mice without the off-tumor toxicity to the brain or skin. We further

investigated the factors important to the function of our AND-CAR and found enhanced in vitro and in vivo function when incorporating a 4-1BB costimulatory domain but no differences when examining combinations of transmembrane domains. Unexpectedly, we found the combination of scFv and signaling domain had a large impact on AND-CAR function. In conclusion, we have developed a model to leveraged cross-reactive scFv's to interrogate logic gating strategies for efficacy and on-target/off-tumor toxicity to healthy tissues and have begun to define the key aspects of CAR design which impact the success of logic gating.



137. Development of Extracellularly Linked Concatemeric Trivalent Cytokine (ELeCTriC) Chimeric Antigen Receptor (CAR) T-Cells as Dual Conditioning and Immunotherapeutic Agents

Quenton R. Bubb[1], Mohammad Balood[2], Gabe E. Seir[2], Leah Swartzrock[3], Ethan Haslett[2], Katie Ho[2], Saida G. Wiltz[3], Elena Sotillo[2], Rebecca M. Richards[4], Tanja A. Gruber[2], Crystal L. Mackall[2], Agnieszka Czechowicz[3]

[1]Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA,[2]Pediatrics, Stanford University, Stanford, CA,[3]Stanford University, Stanford, CA,[4]Pediatrics, University of Wisconsin-Madison, Madison, WI

Pediatric acute myeloid leukemia (AML) is a heterogenous disease of the hematopoietic system in which hematopoietic stem and progenitor cells (HSPCs) acquire genetic or epigenetic lesions that bias them toward a self-renewing, immature differentiation state at the cost of healthy, balanced hematopoiesis. Significant progress has been made toward improving outcomes in pediatric AML in part through advances in Hematopoietic Stem Cell Transplantation (HSCT); however, nearly 40% of children with AML relapse, and in the relapse setting, approximately 50% die due to refractory disease or treatment related morbidities within 5 years. Powerful immunotherapeutic modalities, such as chimeric antigen receptor (CAR) T-Cells, have reached impressive clinical milestones in the treatment of certain hematologic malignancies including acute lymphoblastic leukemia. However, extension of these approaches to AML has been difficult due to immense heterogeneity of target expression in AML and shared antigen expression between AML cells and healthy hematopoietic tissues. This is a unique and significant challenge in AML, as sparing HSPCs can also potentially spare leukemic or pre-leukemic stem cells (LSCs) that can reinitiate disease and cause relapse even after HSCT. Here, we propose that the signaling overlap between HSPCs, LSCs, and leukemic blasts can be exploited as a therapeutic strategy. It is well known that engagement of receptor tyrosine kinases (RTK) KIT, MPL, and FLT3 by their cognate ligands, stem cell factor (SCF), thrombopoietin (TPO), and FLT3 ligand (FLT3LG) and downstream signaling are essential to both healthy and malignant hematopoiesis. By interrogating a large pediatric AML cohort (n=453), we found that despite significant transcriptional heterogeneity among AML subtypes, expression of KIT, MPL, or FLT3 was a feature shared by nearly all pediatric AMLs. Further, after identifying pediatric AML subtypes with high and low interpatient transcriptomic heterogeneity, we found that pediatric AML subtypes with high transcriptional heterogeneity had higher diversity of KIT, MPL, and FLT3 expression and increased expression of stemness-related genes. To bridge this heterogeneity and provide a means of non-genotoxic conditioning for consolidation with an allogeneic HSCT, we have developed a novel, first-in-class Extracellularly Linked Concatemeric Trivalent Cytokine (ELECTRIC) CAR, in which the binding domains of SCF, TPO, and FLT3LG are attached via a flexible linker to enable simultaneous targeting of KIT, MPL, and FLT3 through a single CAR. CD4 or CD8 T-cells endowed with an ELECTRIC CAR specifically target cognate RTKs and effectively killed a panel of diverse AML cell lines and healthy HSPCs *in vitro*. ELECTRIC CARs also exhibited *in vitro* cytoxicity against primary pediatric AML LSCs and leukemic blasts, including CBFA2T3-GLIS2+ leukemias, a dangerous subtype of AML that is not cured with chemotherapy in combination with HSCT. Guided by pre-clinical models of targeted HSC conditioning to enable allogeneic HSCT, we propose that the ELECTRIC CAR system can be applied in the bridge-to-transplant setting to achieve the dual goal of eliminating residual leukemia and effectively providing non-toxic conditioning. Subsequently, the ELECTRIC CARs and the patients' immune compartment could be cleared using FDA-approved immune-depleting agents, thereby enabling a safe and effective allogeneic transplant with potential to effectively cure all AMLs with minimal toxicity.

138. Developing Adoptive Cell Therapy Strategies to Leverage Myeloid Immunity Against AML

Shannon Oda[1,2], Ryma Toumi[1], Simonne Guenette[2], Edison Chiu[1], Aitong Ruan[1], Sofia Nguyen[1]

[1]Seattle Children's Research Institute, Seattle, WA,[2]University of Washington, Seattle, WA

Acute myeloid leukemia (AML) is a rapidly progressing and highly fatal disease, with a 5-year survival rate of 32% (NCI). AML cells alter the programming of immune cells, supporting conversion of anti- to pro-tumorigenic cell types. In particular, AML tumor cells induce pro-tumor macrophages that promote resistance to therapy and phagocytosis. This subset of inhibitory, $CD206^{+}$ macrophages is significantly associated with worse outcomes for AML patients ($^{**}P<0.01$). However, pro-tumor macrophages show significant plasticity and can be polarized to antitumor programming with costimulatory signals, increasing phagocytosis of tumor cells and recruitment of other antitumor immune cells. One such costimulatory signal, CD40, has been explored to induce antitumor myeloid immunity and agonist CD40 antibodies have been tested in clinical trials. However, enthusiasm for agonist antibody therapies has been dampened by dose-limiting toxicities, inadequate pharmacokinetics and tissue penetration, and the non-tumor targeted delivery of the

antibody modality. Another promising approach, adoptive cell therapy (ACT), utilizes living, genetically-engineered T cells that actively localize to sites of tumor and specifically attack tumor cells. ACT is particularly attractive for AML therapy, as ACT is a first-line therapy for other hematological cancers and T cells naturally home to the sites of AML (e.g. blood, bone marrow). We sought to develop a T cell engineering strategy to deliver CD40 costimulatory signals to the AML-BM and reprogram inhibitory macrophages. We engineered membrane-bound fusion proteins, CD40L Dual Costimulatory Receptors (DCR), that pair the CD40L ectodomain with a panel of costimulatory endodomains. We developed both human and murine CD40L DCRs and showed increased T cell antitumor function in *in vitro* studies. We advanced leading CD40L DCRs to proof-of-concept *in vivo* testing in an immunocompetent AML mouse model and found that CD40L DCR-T cell therapy significantly enhanced therapeutic outcomes (***P<0.001). We observed a significant increase in antitumor macrophages (>40%, **P<0.01) as compared to control T cells, suggesting that CD40L DCR expression indeed induced macrophage conversion. Our long-term objective is to develop strategies and define mechanisms to employ both innate and adaptive immunity to provide a diverse and durable antitumor immune response against AML, and support clinical translation.

139. *Ex Vivo* and *In Vivo* Generation of Dual Specific CAR T Cells for Combination CAR T/ Oncolytic Virus Therapy

Olivia Liseth[1], Benjamin Kendall[1], Elizabeth Appleton[1], Thomas Kottke[2], Rosa Diaz[1], Jason M. Tonne[2], Soha Rizk[1], Masahiro Ono[3], Metko Muriel[2], Matthew Coffey[4], Houra Loghmani[5], Ian Parney[2], Webb Mason[2], Alan Melcher[6], Richard G. Vile[2]

[1]Mayo Clinic Minnesota, Rochester, MN,[2]Mayo Clinic, Rochester, MN,[3]Imperial College London, London, United Kingdom,[4]Oncolytics Biotech Incorporated, Calgary, AB,[5]Oncolytics Biotech, Calgary, AB, Canada,[6]The Institute of Cancer Research, London, United Kingdom

We hypothesized that the inflammation induced by oncolytic virus (OV) infection of tumours would enhance the trafficking, activation and cytotoxicity of CAR T cell therapy. However, we showed that a Type I IFN-rich tumour microenvironment induced by OV infection resulted in the attrition of CAR T cells infiltrating the tumour. We also observed that treatment with both OV and CAR T cells generated a population of dual specific (DS) CAR T cells in which the DS CAR T recognised viral epitopes through their T Cell Receptors. These DS CAR T cells were significantly more cytotoxic against tumour and persisted for much longer in vivo than conventional CAR T cells. Moreover, DS CAR T cells could be re-activated *in vivo* by subsequent boosts with the OV. By pre-loading CAR T cells with OV and administering boosts with virus we have shown that it is possible to cure tumours without prior lymphodepletion in pre-clinical models of melanoma and glioma where conventional CAR T therapy is completely ineffective. We show that these DS CAR T cells exhibit a hyper-functional anti-tumor activity associated with a $CD73^{lo}$, $CD62L^{lo}$, $CD127^{lo}$, and $KLRG1^{hi}$ phenotype, suggesting a highly differentiated effector T cell phenotype. Additionally, upon *ex vivo* splenocyte stimulation with EGFRviii CAR targets or viral peptide, DS CAR T produced higher levels of Granzyme B and IFNγ compared to conventional CAR T. Using the Tocky-Nr4a3 transgenic reporter mouse that expresses a blue protein ($t_{1/2}$ ~ 7h) upon Nr4a3 transcription (downstream of the TCR) that then decays to red ($t_{1/2}$ ~ 20h), we found differential TCR activation dynamics between DS CAR T and conventional CAR T cells, with the more potent DS CAR T cells exhibiting shorter, "fast-off" target interactions with a stronger blue protein positive population. Increased prevalence of blue protein positive T cells in the tumor significantly correlated with decreased tumor weight. We are now developing this approach for clinical trials which necessitates the determination of optimal loading of virus onto the CAR T cells to ensure that the CAR T cells are not cleared by anti-viral effectors *in vivo*. Finally we have also developed novel methods to generate a full range of memory and effector DS CAR T cells directly from naive T cells *in vivo* which are also highly active against tumour following *in vivo* boosting.

140. ROR1 CAR Armored with Dominant Negative TGF-Beta Receptor II Demonstrates Enhanced Efficacy in Xenograft Models of Pancreatic Cancer

Bal Krishna Chand Thakuri, Tri Tran, Saule Nurmukhambetova, Jia-Jye Lee, Peirong Hu, Ngoc Tran, Brittany Steimle, Pradyot Dash, Dina Schneider

Lentigen Technology, Inc., a Miltenyi Biotec Company, Gaithersburg, MD

Introduction. The receptor tyrosine kinase-like Orphan Receptor 1 (ROR1) is a membrane receptor highly expressed at the early embryonic stage and rarely expressed in normal adult tissues. ROR1 is broadly expressed in hematologic and solid tumors making it one of the most promising targets for cancer treatment. To date clinically characterized ROR1-CAR T-cells failed to induce durable remissions, in part due to the immunosuppressive nature of tumor microenvironment (TME). Here we report the development of a fully human ROR1-CAR armored with a dominant negative TGFβ receptor II (TGFβRIIDN) which is protective against TGFβ1, one of the major immunosuppressive components of TME. **Methods.** Enriched primary $CD4^+$ and $CD8^+$ T-cells were used for the generation of CAR T-cells by lentiviral transduction. *In vitro*, flow cytometry was used to assess the expression of ROR1-CAR, TGFβRIIDN armor and its effect on TGFβ1 signaling (via pSmad2/3; canonical signaling pathway) in CAR T cells. The xCelligence method was used to measure CAR cytotoxic activity, and elaboration of cytokines IFNγ, TNFα and IL-2 was measured by ELISA. *In vivo* CAR efficacy was evaluated in pancreatic adenocarcinoma (PDAC) NSG xenograft models AsPC-1, and AsPC-1 over-expressing TGFβ1. Cytokines in peripheral blood were assessed by MSD U-plex assay. Immunophenotype of infused T cells were assessed by flow cytometry. Tumor tissues harvested at day 7 post T-cell dosing and at termination of the study were analyzed by immunohistochemistry (IHC) and immunofluorescence MACSima Imaging Cyclic Staining (MICS) methods for biomarkers. **Results.** ROR1 CAR-T cells armored with TGFβ1-protective TGFβRIIDN element attenuated TGFβ1-mediated Smad2/3 phosphorylation in CAR-transduced primary T cells compared to ROR1 CAR alone. When co-cultured with ROR1+ AsPC-1 pancreatic cancer cells in the presence of TGFβ1, armored ROR1 CAR-T cells showed better killing activity and cytokine IFNγ, TNFα and IL-2 secretion. Similarly, armored CAR -T cells co-cultured with AsPC-1 cells overexpressing TGFβ1 maintained high level of cytotoxicity and cytokine response, in contrast

to the CAR alone. *In vivo,* in mice bearing AsPC-1 pancreatic tumor xenografts overexpressing TGFβ1, armored ROR1 CAR treatment achieved complete tumor remissions, higher expansion of CAR$^+$T-cells, prevented elevation of TGFβ1 level in the peripheral blood, with no noticeable toxicity or weight loss, as compared to CAR alone. MICS analysis demonstrated advanced tumor clearance by armored ROR1 CAR T cells at day 7 post T cell infusion. Unexpectedly, in mouse AsPC-1 pancreatic tumor xenografts without TGFβ1 overexpression, IHC and MICS analysis of tumor tissues harvested at day 7 post T cell infusion revealed marked induction of TGFβ1 upon antigen-dependent interaction of ROR1 CAR T cells with ROR1 positive tumor cells. Furthermore, armored ROR1 CAR treatment achieved enhanced tumor clearance in this model. **Conclusion.** The novel ROR1 CAR$^+$T-cells armored with TGFβRIIDN confer resistance to inhibition by TGFβ1 both *in vitro* and *in vivo*. In addition, TGFβ1 induction by CAR T cells represents a novel, CAR-induced checkpoint signaling in the solid TME, which can be overcome by armoring the CAR-T cells with TGβRIIDN, thus enhancing CAR-T therapeutic potential for the treatment of solid cancers.

141. A Phase 1 Dose Escalation Study of a Novel Coupled CAR T Cell Therapy, GCC19CART, for Patients with Metastatic Colorectal Cancer

Bridget P Keenan[1], Christopher H Lieu[2], Marwan Fakih[3], Dongqi Chen[4], Lucy Lu[5], Eric Rowinsky[5], Victor Lu[5], Lei Xiao[5], Alan P Venook[1], Benjamin L Schlechter[6]

[1]Helen Diller Family Comprehensive Cancer Center, San Francisco, CA,[2]University of Colorado Cancer Center, Aurora, CO,[3]City of Hope Comprehensive Cancer Center, Duarte, CO,[4]Innovative Cellular Therapeutics, Shanghai, China,[5]Innovative Cellular Therapeutics, Rockville, MD,[6]Dana Farber Cancer Institute, Boston, ME

Background: GCC19CART, our lead candidate from the CoupledCAR solid tumor platform, aims to overcome limitations of conventional CAR T-cells in solid tumor malignancies by pairing solid tumor CAR T-cells with CD19-targeting CAR T-cells to amplify the proliferation and activation of the solid tumor CAR T component. GCC19CART targets guanylate cyclase-C (GCC), which is reported to be expressed in the metastatic lesions of 70%-80% of subjects with colorectal cancers and largely restricted to the intestinal tract. A Phase 1 investigator-initiated clinical trial conducted in China showed preliminary efficacy with an overall response rate (ORR) at dose level 1 (1x10^6 CAR T-cells/kg) of 15.4% ORR (2/13) and 50% ORR (4/8) in dose level 2 (2x10^6 CAR T-cells/kg). Common side effects included cytokine release syndrome (CRS) and diarrhea/colitis. Immune effector cell-associated neurotoxicity syndrome (ICANS) occurred in a smaller subset of patients. Building on these encouraging findings, we initiated a US Phase 1 study in August 2022 for patients with metastatic colorectal cancer refractory to standard chemotherapy. As of November 1, 2023, four subjects have received treatment at the initial dose level. Methods: Subjects were initially screened for GCC expression; this requirement was later discontinued when immunohistochemistry studies revealed that the frequency of positive GCC expression exceeded 95%. Eligible subjects underwent leukapheresis, a single dose of lymphodepleting chemotherapy (fludarabine 30mg/m2 and cyclophosphamide 300mg/m2) 3 days prior to infusion, and then administration of a single infusion of GCC19CART. Responses were assessed by RECIST v1.1 by site investigator review and an independent third-party imaging contract research organization. Results: 4 subjects have been dosed at level 1 (1x10^6 cells/kg) and completed the DLT period at the time of data cutoff. The most common adverse events were CRS in 4/4 subjects (Grade 1: 2/4 (50%) or Grade 2: 2/4 (50%)) and diarrhea in 3/4 subjects (Grade 1: 1/4 (25%), Grade 2: 1/4 (25%), or Grade 3: 1/4 (25%)). Three out of four patients had a mild tremor (Grade 1) that resolved with corticosteroids and no other evidence of neurotoxicity. The combined overall response rate (ORR) was 50% (2/4) by independent review. Two subjects demonstrated a partial response (PR) while 1 additional subject had partial metabolic response (PMR) on PET/CT with stable disease (SD) and 1 patient had progressive disease per RECIST 1.1. Per site investigator review, the ORR was 25%, with 1 confirmed PR and 3 SD assessed as best response. The median PFS was 3.8 months in the dose level 1 group. Conclusions: Preliminary results from the US study corroborate our finds from the Chinee trial, demonstrating that GCC19CART has meaningful clinical activity and an acceptable safety profile in refractory metastatic colorectal cancer. This trial is ongoing and updated data will be presented, including from patients treated at dose level 2 (2x10^6 CAR T-cells/kg). Clinical trial information: NCT05319314.

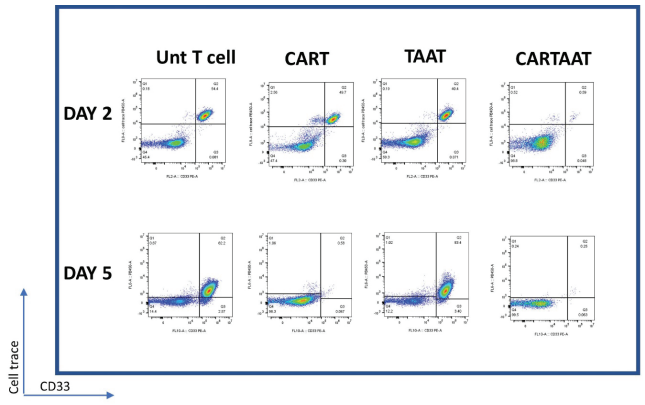
142. Targeting AML with Multi-Antigen Specific T Cells Engineered to Express a CAR Specific for SSX2 Peptide 41-49

Gloria Pezzella[1], Achouak Achour[1], Eric S. Yvon[2], Catherine M. Bollard[1,2], Keri Toner[1,2]

[1]Childrens National Hospital, Washington, DC,[2]The George Washington University, Washington, DC

Background: Acute myeloid leukemia (AML) is the second most common pediatric leukemia, comprising 15-20% of acute hematological malignancies in children. While prognoses have improved due to optimization of chemotherapy and advancements in transplantation, around 30% of patients with AML relapse, and outcomes are poor due to limited treatment options. Targeted therapies, including cellular therapies, are necessary for AML patients. Our group as well as John's Hopkins University (JHU) have shown that tumor-associated antigen specific T cells (TAA-T) can safely prolong event free survival in high-risk patients with solid tumors and leukemia/lymphoma. However, responses in patients with active disease were modest. Therefore, we sought to determine whether CAR-engineering could enhance the potency of the TAA-T platform. Our group has developed a T cell receptor (TCR) like chimeric antigen receptor (CAR) targeting SSX2 peptide (41-49) in the context of HLA-A-0201. We hypothesized that by combining these two cellular therapy modalities-we can enhance the anti-leukemia activity of TAA-T in vitro. **Objectives:** To assess whether PRAME-specific TAA-T expressing $SSX2_{41-49}$ CAR enhanced cytotoxic effects against AML compared to CAR-T cells or unmodified TAA-T cells alone. **Methods:** Mononuclear cells isolated from peripheral blood mononuclear (PBMCs) of healthy donors were co-cultured with antigen presenting cells (APCs) pulsed with PRAME pepmix in the setting of exogenous cytokines. After two weekly stimulations with PRAME pepmix pulsed dendritic cells as APCs, TAA-T cells were transduced with $SSX2_{41-49}$ CAR and stimulated once more the

following week with the same APCs. TAA- T products were analyzed for antigen specificity using IFN-γ ELISpot assay by reintroducing PRAME or T2 cells loaded with $SSX2_{41-49}$ peptide. Immunophenotype and transduction efficiency was determined by flow cytometry. Cytotoxicity of TAA-Ts was determined by flow based coculture with cell trace violet and CD33 staining used to identify the THP1 tumor cells. $SSX2_{41-49}$ CAR TAA-T, $SSX2_{41-49}$ CAR-T, and unmodified PRAME-specific TAA-T cells were cocultured with THP1 tumor cells at 1:5 target to effector ratio. Cells cultures were analyzed by flow cytometry at 0, 24 hours, 48 hours, and 120 hours. **Results:** TAA-T targeting PRAME elicited the following responses on ELISPOT (means measured in SFC/100,000 cells): PRAME=298 and T2+SSX2=35. TAA-T from the same healthy donors (n=5) transduced and stimulated with PRAME elicited: PRAME=62 and T2+SSX2=219. Flow cytometry analysis showed that mean 71.6% (1.53-71.6%) of TAA-T were CD3+CD8+ T cells and mean 79.9% (1.83-90.0%) of $SSX2_{41-49}$ CAR transduced TAA-Ts were CD3+CD8+ T cells. The mean transduction efficiency of TAA-T was 83.9% (range 48.7-93.5%). At 120 hours, 35.4% (0.25-80.9%), 45.4% (0.58-98.2%), and 57.1% (2.82-83.4%) of the original THP1 tumor cells were still present in culture for transduced TAA-T, CAR-T, and unmodified TAA-T respectively. In conclusion, our novel cellular therapeutic of TAA-T transduced with $SSX2_{41-49}$ CAR display enhanced potency against AML tumor cells than unmodified TAA-T or CAR-T cells. Future studies are needed to optimize the expansion of $SSX2_{41-49}$ CAR transduced TAA-T and evaluate the potency of this novel cell therapy approach in an *in vivo* mouse model and ultimately in the clinic.



SSX2 CAR TAA-T demonstrate cytolytic activity after 5 days of coculture with THP1. Unmodified T cells, SSX2 CAR-T, TAA-T and SSX2 CAR TAAT were co-cultured with THP1 tumor cell line at 1:5 to target effector ratio. After 5 days, SSX2 CAR TAA-T showed effective tumor clearance by flow cytometry.

In Vivo Gene Therapy Clinical Trials

143. Long-Term Efficacy and Safety in Adults with Glycogen Storage Disease Type IA (GSD IA) from a Phase 1/2 Clinical Trial and Long-Term Follow-Up Study of DTX401, an AAV8-Mediated, Liver-Directed Gene Therapy

John J. Mitchell[1], Rebecca Riba-Wolman[2], David Rodriguez-Buritica[3], Ayesha Ahmad[4], Maria-Luz Couce Pico[5], Terry G. Derks[6], David A. Weinstein[2], Deepali Mitragotri[7], Richard Collis[7], Andrew Alexander Grimm[7]

[1]Montreal Children's Hospital, Montreal, QC, Canada,[2]University of Connecticut, Farmington, CT,[3]University of Texas McGovern Medical School, Houston, TX,[4]University of Michigan, Ann Arbor, MI,[5]Hospital Clínico Universitario de Santiago de Compostela, Santiago de Compostela, Spain,[6]University of Groningen, Groningen, Netherlands,[7]Ultragenyx Pharmaceutical Inc., Novato, CA

Background*:** Glycogen storage disease type Ia (GSD Ia) results from a deficiency of glucose 6-phosphatase (G6Pase) which is essential for glycogenolysis and gluconeogenesis. DTX401 is an investigational adeno-associated virus serotype 8 (AAV8) vector expressing the human *G6PC* gene. ***Methods*:** An open-label, phase 1/2, dose escalation gene therapy trial (NCT03517085) evaluated the safety and efficacy of a single DTX401 intravenous infusion in 12 adults with GSD Ia for 52 weeks. Three participants in Cohort 1 received DTX401 2.0 x 10^{12} genome copies (GC)/kg, and three participants each in Cohorts 2, 3, and 4 received 6.0 x 10^{12} GC/kg. Participants in Cohorts 1 through 3 received reactive steroids, and participants in Cohort 4 received a prophylactic steroid regimen to prevent transaminase elevations. All participants entered a long-term follow-up study (NCT03970278) to monitor safety and efficacy for up to 312 weeks after DTX401 administration. The data cutoff for this analysis was 21-Dec-2023. ***Efficacy Results*:** From baseline to Week 52, mean (SD [range]) change in actual total daily cornstarch intake was -68.1% (19.5 [-100 to -42%]), p<0.0001 among 10 participants with a cornstarch assessment within the ±14-day analysis window for the Week 52 visit. From baseline to last available timepoint in the follow-up study (including to Year 5 for three participants in Cohort 1 and to earlier timepoints for subsequently enrolled Cohorts 2 through 4), mean (SD [range]) change in actual total daily cornstarch intake was -74.1% (21.8 [-100 to -36%]), p<0.0001 for all 12 participants. ***Safety Results: All participants experienced a treatment-emergent adverse event (TEAE) and a related TEAE; however, no infusion-related or treatment-related serious adverse events (SAEs) were reported. There were three participants with five related TEAEs of hypertriglyceridemia and one participant with two related TEAEs of proteinuria. All SAEs were Grade ≤3 in severity except for one Grade 4 SAE of multisystem organ failure in the setting of cholecystitis. All SAEs were determined to be unrelated to study drug by both the investigator and study sponsor. ***Conclusions*:** DTX401 showed efficacy and an acceptable safety profile in all treated participants at Week 52 that was durable and sustained for up to five years. Participants in all cohorts showed a significant reduction in cornstarch needs from baseline to both Week 52 and to the last available timepoint. Participants treated with DTX401 should continue to be

monitored long-term for overall metabolic control. All participants remain in the follow-up study and updated efficacy and safety results will be reported.

144. AAV1-hOTOF Gene Therapy for Children with Autosomal Recessive Deafness 9

Yilai Shu[1], Jun Lv[1], Hui Wang[1], Xiaoting Cheng[1], Yuxin Chen[1], Daqi Wang[1], Longlong Zhang[1], Qi Cao[1], Honghai Tang[1], Shaowei Hu[1], Kaiyu Gao[2], Mengzhao Xun[1], Jinghan Wang[1], Zijing Wang[1], Biyun Zhu[1], Chong Cui[1], Ziwen Gao[1], Luo Guo[1], Sha Yu[1], Luoying Jiang[1], Yanbo Yin[1], Jiajia Zhang[1], Bing Chen[1], Wuqing Wang[1], Renjie Chai[3], Zheng-Yi Chen[4], Huawei Li[1]

[1]Eye & ENT Hospital of Fudan University, Shanghai, China,[2]Shanghai Refreshgene Therapeutics, Shanghai, China,[3]Southeast University, Nanjing, China,[4]Harvard Medical School, Eaton-Peabody Laboratory, Massachusetts Eye and Ear, Boston, MA

Background Autosomal recessive deafness 9 (DFNB9), caused by mutations of the *OTOF* gene, is characterized by congenital or prelingual, severe-to-complete bilateral hearing loss. However, no drug treatment is available for hereditary deafness. AAV1-hOTOF, adeno-associated virus (AAV) serotype 1 carrying a human *OTOF* transgene, was designed to treat patients with DFNB9. Here, we report the safety and efficacy of AAV1-hOTOF gene therapy as the treatment of children with DFNB9 via unilateral or binaural injection. **Methods** In this single-arm trial, patients (1-18 years of age) with severe-to-complete hearing loss were eligible. AAV1-hOTOF was administered into one or two cochleas through the round window. The primary outcome was dose-limiting toxicity (DLT). Adverse events (AEs), immune response, auditory function and speech perception were evaluated. **Results** From December 2022 to September 2023, 9 patients with 95 dB of average auditory brainstem response (ABR) thresholds at 0.5-4 kHz were enrolled. Six patients received unilateral injections, of which patient #1 received a 9×10^{11} vg dose and patients #2-6 received a 1.5×10^{11} vg dose. Subsequently, patients #7-9 received a binaural injection at a dose of 1.5×10^{11} vg. Follow-up visits ranged from 6 to 26 weeks. No DLT was observed. 61 adverse events were observed, with 97% (59/61) being grade 1 or 2, and 3% (2/61) being grade 3. In patient #1, the average ABR threshold was recovered to 68 dB at 4 weeks, 53 dB at 13 weeks, and 45 dB at 26 weeks. In patients #2-5, the average ABR thresholds were >95, 48, 38, and 40 dB at 26 weeks, respectively, while the average ABR threshold was 60 dB in patient #6 at 13 weeks. In patients #7-8, the average ABR thresholds of the left (right) ear was reduced to 63 (63), 88 (83) at 13 weeks. In patients #9, the average ABR thresholds of left (right) ear was reduced to 60 (63) at 13 weeks. Speech perception was improved in 5 patients who had hearing recovery. The ability of sound source localization was improved in all patients who received binaural injection. **Conclusions** The AAV1-hOTOF gene therapy is safe and efficacious as a novel treatment for patients with DFNB9.

145. VGN-R08b Gene Therapy for Neuronopathic Gaucher Disease

Huiwen Zhang[1], Yueniu Zhu[1], Weiwei Mao[1], Xiaoqiang Wang[1], Dake He[1], Xia Zhan[1], Yi Yang[1], Mengni Yi[1], Xiaodong Zhu[1], Hua LV[2], Shan Ding[2], Xiaoping Zhao[2]

[1]Xinhua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China,[2]Shanghai Vitalgen BioPharma Co., Ltd., Shanghai, China

Objective: Gaucher disease is caused by biallelic mutations in the GBA1 gene, with severe mutations leading to neurological manifestations (nGD). The GBA1 encoded enzyme GCase is a key lysosomal enzyme required for normal metabolism of glycolipids. GCase deficiency causes the accumulation of glycolipid substrates, resulting in lysosomal dysfunction that leads to inflammation and other pathological changes. Acute nGD (GD2) is of the most severe type, which is characterized by hepatosplenomegaly and central nervous system involvement within first year of life. And the disease progresses rapidly leading to death by the age of 2 years. The most significant signs, including hyperextension of the neck, myoclonic seizures and swallowing impairment, followed by prolonged spontaneous apneas and chronic/subacute pulmonary disease, would be the main reasons of death. Here we present the preliminary results from an early dose escalation trial to evaluate tolerability and safety of VGN-R08b, AAV9-GBA1, delivered into the central nervous system via ICV injection. **Methods**: Six infants (4F, 2M; 3-26 months) were diagnosed as GD2, 4 on invasive ventilator and ERT, 1 with ERT, and 1 without specific treatment. They were implanted with Ommaya reservoir for drug delivery prior to the single dose injection. The first patient received 6.0×10^{10} Vg/g and other 5 patients received 2.0×10^{10}Vg/g accompanied with prophylactic glucocorticoid. The GCase activity and Lyso-GL1 levels in CSF were analyzed, and neurologic symptoms were assessed. **Results**: Results were analyzed for the 6 subjects who were followed not less than 4 weeks (4-26 weeks). The injections were well tolerated, without any AE related to the procedure or VGN-R08b. GCase activity in CSF of the subject dosed 6.0×10^{10}Vg/g increased to 4~8 folds of normal upper limits during follow-up (baseline 0.3 increased up to 68.3 μmol/d/L); GCase activity in subjects dosed 2.0×10^{10}Vg/g increased to 2~5 folds of normal upper limits (baseline 0.3~1.6 increased up to 17.6~43.7 μmol/d/L). The Lyso-GL1 levels in CSF decreased dramatically, and during a longer follow-up in the first two subjects, it maintained below 26.6pg/mL (Lyso-GL1 levels in CSF of GD patients range 26.6~2000 pg/mL). In further, their neurological symptoms, including neck hyperextension, peripheral hypertonia and myoclonic seizures, improved in severity or disappeared soon within a 6-month's follow-up. Additional improvements were also noticed. The upper level of body temperature on episodes of fever dropped by one degree in 3 patients who's on invasive ventilator. The frequency of fever decreased from 2.25 to 0.75 times per month in one subject who's on invasive ventilator. Of the 4 subjects whose protein quantifications in CSF were abnormally decreased at baseline, the levels in 3 subjects increased to the normal range 4 or 8 weeks after treatment, indicating an increased neuronal activity. The dosages of Ambroxol in the 4 subjects, as for controlling neurological symptoms at enrollment, has been discontinued 4 weeks to 6 months after VGN-R08b treatment. We are also grateful to observe that the brain MRIs keep stable in morphology when checked on the 12 weeks'follow-up in 4 of 5 patients. **Conclusions**: VGN-R08b gene

delivery via ICV has now been performed in total of 6 patients. To our knowledge, this is the first time in the world to report changes of GCase and its metabolite Lyso-GL1 levels in CSF after gene therapy in GD. The procedure is safe and the expression of GCase in CSF increased significantly. Furthermore, Lyso-GL1 levels in CSF decreased significantly during the follow-up period, accompanying continuous improvements of neurological symptoms in GD2 infants that had never been achieved before.

146. Interim Review of Safety, Tolerability, and Efficacy From a First-In-Human Phase 1/2a Clinical Study of ICM-203, an Intra-Articular, AAV Gene Therapy for Osteoarthritis

Alison E. Heald[1,2], Bogdan Solomon[3], Richard Page[4,5], Yoenhee Ahn[1], Young Na Yum[1], Jayhyuk Myung[1], Jamie E. Collins[6], Ali Guermazi[7], Dae-Won Kim[1,8]

[1]ICM Co., LTD, Seoul, Korea, Republic of,[2]University of Washington School of Medicine, Seattle, WA,[3]Royal Adelaide Hospital, Adelaide, SA, Australia,[4]Barwon Health, University Hospital Geelong, Geelong, VIC, Australia,[5]Deakin University, Geelong, VIC, Australia,[6]Brigham and Women's Hospital, Boston, MA,[7]Boston University School of Medicine, Boston, MA,[8]Yonsei University, Seoul, Korea, Republic of

Introduction: This first-in-human phase 1/2a study is being conducted to assess the safety, tolerability, and activity of ICM-203, a recombinant AAV vector designed to express a truncated form of human Nkx3.2, a transcription factor which plays an important role in both chondrocyte and synoviocyte activity. **Methods:** In this double-blind, placebo-controlled, dose escalation study (NCT04875754), subjects with Kellgren-Lawrence grade 2 or grade 3 knee osteoarthritis and minimum Joint Space Width (mJSW) > 1mm receive a single intra-articular injection of ICM-203 or placebo in a 3:1 ratio, with planned dose escalation of ICM-203 from $6x10^{12}$ vector genomes (vg) to $2x10^{13}$ vg and then $6x10^{13}$ vg. The primary safety endpoint is safety and tolerability through assessment of treatment-emergent adverse events. The primary efficacy endpoints are changes in knee pain as assessed on a numerical rating scale (NRS); changes in knee function as measured using the Knee Injury and Osteoarthritis Outcome Score (KOOS) activities of daily living (ADL) subscore, as well as structural knee changes, including change in mJSW on knee radiograph and changes in Magnetic Resonance Imaging (MRI) Osteoarthritis Knee Score (MOAKS). Here, 12-month blinded follow-up data from 8 subjects in the low-dose cohort treated with ICM-203 (n=6) or placebo (n=2) are reported. **Results:** No significant concerns about safety and tolerability arose, although transient increases in injected knee pain 6-12 days after study drug administration were observed in 4 of 8 subjects. Knee pain (NRS) decreased in 6 of 8 subjects and knee function (KOOS ADL) improved in 4 of 8 subjects between Day 1 and Week 52. Improvement in medial mJSW was observed at Week 52 in 3 of 5 subjects with predominantly medial space disease and medial mJSW > 1mm. Cartilage thickness was preserved or improved in 5 of 8 subjects and bone marrow lesions (BML) improved in 3 of 8 subjects at Week 52 (Fig. 1). Osteophytes were unchanged in 7 of 8 subjects and only worsened minimally in 1 of 8 subjects at Week 52. Synovitis (Hoffa + effusion) improved at Week 52 in 2 of 2 subjects with more inflammation (synovitis score >4) at baseline (Fig. 2). **Conclusions:** Intra-articular injections of ICM-203 $6x10^{12}$ vg or placebo were safe and well tolerated. Improvement in joint pain and/or function were observed in 6 of 8 subjects. Favorable structural changes were observed on MRI, such as preservation of cartilage thickness, decrease in BML, and improvement in synovitis in subjects with more inflammation at baseline. Changes in osteophytes were minimal, suggesting no adverse impact on bone remodeling. Investigation of higher doses of ICM-203 is in underway.

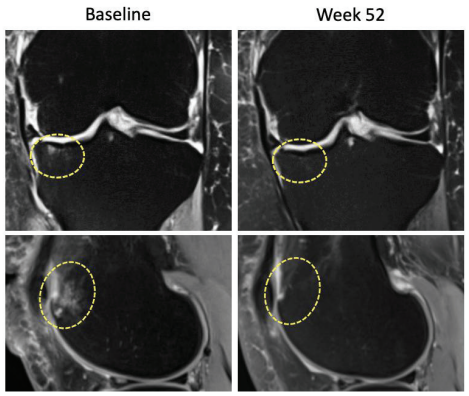


Fig. 1: Reduction in BML (yellow circles) in representative 2D IW MR images of subject R1007.

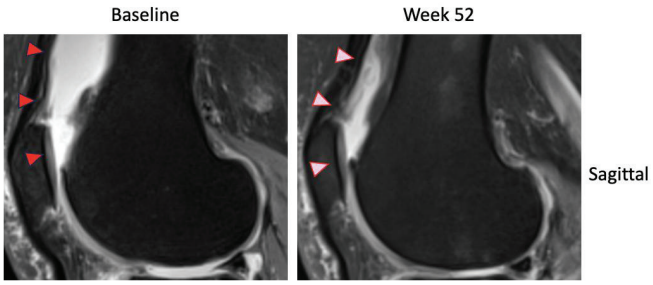


Fig. 2: Reduction in synovitis (red arrowheads) in representative 2D IW MR images of subject R1008.

147. Demonstration of Nuclease-Free Genome Editing (GeneRide™) in Pediatric Patients with Methylmalonic Acidemia

Marie Payton[1], Thomas Morgan[2], Jirair K. Bedoyan[3], Angela Sun[4], Hong Li[5], Daniel Gruskin[6], Mark A. Kay[6], Frederic Chereau[1], Jerry Vockley[3]

[1]Alexion AstraZeneca Rare Disease, Lexington, MA,[2]Vanderbilt Children's Hospital, Nashville, TN,[3]UPMC Children's Hospital of Pittsburgh, Pittsburgh, PA,[4]Seattle Children's Hospital, Seattle, WA,[5]Emory University, Atlanta, GA,[6]LogicBio Therapeutics, Lexington, MA

Methylmalonic acidemia (MMA) is an autosomal recessive inborn error of metabolism where certain amino acids and fats are not properly processed due to deficient activity of the mitochondrial enzyme methylmalonyl CoA mutase (MMUT). Therapeutic options include strict dietary management or liver transplantation. A phase 1/2 dose escalation trial (NCT04581785) evaluated the safety and efficacy of a single hLB-001 intravenous infusion in pediatric MMA patients. Liver-targeted hLB-001 utilizes the AAV-LK03 capsid to deliver a functional copy of the human *MMUT* coding sequence for homologous-recombination induced, non-disruptive integration

into the 3' end of the albumin (*ALB*) locus of hepatocytes. Using the GeneRide gene editing technology, LB-001 introduces a P2A ribosome skipping sequence peptide allowing edited alleles to produce ALB and MMUT as two separate proteins, and in doing so tags the C-terminus of ALB with the P2A peptide, which becomes a technology-dependent circulating biomarker for target-site integration events, albumin-2A (ALB2A). The hLB-001-edited hepatocytes express functional MMUT protein. Over time, the MMUT expressing hepatocytes selectively survive and progressively repopulate the MMA-diseased liver. This is observable by detecting increasing amounts of circulating ALB2A, a surrogate for the edited hepatocytes expressing mitochondrial MMUT protein. The clinical trial enrolled patients between 6 months and 12 years of age. Four patients enrolled in the study and received hLB-001 at a dose of $5x10^{13}$ vg/kg along with prophylactic corticosteroids. All patients entered a long-term follow-up study (NCT05506254); two years of post-treatment data are presented here. Two patients in the younger age group experienced thrombotic microangiopathy, with one case being life-threatening. All patients experienced transient increases in transaminases with hLB-001 administration. Although there is a theoretical risk of steroids leading to a catabolic episode in these patients, only one mild case of hyperglycemia was experienced. All patients recovered from these events. All patients showed evidence of targeted integration via measurable serum ALB2A. Persistently increasing levels of ALB2A throughout the study were observed in 2 patients, suggesting continued expansion of the edited hepatocytes. However, at the 2-year timepoint, serum ALB2A had not yet reached levels that would be predicted to reflect the expected necessary number of MMUT-producing cells required to provide a therapeutic benefit. Of the 2 patients without sustained increases in ALB2A, one patient's loss of expression may have been associated with an increase in liver enzymes that occurred immediately prior to the elimination of ALB2A levels. While the reason for the lack of sustained increase in ALB2A in the other patient is unclear, it is possible that this patient's disease was sufficiently mild and controlled to reduce the selective pressure for edited hepatocytes. This study was the first to demonstrate successful *in vivo* homologous recombination genome editing without the use of nucleases or promotors in a pediatric population. Future efforts will focus on methods to increase the integration rate of a corrective gene to accelerate time to efficacy with increased safety.

148. Phase 1/2 Trial of Combined Intrathalamic/Intracisternal/Intrathecal Gene Therapy for Tay-Sachs and Sandhoff Diseases

Terence R. Flotte[1], Allison M. Keeler-Klunk[2], Meghan Blackwood[2], Rebecca Artinian[2], Heather L. Gray-Edwards[2], Toloo Taghian[2], Oguz Cataltepe[3], Miguel Sena-Esteves[2], Florian Eichler[4]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]UMass Chan Medical School, Worcester, MA,[3]University of Massachusetts Medical School, Worcester, MA,[4]Neurology, Massachusetts General Hospital, Boston, MA

The GM2 gangliosidoses, Tay-Sachs and Sandhoff diseases, are caused by deficiency of lysosomal hexosaminidase A activity due to mutations in either the alpha subunit (encoded by HEXA) in Tay-Sachs or the beta subunit (encoded by HEXB) in Sandhoff. Severe deficiency states ($\leq$ 0.1% activity) result in infantile forms of these diseases in which infants begin to show normal neurodevelopmental progress, but then begin to relentlessly regress around 5 to 6 months of age leading to global neurological deficits, seizures and ultimately death around 3 to 4 years old. Our group has developed rAAVrh8 vectors encoding HEXA and HEXB, which when injected simultaneously into the thalami bilaterally and into the CSF of deficient animals (Sandhoff mice and cats and Tay-Sachs sheep) can restore CNS Hexosaminidase enzyme activity, decrease GM2 levels in CSF, and rescue most or all of the phenotypic consequences of the disease. Following upon a small (N of 2) expanded access (EA) trial, we initiated a phase 1/2, single-dose, dose-escalation of combined biThalamic (BiT), intra-cisterna magna (CM), and intrathecal (IT) infusion of rAAVrh8-HexA/HexB at four dosage levels ranging from 5.87E+12 vg/kg per patient to 4.08E+13vg/kg. Most importantly, the BiT injection volume (and vector dose) were doubled between each cohort (starting dose (SD) 180mcl, low dose (LD) 360mcl, mid dose (MD) 720 mcl, and high dose (HD) 1250 mcl) with the SD matching the dose used in the prior EA trial. Patients were immune suppressed with rituximab, sirolimus, and corticosteroids (10mg/kg solumedrol). Post-dose, patients continued with sirolimus for 6 months followed by a taper and prednisolone (1 to 2 mg/kg/day) for 3 months followed by a taper. All 9 subjects have been enrolled in this trial. Interim results of CSF HexA enzyme activity and GM2 levels show a dose-related, partial biochemical correction of the disease. Positive clinical findings in treated infantile subjects indicate prolonged oral feeding without aspiration until age 3 to 3.5 years. The data on dose-response effects on the CSF biomarkers and clinical outcomes will support later development. Multiple aspects of the immune response were evaluated in these subjects, including cytokine responses, anti-capsid antibodies, anti-capsid and anti-transgene ELISPOT responses, and development of vector-specific Tregs. The timing of these responses and their treatment will be discussed in the context of broader application to other rAAV clinical trials.

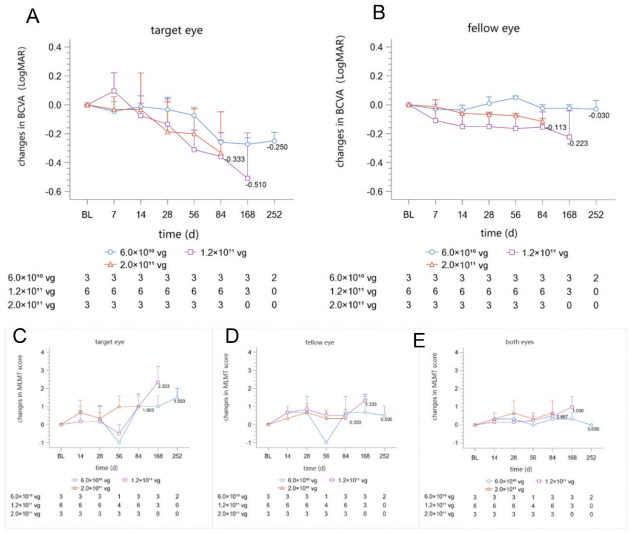
149. Subretinal Gene Therapy in Patients with Bietti Crystalline Dystrophy: Preliminary Results of a Phase 1/2 Clinical Trial

Shiyi Yin[1], Haihan Zhang[1], Jieqiong Chen[2], Jinyuan Wang[1,3], Yang Li[1], Qiong Yang[1], Ying Sha[1], Hua Lv[4], Xiaoping Zhao[4], Ning Guan[4], Ruixue Chen[4], Hongxia Mao[4], Xiaodong Sun[2], Wenbin Wei[1]

[1]Beijing Tongren Hospital, Capital Medical University, Beijing, China,[2]Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China,[3]School of Clinical Medicine, Tsinghua University, Beijing, China,[4]Shanghai Vitalgen BioPharma Corporation Limited, Shanghai, China

Introduction: Bietti crystalline dystrophy (BCD) caused by CYP4V2 biallelic mutation is a severe and visually threatening genetic disorder with no effective treatment. This study aimed to show the preliminary results of BCD patients received a single unilateral subretinal injection of VGR-R01, an AAV8 mediated CYP4V2 gene therapy. **Methods:** An open-label, phase1/2, dose-escalation clinical trial (NCT05694598) evaluated the safety and efficacy of a single VGR-R01 subretinal injection in 12 patients with BCD. Three patients each in Cohort 1 and 3 received VGR-R01 6.0×10^{10} and 2.0×10^{11} vg/ege, and six patients in Cohort 2 received 1.2×10^{11} vg/eye. All patients received prophylactic glucocorticoid to reduce the risk of vector-related immune responses.

The data cutoff for this analysis was Dec. 20, 2023. **Results:** 12 patients (mean age,42 years; range, 32~52 years; 7 [58%] male) were included in the study. The median observation period was 128 days (range, 78 ~ 247 days). Compared with the baseline (range, hand move to 0.61 LogMAR) , 58.3% (7/12) of patients had a significant improvement in visual acuity of the injected eyes (decrease of ≥ 0.3 LogMAR in BCVA). 3 patients in the Cohort 1 had a mean change from baseline in BCVA in the target eyes of −0.270 logMAR at D168 visit. 6 patients in the Corhort 2 had a mean change in BCVA of −0.345 logMAR at D84 visit, 3 of whom completed D168 visit with a mean change from the baseline of −0.510 logMAR. Cohort 3 (at D84 visit) had a mean change of −0.333 logMAR (Figure A, B). All patients had an average increase in Multi-luminance mobility testing (MLMT) of 1-2 points (-1 to +4), with 33% (4/12) of patients having an improvement two weeks after injection of VGR-R01(Figure C~E). There was no significant anatomical change or mean foveal thickness in the both eyes. All patients experienced a treatment-emergent adverse event (TEAE) but just one eye inflammation was related to VGR-R01. Most common AEs were related to administration procedure. **Conclusions:** VGR-R01 is safe and well tolerated, and efficacy is observed. More importantly, for some subjects with extremely low vision, the VGR-R01 administration also significantly improved their visual function.



Gene Disruption and Excision

150. Identification and Engineering of ABR-004, a Compact, High-Fidelity Nuclease for Therapeutic Gene Editing

Jeffrey R. Haswell, Michael S. Kuefner, Tanya Jain, Anthony J. Garrity, Linh B. Truong, Sneha Halwasia, Kyle Rehberger, Lauren E. Alfonse, Alexa Ornstein, Erin Doherty, Jenna M. Rachman, Gabrielle Z. Munoz, Gillian A. Mehigan, Tomoaki Sasaki, Zachary Maben, Blake D. McMahon, Carl Co, Gregory R. Hoffman, John E. Murphy, Tia DiTommaso

Arbor Biotechnologies, Cambridge, MA

Achieving widespread therapeutic gene editing will require a broad toolbox of editing enzymes. Here we describe an end-to-end pipeline for optimization of CRISPR gene editing nucleases towards greater efficiency and specificity in therapeutic applications, demonstrated through the development of ABR-004, a highly active <750 amino acid nuclease guided by a small (<60) nucleotide guide RNA. Through a metagenomic search, we initially identified a family of miniature CRISPR-Cas type V-L systems capable of RNA-guided double-stranded DNA target cleavage without requiring a tracrRNA. Focusing on one ortholog, we solved the cryo-EM structure in complex with its guide RNA and target DNA, and then combined structure-guided design and unbiased mutational scanning to rationally engineer a variant, ABR-004, for increased activity, achieving >70% indels at a number of targets within the *PCSK9* gene in primary human hepatocytes when delivered as an RNP. We further optimized additional parameters, using information from the cryo-EM structure, including guide length and chemical modifications, to increase editing specificity. To demonstrate potency in an *in vivo* model, ABR-004 mRNA and guide were formulated in an LNP and delivered at a low dose (0.5 mg/kg) to transgenic mice expressing a human *PCSK9* transgene, resulting in a >80% reduction in PCSK9 serum protein levels two weeks after treatment. Next, ABR-004 LNPs targeting *PCSK9* were delivered to cynomolgus macaques, resulting in a 65% reduction of serum PCSK9 and a 66% reduction in serum LDL-C after 4 weeks. This work demonstrates the feasibility of developing new gene editing tools, starting from computational identification, through molecular and structural characterization, engineering, and ending with *in vivo* application in non-human primates. Given its small size (<2.3kb combined size of guide + nuclease), specificity, and potency, ABR-004 is an attractive option for therapeutic applications, particularly those relying on AAV delivery.

151. Toward an *In Vivo* Approach to Knock-Out the HIV Co-Receptor CCR5 in Hematopoietic Stem Cells Using HDAd Vectors Expressing Base Editors

Anna K. Anderson[1], Aphrodite Georgakopoulou[1], Karthik Karuppusamy[1], Anne-Sophie Kuhlmann[2], Andrew Riker[3], Hans-Peter Kiem[2], Andre Lieber[1], Chang Li[1]

[1]University of Washington, Seattle, WA,[2]Fred Hutch, Seattle, WA,[3]Fred Hutchinson, Seattle, WA

We have developed an *in vivo* HSC transduction strategy that allows for efficient and stable gene transfer into mobilized HSCs. Our approach utilizes helper-dependent adenovirus (HDAd) vectors that target therapeutic gene delivery to HCSs. Here, we employ this approach using base editors to knock-out the HIV co-receptor CCR5 in HSCs and their progeny. Base editors can introduce precise cytidine (CBE) or adenine (ABE) substitutions with minimal occurrence of undesired DNA changes at the genomic target site. We screened a panel of single guide RNAs paired with base editors in TZM-bl cells to achieve CCR5 knockout. TZM-bl cells are susceptible to HIV infection (through CD4 and CCR5) and HIV infection can be measured as a function of Tat-induced luciferase reporter gene expression. In these screening studies, we found that a sgRNA targeting the CCR5 start codon (referred to as SILENCE2) combined with an ABE (ABE8e) and a sgRNA that creates a premature stop codon (referred to as STOP1) combined with a CBE (BE4max) or a laboratory-evolved CBE (TadCBE) exhibited the highest on-target editing rates as well as CCR5 down-regulation. Notably, editing rates were significantly higher with the TadCBE than with the BE4max. We then produced all-in-one base editor HDAd vectors containing the sgSTOP1 and sgSILENCE2 alone or in combination with a Dual editor (TadDE). In addition to the editing machinery, the vectors contained an expression cassette for $mgmt^{P140K}$, which allows for enrichment of edited cells by selection with low doses of O^6BG/BCNU. Downregulation of CCR5 (measured by flow cytometry) was highest for vectors containing the STOP1 guide (HDAd-STOP1-TadCBE and HDAd-STOP1-SILENCE2-TadDE) indicating that editing using this guide blocks CCR5 expression more efficiently. HDAd-transduced/edited TZM-bl cells were challenged with the HIV-BaL strain. CCR5 base editing by the HDAd-SILENCE2-ABE8e vector conferred 64% inhibition and both HDAd-STOP1-TadCBE, and HDAd-STOP1-SILENCE2-TadDE vectors conferred 98% inhibition of HIV-BaL infection/replication based on luciferase expression levels. Next, we transduced human $CD34^+$ cells from three healthy donors with HDAd-STOP1-SILENCE2-TadDE or HDAd-STOP1-TadCBE followed by treatment with O^6BG/35 μM BCNU. On average, target site editing rates were 25% for HDAd-STOP1-TadCBE and 13% for HDAd-STOP1-SILENCE2-TadDE. To allow for infection studies with HIV, we subjected transduced/edited $CD34^+$ cells (cord blood derived) to *in vitro* differentiation over 42 days resulting in $CD4^+CCR5^+$ T-cells. At the end of differentiation, cells were exposed to HIV. Three days after HIV infection, HIV genome titers in supernatants of T-cells derived from HDAd-STOP1-TadCBE and HDAd-STOP1-SILENCE2-TadDE-transduced $CD34^+$ cells were 21-fold and 4-fold lower, respectively, compared to T-cells derived from untransduced $CD34^+$ cells (which had titers of $>2x10^9$ copies/ml, based on qRT-PCR). Based on HIV-Gag flow cytometry of T-cells, inhibition of HIV replication was 84% and 70%, respectively. For *in vivo* studies, we are generating humanized mice with human $CD34^+$ cells that can differentiate into $CD4^+/CCR5^+$ T-cells. We plan to mobilize these mice, transduce human HSCs with HDAd-sgR5-1 *in vivo* and challenge the animals with HIV. We are also preparing *in vivo* HSC transduction studies in non-human primates by selecting appropriate sgRNAs for CCR5 knock-out in rhesus cell lines. In addition, we are generating an HDAd vector with two functional modules, e.g. the HIV decoy receptor eCD4Ig and the CCR5-knockout base editor to minimize the development of HIV escape mutants.

152. Targeting the Hepatitis B Virus cccDNA via the LNP-Delivered hfCas12Max Nuclease to Eliminate Hepatitis B Virus *In Vitro* and *In Vivo*

Yinghui Wei[1], Zikang Wang[1], Weiya Bai[1], Haosha Tang[1], Tong Li[1], Shaoran Wang[1], Xuan Yao[1], Alvin Luk[1], Yingsi Zhou[1], Hui Yang[1,2], Linyu Shi[1]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: The hepatotropic, non-cytopathic DNA virus, Hepatitis B virus (HBV) generates a covalently-closed-circular-DNA (cccDNA) intermediate in the nucleus of infected cells, as well as integrated sequences that act as templates for viral transcription and protein production. Although current antiviral therapies with nucleoside analogues inhibit replication of HBV DNA in capsids present in the cytoplasm of infected cells, they fail to reduce or eliminate nuclear cccDNA as well as cure chronic hepatitis B. Here, we present a potential curative strategy employing high-fidelity Cas12Max (hfCas12Max), an engineered Cas12i variant with high activity and specificity, delivered by lipid nanoparticle (LNP) which targets the HBV genome. **Methods**: Effective guide RNAs (gRNAs) were initially selected and screened within the PiggyBac(PB)-HBV-HEK293 system. Furthermore, a functional gRNAs screen was performed in HBV-integrated cell lines and primary human hepatocytes (PHHs) infected with HBV. To evaluate the *in vivo* efficacy of the hfCas12Max nuclease, we developed an episomal adeno-associated virus (AAV) mouse model harboring a 1.3-fold HBV genome, which serves as a surrogate for cccDNA. Clinically relevant delivery was achieved through the systemic administration of LNPs encapsulating hfCas12Max mRNA and gRNAs. **Results**: Leading gRNAs targeting the hepatitis B surface antigen (HBsAg) and associated genes were identified within HBV-integrated hepatic cell lines. Upon delivery via the LNPs, the hfCas12Max targeting led to a substantial decrease in cccDNA, HBV-DNA, and HBsAg levels in HBV-infected PHHs (Fig. 1A-E). In the AAV-HBV mouse model, we observed sustained reductions in HBsAg expression (up to 2.5 log) and serum HBV DNA (~3log) following systemic delivery of varying doses of the CRISPR-hfCas12Max system mediated by LNP (Fig. 2A-D). **Conclusions**: Our findings suggest that the CRISPR- hfCas12Max system could disrupt the HBV-expressing templates both *in vitro* and *in vivo*, which underscores its potential for the eradication of cccDNA, paving the way toward a potential cure for HBV.

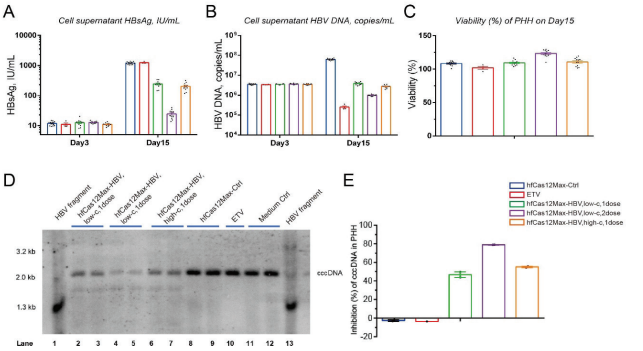


Figure 1. LNP-mediated delivery of hfCas12Max mRNA and gRNAs reduces HBV viral parameters in primary human hepatocytes (PHH). (A, B) HBV replication was assessed by HBsAg and HBV DNA in PHH supernatant. hfCas12Max targeting leads to efficient reduction of HBsAg (A) and HBV DNA (B). (C) The viability of PHH cells in each group was unaffected on day 15. (D) Hirt DNA samples were isolated from each sample and then subjected to Southern blot (n=2). Lanes 1 and 13 were loaded with HBV DNA fragments of 25 pg for each fragment (3.2kb, 2.0kb) and 12.5 pg for the 1.3kb fragment. Lanes 2 to 12 were loaded with 20 µL of Hirt DNA samples for each lane. (E) hfCas12Max nuclease treatment results in a significant reduction in cccDNA at day 15 post-transduction in PHH.

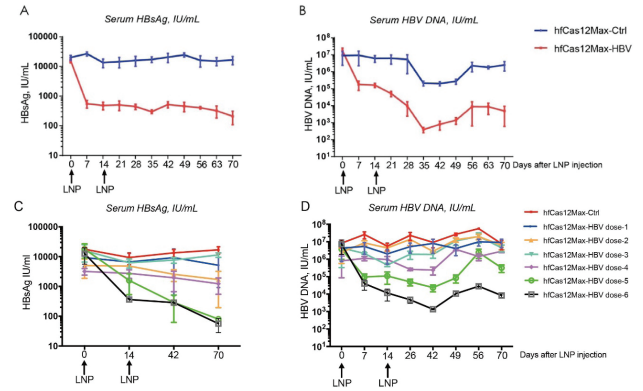


Figure 2. LNP-mediated delivery of hfCas12Max mRNA and gRNAs leads to sustained reduction of viral markers in AAV-HBV mouse model. (A, B) The AAV-HBV mouse model was used in the in-vivo pilot study. 4 weeks after hydrodynamic injection with AAV8-1.3-fold HBV genome, mice received two doses (2x) of the hfCas12Max-targeting reagents (mRNA & gRNA formulated into LNP). hfCas12Max nuclease treatment resulted in sustained reductions in HBsAg expression (~2 log) (A) and HBV DNA (~3 log) (B) in serum. (C, D) The antiviral activity was explored in the AAV-HBV mouse model by assessing serum HBsAg (C) and HBV DNA levels (D) following the administration of escalating doses of LNP, ranging from dose-1 to dose-6.

153. Non-Viral Gene Editing with Dual Guide RNAs for Chronic Hepatitis B Infection

Samuel Slattery, Sujeewa Sampath Lellupitiyage Don, TJ Cradick, Jennifer Gordon, Ryo Takeuchi

Excision BioTherapeutics, Watertown, MA

Hepatitis B virus (HBV) infection is one of the most prevalent infectious diseases worldwide. In hepatocytes with chronic HBV infection, a subset of HBV DNA is present as covalently closed circular DNA (cccDNA) and serves as the template for a series of viral transcripts. Currently approved treatment with antivirals and immunomodulators reduces the viral load to slow down the progress of liver injury but fails to eliminate the cccDNA that enables persistent viral infection. Gene editing holds the great potential to target all forms of viral DNA, including cccDNA, and may offer a new option for functional cure or even better clinical outcomes. We have developed multiple candidate therapeutics with paired guide RNAs, because we hypothesize that DNA strand breaks generated at multiple sites can lead to large deletions (excisions) that eliminate a large region of the viral genome, effectively inactivating and preventing the emergence of escape variants. Here, we evaluated a dual guide RNA-mediated editing strategy against HBV in cell-based assays and in vivo. Building on a proprietary computational approach, we identified multiple nucleases and guide pairs to target well-conserved sites within the HBV genome. We then cross referenced these selected guide target sites against human reference genomes to select sites that shared the least sequence identity with human genetic sequences, thereby minimizing the chance for off-target editing. Using reporter cell lines with the partial HBV genome integrated in the chromosomal DNA, we identified pairs of guide RNAs that efficiently deleted sequences between target sites found in a wide range of HBV isolates. We next examined a representative combination of editing nucleases and paired guide RNAs in HBV-infected cells and verified that it decreased copies of total HBV DNA. To demonstrate proof-of-concept in vivo, we intravenously administered a lipid nanoparticle (LNP) that encapsulated an editing nuclease-encoding mRNA and a pair of guide RNAs in the HBV mouse model established with AAV-HBV. A single LNP administration significantly lowered HBV biomarkers in serum. Consistent with that observation, we detected reduced levels of intrahepatic HBV, suggesting that cutting of HBV DNA facilitated its degradation in vivo. These results laid the foundation for advancing a potential curative CRISPR-Cas HBV treatment into IND enabling studies and human clinical studies.

154. A Comparison Study Between Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Guide RNAs and Discovery of LgRNA-33 as an Anti-HBV Agent of Multiple Mechanisms of Action (MOA)

Qiong Zhao[1], Xiaoyun Lin[2], Ju-tao Guo[1], Minghong Zhong[2]

[1]Baruch S Blumberg Institute, Doylestown, PA,[2]GeneLancet Biosciences, Inc., Doylestown, PA

Cure of chronic hepatitis B requires elimination or inactivation of HBV cccDNA and integrated DNA. Several CRISPR/Cas DNA editing-based therapeutics are currently under preclinical development to achieve this therapeutic goal. To improve the gene editing efficiency and accuracy, we optimize guide RNAs through chemical ligation of short RNA segments via non-phosphoramidite chemistry, i.e., ligated guide RNA (LgRNA) and chemical modifications. Compared to the linear chemical synthesis of a classical single guide RNA (sgRNA), our proprietary LgRNA technology provides high-quality products (>99% purity by HPLC) containing a single spacer sequence and enables cost-effective scaleup production (potentially multiple kilograms), diverse chemical modifications, targeted delivery by conjugations and various formulation technologies. We have shown that LgRNAs more efficiently cleave/edit target integrated HBV DNA and HBV cccDNA at multiple sites in hepatocytes (Fig. 1 (A)). To our surprise, in addition to the on-target editing of 50 to 80% of integrated HBV DNA in HepAD38 cell culture in the presence of tetracycline (tet) to suppress HBV pgRNA transcription as determined by next-generation sequencing (NGS), the LgRNAs targeting different regions of HBV genome (preC, core, HBsAg or HBx region) significantly reduced the levels of subgenomic HBV RNAs and secreted HBsAg, and upon removal of tet from culture media of the LgRNA transfected HepAD38 cells, significant reduction of HBV pgRNA was observed in cells transfected with all LgRNAs. Moreover, a single transfection of LgRNA into HBV-infected HepG2-NTCP cells expressing Cas9 achieved on-target editing of 10 to 40% of cccDNA as determined by NGS. Similar to that observed in HepAD38 cells, all tested LgRNAs targeting different regions of HBV genome

significantly reduced subgenomic HBV RNA and secreted HBsAg. In addition, LgRNAs targeting HBx, PreC, or core regions reduced both secreted HBeAg and the amount of cccDNA in the infected cells. Exceptionally, LgRNA-33 showed anti-HBV activity by multiple mechanisms of action (MOA). By a single treatment (10 pmol) of HBV-infected HepG2-NTCP cells-expressing Cas9, LgRNA-33 reduced HBV cccDNA level by >60%, while inactivated the remaining cccDNA by 30% (as indicated by NGS data), decreased sub-genomic RNAs by >70% and pgRNA by ~65%, and removed >90% of HBsAg and ~80% of HBeAg viral proteins in the infected cells. LgRNA-33 also showed dose-dependent activity in HepAD38 cells containing a full copy of integrated HBV DNA and nearly cleared both HBsAg (Fig. 1 (B)) and subgenomic RNA at a ten pmol dose (Fig. 1 (C)).

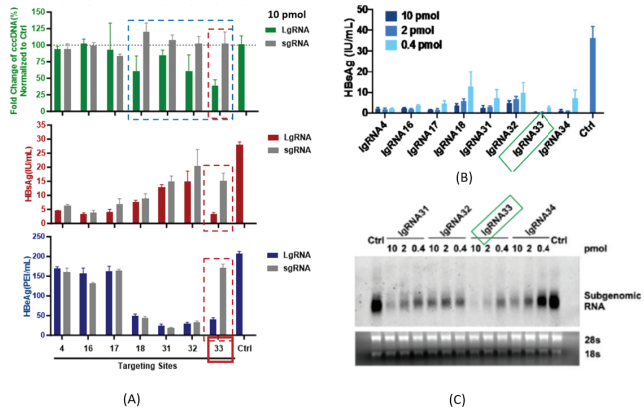


Figure 1. Anti-HBV activities of sgRNAs and LgRNAs in hepatocytes.

(A) Comparison study between sgRNAs and LgRNAs in HBV-infected hepatocytes. LgRNA-33 showed dramatically better efficiencies than sgRNA-33.
(B) Dose-dependent decreases of cellular HBsAg levels by LgRNAs in HepAD38 cells containing a full copy of integrated HBV DNA.
(C) Dose-dependent decreases of viral subgenomic RNA by LgRNAs in HepAD38 cells containing a full copy of integrated HBV DNA.

155. Engineering CasX to Create a Gene Editor with Potent Activity in Non-Human Primates

Addison Wright, Maroof M. Adil, Shyam Sundhar Bale, Katherine Baney, Isabel Colin, Fred Deiter, Benjamin Demaree, Sarah K. Denny, Michelle Eggers, Sean Higgins, Santhosh Karanth, Steven Keller, Kirsten A. Reimer, Han Spinner, Brett T. Staahl, Keith E. Szulwach, Kian Taylor, Ross White, Benjamin L. Oakes

Scribe Therapeutics Inc, Alameda, CA

The application of RNA-guided endonucleases from CRISPR-Cas systems has revolutionized the gene editing field. The need for Cas enzymes with reduced size, increased specificity, or distinct editing profiles or PAM preferences has driven the identification and characterization of diverse novel Cas effectors. However, many of these naturally occurring effectors have poor translatability into mammalian systems. Here, we present our work engineering the CasX (Cas12e) holoenzyme for improved activity and demonstrate its efficacy in human cells and animal models, including non-human primates. CasX is a Class 2 Cas effector enzyme that binds and cuts dsDNA substrates when loaded with a matching guide RNA. Unlike Cas9 and Cas12a variants, however, CasX is small enough (<1000 amino acids) to fit efficiently in an AAV vector with multiple guides or complex regulatory elements, generates distinct deletion patterns, and has less tolerance for mismatches between the guide RNA and dsDNA substrate. By building and screening hundreds of thousands of distinct CasX nucleases and guide RNAs, we have created novel and dramatically improved variants. The resulting "X-Editor" (XE) ribonucleoprotein (RNP) exhibits editing activity orders of magnitude higher than wild-type CasX molecules. We characterized XE editing efficiency compared to other engineered small Cas systems across dozens of loci in human cells, and we further show that XE can achieve knock-out rates of immuno-oncology targets equal to or better than SpyCas9 when delivered as RNP to primary human immune cells. We also show that XE achieves near-saturating levels of gene editing across the CNS, muscle, eye, and liver in mice when delivered via AAVs or LNPs. Finally, we show that XE is well-tolerated and saturates editing in the liver of non-human primates when delivered via LNPs. We demonstrate that XE is a potent and flexible platform for gene editing, with robust activity and specificity across a range of delivery modalities and targets.

New Avenues for Viral Vectors

156. HDAd6/3+-cxcr4 Vectors: A Safe and Efficient Vector Platform for *In Vivo* HSC Transduction in Rhesus Macaques

Chang Li[1], Hongjie Wang[1], Anna K. Anderson[1], Lishan Huang[1], Aphrodite Georgakopoulou[1], Theo Koob[1], Sucheol Gil[1], Donna J. Palmer[2], Philip Ng[2], Hans-Peter Kiem[3], Andre Lieber[1]

[1]University of Washington, Seattle, WA,[2]Baylor College of Medicine, Houston, TX,[3]Fred Hutch, Seattle, WA

We generated helper-dependent adenovirus vectors (HDAds) targeting receptors present at high levels on hematopoietic stem cells (HSCs), namely CD46 and desmoglein 2 (DSG2). We used these vectors for an *in vivo* HSC gene therapy approach that we recently developed. This approach involves subcutaneous injections of G-CSF/plerixafor (AMD3100) to mobilize HSCs from the bone marrow into the peripheral blood stream, followed by intravenous injection of HDAd vectors. A large fraction of transduced HSCs returned to the bone marrow and can be expanded using $mgmt^{P140K}$ expression and O^6BG/BCNU treatment. Here we compared two HDAd vector platforms: *i)* HDAd5/35++, an Ad5 derived vector with affinity-enhanced fibers from Ad35 that targets CD46 and has been used in most of our previous studies; *ii)* HDAd6/3+-cxcr4, our latest HDAd vector platform derived from the relative rare serotype 6 and containing fibers from Ad serotype 3 with enhanced affinity to DSG2. The vector also transiently expresses cxcr4, which facilitates the return of mobilized HSCs to the bone marrow. In *in vitro* studies with human and rhesus $CD34^+$ cells, GFP-expressing HDAd6/3+-cxcr4 transduced $CD34^+$/$CD45RA^-$/$CD90^+$, a subfraction that is enriched for primitive HSCs, at least twice as efficiently as HDAd5/35++. For *in vivo* studies, rhesus macaques were mobilized with G-CSF/AMD3100 and intravenously injected with HDAd6/3+-cxcr4 vectors at a dose of 3.2×10^{12} vp/kg after cytokine prophylaxis with IL-6 and IL-1 blockers (N=6). At the peak of mobilization, the time point when HDAd vectors were injected, 40

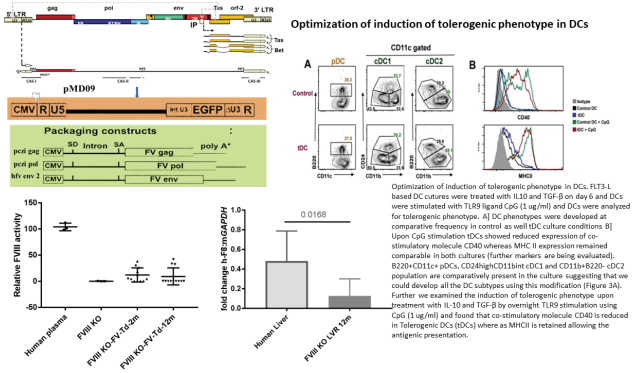
- 150 x 10^3 $CD34^+/CD45RA^-/CD90^+$ cells per ml (~100-fold increase compared to non-mobilized animals) were detected in peripheral blood, indicating efficient mobilization. HDAd6/3+-cxcr4 vector clearance in peripheral blood was slower than that of HDAd5/35++, most likely because HDAd6/3+-cxcr4 was less sequestered by blood cells, specifically erythrocytes. While HDAd5/35++ injected animals showed low serum levels of IL-6 and TNFα at 6 hours after HDAd injection, no cytokines were detectable by cytometric bead array after HDAd6/3+-cxcr4 injection, again pointing toward less interaction of HDAd6/3+-cxcr4 particles with granulocytes and tissue macrophages. Furthermore, the HDAd6/3+-cxcr4 vector design, specifically the use of the Ad6 fiber shaft, minimized liver transduction as reflected by normal levels of liver enzymes. These data indicate that HDAd6/3+-cxcr4 vectors are safe. The efficacy of *in vivo* HSC transduction was measured based on vector copy number (VCN) in bone marrow aspirates. The VCN in HDAd6/3+-cxcr4-injected animals increased during the first weeks, most likely due to cxcr4-mediated translocation from the spleen to the bone marrow, reaching ~0.3 copies per $CD34^+$ cell; a transduction level that was 20-fold higher than that in HDAd5/35++ injected animals. In NHP studies with HDAd6/3+-cxcr4 vectors containing an integrating transgene expression cassette, stable VCN levels in bone marrow $CD34^+$ cells and PBMCs were demonstrated after two rounds of O^6BG/BCNU *in vivo* selection at 16 weeks after *in vivo* transduction (study is ongoing). The bone marrow and PBMC lineage composition (CD3, CD20, CD14) returned two pre-mobilization levels by week 6 after *in vivo* transduction. In summary, the DSG2-targeting HDAd6/3+-cxcr4 vector platform allows for safe and efficient HSC *in vivo* transduction in rhesus macaques. Our current efforts are focused on developing a strategy for HSC *in vivo* expansion that does not require chemotherapy drugs, including epitope editing in combination with non-genotoxic antibodies/antibody-drug conjugates or the expression of truncated versions of the erythropoietin receptor of HMGA2.

157. Foamy Viral Gene Therapy Using a Foamy Viral Vector and Strategies to Induce Transgene Tolerance

Sanal MG[1], Sarita Gupta[1], Rahul Saha[1], Prafullakumar B. Tailor[2], Nagarajan Perumal[2]

[1]Cellular and Molecular Medicine, Institute of Liver and Biliary Sciences, New Delhi, India,[2]National Institute of Immunology, New Delhi, India

Background: Haemophilias are genetic diseases where the patient tends to bleed excessively as a result of a defect in blood clotting. Currently, there is no cure but the condition is managed by the supplementation of the deficient factor such as recombinant factor 8/ 9. Here we attempted to cure Hemophilia A using a gene therapy approach [with a foamy viral vector (FVV) to transduce factor 8] in liver cells in a haemophilia-A (factor 8 Knock Out) mouse model. FVV can deliver larger cargos such as the factor 8 gene. Foamy virus as it is not endemic and is non-pathogenic in humans. Host immunity against transgene is a problem in gene therapy. Here we also explored tolerogenic DCs induced by treatment with immunosuppressive cytokines such as IL-10 in mitigating host immunity. Methods: We cloned the Factor 8 gene in a modified FVV pMD09 under a liver-specific promoter (TTR). The virus was generated in HEK293 cells. We injected retro-orbitally 2-6 × 10^6 infectious units of FVV in 12 mice and 6 animals (untreated) served as controls. At 2-12 months we estimated factor 8 activity in citrated plasma using the Electrachrome™ FVIII kit (Cat. #49730503 HemosIL® Wefen, MA, USA) following the manufacturer's instruction. We also isolated total RNA and gDNA from mouse liver. RNA was used to prepare cDNA to estimate human factor 8 transcripts by Real-Time PCR (normalized to mouse GAPDH). The gDNA was used to estimate integrated viral genetic elements by PCR. Results: We observed expression of factor 8 in 7 animals after two months and 3 animals at 1 year. The expression levels ranged from 2% of the positive controls to 38% at two months. All three animals that retained the expression had factor 8 levels of 30% or above compared to the control (human plasma). All animals that were positive for plasma factor 8, also had detectable factor 8 transcripts in liver tissue although the transcript levels did not correlate well with factor 8 plasma levels estimated (functional assay). We found FVV integrated into the genome of all 3 mice which expressed factor 8 at 1 year. Conclusion: FVV can transduce mice liver and express factor 8 up to 1 year. We are yet to see if tolerogenic DCs can improve the levels and duration of factor 8 expression in mice.



158. A Novel Method for Generating Unprecedented Unbiased, Ultra-High-Sequence Diversity Libraries of Plasmids and Recombinant Viruses (With Validation) for High-Throughput Screening, Data for Machine Learning (AI) and Targeted Therapy

Praveensingh B. Hajeri[1], Masato Yamamoto[2]

[1]Department of Surgery, University of Minnesota, Minneapolis, MN,[2]University of Minnesota, Minneapolis, MN

Background: A typical drug discovery pipeline needs good libraries of potential therapeutic candidates with high-sequence diversity. The probability of finding a good hit primarily depends on the quantity (diversity) and quality (distribution) of candidates in libraries used for screening. They include libraries of peptides, single-chain antibodies, nanobodies, CRISPRs, shRNAs, phage display, yeast display, Ribosome display, DARPins, BiKEs, TriKEs, CAR-T cells, and recombinant viruses (including adenoviruses, lentiviruses, AAVs, and other oncolytic viruses, etc.). Screening them for specific interaction with the target of interest to narrow down to fewer candidates, which can be further validated, are the basic steps in the generic discovery pipeline. Lately, artificial intelligence (AI) based designing has also been used to design candidates for targeted interaction. However, the quality of

such prediction depends on the data used in machine training. Since the current libraries are known to be biased, such libraries may not be as good as unbiased libraries. ***Current limitations:*** Generating high-sequence diversity libraries is very challenging. Several published studies and industries claim to have very high diversity libraries ranging over 10^9 sequence diversity. However, most of those libraries lack validation by stringent experimental standards, and the diversity claimed is mainly based on sequencing a small number of candidates in the library and statistical extrapolations. Most of the time, we found that those who claim very high diversity (over 10^8) did not validate their libraries with stringent next-generation sequencing (NGS) based methods, and those who used stringent NGS methods for validation usually do not claim such big diversity. Therefore, there is an imminent need for a method to generate genuine high-sequence-diversity libraries of high quality. ***Methods and results:*** We have developed a novel method to generate such libraries with very high sequence diversity and validated them with stringent NGS-based methods. Compared to other established methods, our libraries are at least 100 to 1000 times better in diversity (quantity) and have a more uniform and unbiased distribution (quality). Through benchmarking studies, we validated total diversity, duplication rate, sequence bias, and the ability to scale up. As a proof of concept, we generated libraries of plasmids (shuttle vectors to make recombinant adenoviruses) and recombinant adenoviruses with high sequence diversity in a structural protein (Fiber). We also used them for screening against a cancer-specific cell-surface protein. ***Applications:*** Our methods can generate quantitatively and qualitatively better (100-1000 times) libraries. They can be used in high-throughput screening against targets of interest to narrow down to fewer candidates. Smaller libraries resulting after serial and multiple rounds of screening can serve as a dataset for machine training to aid AI-based design. Our unbiased, high-diversity libraries provide a high-quality dataset to improve Ai-based design criteria. We expect our libraries may help in avoiding AI-based design altogether in some cases.

159. SV40 Vector-Based Gene Therapy: A Groundbreaking Platform for Genetic Disorders and Autoimmune Diseases

Petrus De Haan, Petrus De Haan

Amarna Therapeutics, Leiden, Netherlands

To date, adeno-associated virus (AAV) vectors are the most popular for use in *in vivo* gene therapy. However, clinical studies using AAV vectors revealed that administration of vector particles elicits innate and adaptive immune responses against the viral and transgene proteins in the vast majority of treated patients. For these reasons, an alternative non-immunogenic gene delivery vector is highly desired. Vectors derived from Simian Virus 40 (SV40) could be an attractive alternative to AAV vectors for clinical gene therapy. SV40 is a polyomavirus with icosahedral capsids containing a 5.25 kb long circular double-stranded DNA covered with histones to form euchromatic mini chromosomes. The virus strictly replicates in macaques, where it causes chronic asymptomatic infections. Unlike other viruses, polyomaviruses are able to evade the host's immune system after cell entry and since humans can be considered naive to SV40, it is expected that replication defective SV40 vectors are non-immunogenic and tolerogenic when applied in clinical settings. We at Amarna have developed a replication-defective SV40-based gene delivery platform named SVec. SVec particles are produced at high titers using a proprietary engineered Vero producer cell line named SuperVero. Because of its non-immunogenicity and tolerogenicity in humans SVec particles can be repeatedly administered to the same patient without the induction of any immune response. We tested SVec for its feasibility in developing gene replacement therapies for two genetic disorders, hemophilia B (HemB) and primary hyperoxaluria type 1 (PH1). For both diseases the genetic defect was successfully corrected by hepatic expression of SVec vectors encoding functional blood clotting factor IX in the F9 knockout HemB mouse model and alanine-glyoxylate aminotransferase in the AGxT knockout PH1 mouse model respectively. We next explored the potential of SVec to develop effective tolerization therapies for autoimmune diseases. There is an accumulating body of evidence that most of todays' major degenerative and inflammatory diseases are autoimmune diseases and it is generally accepted that the ultimate curative intervention for this cluster of diseases would be the induction/restoration of immune tolerance to the primary self-antigen of the disease. For two established autoimmune diseases, diabetes mellitus type 1 (T1DM) and multiple sclerosis (MS) we recently obtained prophylactic and therapeutic proof-of-principle in the relevant mouse models of both diseases. Using the inducible experimental autoimmune encephalitis (EAE) mouse model of human MS, hepatic expression of an SVec vector encoding myelin oligodendrocyte glycoprotein (MOG), the primary self-antigen of MS, both protects the tolerized animals from developing the autoimmune disease symptoms or ameliorates disease symptoms in symptomatic mice. Expression of an SVec vector encoding proinsulin, the primary self-antigen of T1DM in the liver stops the autoimmune destruction of pancreatic β-cells and hence disease progression in non-obese diabetes (NOD) mice that were on their way to develop T1DM. These remarkable therapeutic effects have been obtained using relatively low vector doses ($2x10^7$ infectious units per kilogram of body weight). Overall, we have shown in relevant animal disease models that SVec vectors are ideally suited for developing safe and effective gene replacement and tolerization therapies to treat genetic disorders and autoimmune diseases. On the basis of these encouraging results we are building a pipeline of SVec-based gene therapy products for treating todays major diseases including genetic disorders (PH1, HemB and hemophilia A), metabolic diseases (T1DM, diabetes mellitus type 2 (T2DM)/obesity) and neurodegenerative diseases (MS, Alzheimer's dementia, Parkinson disease and amyotrophic lateral sclerosis).

160. Delivery of Large Gene Circuits *In Vivo* Using an Engineered Baculovirus Vector for Multifactorial Control of Therapeutic Gene Expression

Lucas B. C. Brown, Linlin Zhang, Gang Bao, Caleb J. Bashor

Bioengineering, Rice University, Houston, TX

Many of the viral vectors used for gene therapy are limited by the cargo size they can deliver into cells in tissue. As a result, most therapies being actively considered today tend to consist of monomodal expression of one or two genes. While this modality is undoubtedly effective for many applications, there remains advantages to being able to deliver more genetic cargo. A viral vector with an increased cargo capacity could allow room not only for more and larger therapeutic genes,

but also regulatory elements that permit complex, multifactorial regulation of therapeutic gene expression. Here we use the insect-derived baculovirus capable of packaging and delivering >100kb of transgene DNA as a vector for complex gene circuits that regulate and enhance in vivo gene therapy. Baculovirus has many advantages over other vectors: the ability to transduce a broad spectrum of mammalian cells, a large packaging capacity, no replication in mammalian cells, and a low toxicity *in vivo.* However, while baculovirus has been used as a gene therapy vector previously, its potential has been limited by its transient expression, as well as its susceptibility to inactivation by the complement system. We first address several shortcomings of the baculovirus by pseudo-typing the AcMNPV baculovirus with two proteins, the Vesticular stromatitis virus protein G and a fusion protein consisting of several complement regulatory domains. This engineered vector has increased transduction and persistence in mouse liver, muscle, and brain tissue. To our knowledge, this is the first time systemic delivery of baculovirus has been shown to be an effective delivery route. We further increased the length of baculovirus transgene expression to more than a month *in vitro* and *in vivo* through the screening of promoters and other genetic elements such as the Ebna1-OriP system. We then implemented a hierarchical cloning scheme for the rapid generation and prototyping of baculovirus vectors containing up to 10 different expression units. Using this system, we screened a library of 24 variations of a tamoxifen inducible circuit in order to select the architecture with the highest dynamic range, up to a 67-fold increase over uninduced. Finally, we demonstrate two orthogonal small molecule inducible systems (grazoprevir and tamoxifen) delivered by baculovirus *in vivo*. Our findings will demonstrate the usefulness of complex regulation for the gene therapy field, as well as the utility of the baculovirus as a therapeutic vector.

161. Defining the Role of Viral and Cellular Insulators in Promoting Durable HSV-1 Vector Mediated Transgene Expression in Muscle and Brain

Selene Ingusci, Justus B. Cohen, Joseph C. Glorioso
Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA

Herpes simplex virus 1 (HSV-1) replication defective vectors are promising new gene therapy vectors for expression of large or multiple genes. We have developed safe HSV-1 gene transfer vectors by functional deletion of all immediate-early (IE) genes and the reiterated JOINT region flanking the unique long (U_L) and unique short (U_S) genomic components, together providing a payload capacity of ~30kb. Deletion of the gene encoding the highly cytotoxic infected cell protein 0 (ICP0) completely abrogated viral gene expression and eliminated vector cytotoxicity and recruitment of inflammatory cells *in vivo.* While this enhanced vector safety, the transgene payload was also rapidly silenced. Transgene expression was rescued by replacing either the latency associated transcript (LAT) or the ICP4 lytic gene with the transgene cassette in close proximity to naturally occurring viral insulator/boundary elements that help maintain euchromatin. The viral insulators are enriched in binding sites for the CTCF protein, a master regulator of chromatin structure. Surprisingly, the LAT locus was more permissive for transgene expression in myotubes *in vitro* whereas the same cassette in the ICP4 locus was more active in differentiated SH-SY5Y neuroblastoma cells. *In vivo* delivery to skeletal muscle demonstrated stable transgene expression from the LAT locus for at least 1 year, whereas persistent transgene expression from the ICP4 locus was documented over a 5-month period in mouse hippocampus. However, the level of expression in brain neurons tended to decline over time. To remedy this decline and further enhance transgene expression, we designed a novel insulator construct by combining cellular and viral endogenous insulator elements. The new design included mouse tRNA genes and scaffold/matrix attachment regions (S/MARs) known to function as chromatin barrier elements shielding the transgene from the spread of repressive heterochromatin. This novel combination of insulator elements provided significantly higher levels of transgene expression in the brain and also eliminated the difference in transgene activity observed between the two loci in both muscle and neurons. This new cassette design will ensure high and durable transgene activity in multiple tissue types and should prove beneficial for treating a wide range of muscle and brain disorders.

162. Adenoviral DNA Replication of HC-AdV Genomes

Jonas Kolibius, Patrick Freitag, Fabian Weiss, Andreas Plückthun
Department of Biochemistry, University of Zurich, Zurich, Switzerland

Introduction: High-capacity adenoviral vectors (HC-AdVs) have shown to be a promising vector type for the nuclear delivery of large DNA cargoes. This includes the coding sequence of therapeutic antibodies, cytokines, genome editors, and combinations of immunotherapeutics. As the HC-AdV is devoid of all viral genes, the viral genome does not undergo viral DNA replication after nuclear translocation. This often results in weak payload expression, loss of transgene expression due to cell division, genome degradation, or cellular silencing effects. To circumvent these limitations, we are developing a new adenoviral vector type featuring intrinsic amplification of its delivered vector genome without viral particle formation and lysis of the host cell while preserving a large transgene capacity of more than 20 kb. Methods: We established a transfection-based complementation assay to identify the individual adenoviral proteins that are required for efficient viral genome replication in HEK293 and A549 cells. After transfection of plasmids encoding the viral replication machinery and followed by transduction of a HC-AdV, the expressed viral replication machinery triggers amplification of the delivered HC-AdV genome. Total DNA can then be extracted, and adenoviral genome replication can be precisely quantified by qPCR. When encoding a reporter on the HC-AdV genome, transcription and payload expression can be monitored via RT-qPCR and flow cytometry. Results: By applying the established *trans*-complementation assays, we were able to identify the minimal set of viral components necessary for adenoviral DNA replication. Expression of these proteins results in potent amplification of incoming HC-AdV genomes upon translocation to the nucleus. Replication of the delivered vector genome leads to a greatly increased expression of the encoded payload. There was no induction of a cytopathic effect observed, suggesting that killing of the host cell is successfully prevented by careful selection of the delivered viral genes. Conclusion: We have screened various adenoviral genes for the involvement of their respective gene products in the adenoviral DNA replication. As expected, the presence of a minimal set of adenoviral gene products

is crucial for the replication machinery to successfully amplify the genomes of HC-AdVs, while other genes mostly modulate the cellular environment to further increase the efficiency of the replication machinery so that more viral genome copies can be generated. Replication of the payload-encoding HC-AdV genome results in an elevated payload expression which potentially allows a dose reduction of HC-AdVs in a future therapeutic application to alleviate vector-mediated immunogenicity. Importantly, genome replication and increased payload expression does not induce a cytopathic effect, resulting in persistent payload expression due to the prevention of host cell death, which is usually a limitation when working with other replicating adenoviral vector classes, such as replicating single-cycle or fully replication competent adenoviral vectors.

Gene Transfer and Genome Editing for Inherited Metabolic Disorders

163. Long-Term Efficacy, Safety, and Extrahepatic Benefit of Liver-Directed Lentiviral Gene Therapy in a Methylmalonic Acidemia Mouse Model

Elena Barbon[1], Camilla Negri[1], Andrea Raimondi[1], Chiara Simoni[1], Elisabetta Manta[1], Francesca Sanvito[1], Giancarlo la Marca[2], Amy Argabright[3], Angelo D'Alessandro[3], Alessio Cantore[1,4]

[1]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Department of Experimental and Clinical Biomedical Sciences, University of Florence, Italy, Florence, Italy,[3]University of Colorado Anschutz Medical Campus, Aurora, CO,[4]Vita-Salute San Raffaele University, Milan, Italy

Methylmalonic acidemia is an inherited metabolic disease due to deficiency of the mitochondrial enzyme methylmalonyl-coA mutase (MUT), resulting in the build-up of toxic circulating methylmalonic acid (cMMA). The disease-related morbidity and mortality in infancy led to liver transplantation as elective treatment. Integrative lentiviral vector (LV)-based gene therapy may represent an alternative to liver transplantation, allowing for stable gene transfer even in young children at initial disease stage. We assessed the long-term efficacy and safety of a liver-directed LV gene therapy in a mouse model of the disease (MCK-Mut-/-). MCK-Mut-/- mice present hepatic and renal cMMA accumulation early in life which further increase with aging, thus indicating a progressive worsening of the disease. The treatment of juvenile MCK-Mut-/- mice with a LV encoding a human MUT transgene under the control of a hepatocyte-specific expression cassette (LV.MUT, 5E10 TU/kg) showed rapid, substantial, and long-lasting (> 1 year) decrease in cMMA (mean of 146 μM and 979 μM in treated vs. untreated animals 12 months post LV) with normalization of FGF-21, a biomarker of mitochondrial function. Overexpression of MUT enzyme resulted in almost complete normalization of intrahepatic MMA concentrations (0.3 μM, 5.3 μM and 300 μM in wild-type, treated and untreated animals respectively). In line with this finding, liver histology and mitochondria ultrastructure of treated mice was fully normal while age-matched MCK-Mut-/- untreated mice displayed major disease-related alterations. Importantly, we observed significant MMA reduction in the brain (28 μM and 93 μM in treated vs. untreated animals) and in the kidney (81 μM and 552 μM in treated vs. untreated animals), indicating an extrahepatic benefit achieved by liver-mediated detoxification. Multiomic analysis on liver and serum samples of MCK-Mut-/- mice showed a major dysregulation of metabolic intermediates, lipids and proteins specifically related to the mitochondrial energy metabolism (i.e., carnitine and fatty acid metabolism and tricarboxylic acid cycle), which were partially to completely rescued upon gene therapy in both compartments. We treated additional MCK-Mut-/- mice at different LV doses (2.5, 5 or 10E10 TU/kg) and observed a dose-dependent correction of disease biomarkers. Mice treated at the lower LV dose displayed a progressive rescue, with full weight normalization and a decrease in cMMA comparable to those of mice injected at higher LV doses, only around 3 months post-LV. This kinetics suggest a selective advantage of the corrected cells, especially when starting from an initial lower LV dose/liver transduction. To further improve our approach, we tested a LV encoding a codon optimized MUT transgene (LV.MUTco) which was administered to juvenile MCK-Mut-/- mice at a dose of 2E10 TU/kg, lower than previously tested doses. LV.MUTco treatment resulted in a liver MUT transgene overexpression 6-8 fold higher compared to that obtained with LV.MUT at a similar vector dose, paralleled by a greater reduction in cMMA (199μM and 369μM in LV.MUTco and LV.MUT treated mice, respectively, vs 1120 μM in untreated mice, 6 months post LV), thus potentially allowing to reduce LV dose to achieve the same therapeutic efficacy. Overall, these data provide evidence for the efficacy, safety, and extrahepatic benefit of liver mediated LV gene therapy for methylmalonic acidemia.

164. Efficient Gene Therapy via Retrograde Injection into the Ductal Systems of Liver and Pancreas

Amita Tiyaboonchai[1], Leslie Wakefield[1], Anne G. Vonada[1], Sunghee Chai[1], Yulia Eygeris[2], Gaurav Sahay[2], Markus Grompe[1]

[1]Pediatrics, Oregon Health and Science University, Portland, OR,[2]Pharmaceutical Sciences, Oregon State University, Portland, OR

Gene therapy vectors targeted to the liver and pancreas are typically delivered intravenously via the systemic circulation. The biliary system connects to the liver via the common hepatic duct and to the pancreas via the pancreatic duct providing a direct localized route for targeted delivery of vectors to the liver and pancreas. In humans, retrograde injection is routinely performed using Endoscopic Retrograde Cholangiopancreatography (ERCP), enabling access to both liver and pancreas through the ampulla of Vater. Retrograde injection via the duct system has several potential advantages over systemic gene therapy including minimization of off-target transduction, increasing the local concentration of the gene therapy vector, and bypassing any liver fibrosis to potentially allow efficient transduction of hepatocytes even in the presence of sinusoidal capillarization. To test the feasibility of this strategy, we performed proof of principle experiments for ductal delivery of gene therapy vectors in mice. Here, we successfully delivered recombinant adeno-associated virus vectors (rAAVs) and lipid nanoparticles (LNPs) to both liver and pancreas through retrograde ductal injection.To target the liver, rAAV (serotype DJ) expressing

human Factor 9 (hF9) was injected into the common bile duct. Control animals received the same vector intravenously. Circulating hF9 was comparable in both the mice that received hF9 via the common bile duct or intravenously. Similarly, ductal injection with an rAAV of a different serotype (KP1) expressing a RFP reporter resulted in ~ 40% hepatocyte transduction. To test whether LNPs could also be delivered to the liver in this fashion, LNPs containing Cre-mRNA were administered via the bile duct to mTmG mice. mTmG mice express a tdTomato reporter flanked by LoxP sites upstream of a GFP reporter. Following Cre recombination, transgene expression in these mice switches from tdTomato to GFP. Retrograde ductal injection of the Cre-mRNA LNPs resulted in near 100% conversion of hepatocytes from red to green fluorescence, confirming successful ductal delivery of LNPs to the liver.The pancreas also has a ductal system that connects to the biliary network at the ampulla of Vater. Unlike the liver, it is very difficult to target gene therapy to the pancreas by systemic injection. An RFP-expressing rAAV (serotype KP1) was delivered by injection into the ampulla of Vater, resulting in a ~45% transduction of acinar cells. Next, Cre-mRNA containing LNPs were delivered to mTmG mice by injection into the ampulla of Vater. Approximately 20-30% of acinar cells were found to be GFP-positive indicating the successful delivery of the mRNA to the pancreas. Islet transduction was very rare. Together these data show that retrograde ductal injection into the liver or pancreas can provide an efficient route for gene therapy by rAAV or LNP-mediated mRNA. Experiments to compare vector dose responses, off-target transduction and immunogenicity of ductal vs. systemic administration are ongoing.

165. Development of ABO-101, a Novel Gene Editing Therapy for Primary Hyperoxaluria Type 1

Tia DiTommaso, Michael S. Kuefner, Sneha Halwasia, Anthony J. Garrity, Sejuti Sengupta, Rachel Wollacott, Raisa Lowe, Lijun Liu, Daniel S. Ory, Pamela Stetkiewicz, John E. Murphy

Arbor Biotechnologies, Cambridge, MA

Primary Hyperoxaluria, or PH, is a group of rare genetic metabolic disorders characterized by the overproduction of oxalate by the liver. Oxalate builds up and accumulates in the kidneys and other organ systems, causing kidney damage and progression towards renal failure. PH1, the most common and clinically severe subtype of PH, results from a mutation of the *AGXT* gene which is expressed primarily in the liver. Here we present pre-clinical *in vivo* evidence supporting ABO-101 as a novel CRISPR-Cas-mediated gene editing therapy for the treatment of PH1. The current standard of care siRNA therapy for PH1 requires lifelong treatment and targets *HAO1*, which encodes the glycolate oxidase enzyme (GO) and is upstream of the mutant *AGXT*. Inhibition of *HAO1* lowers the availability of oxalate precursors and thus results in a therapeutically meaningful reduction of oxalate levels and a corresponding increase in serum glycolate, a metabolic byproduct of *HAO1* inhibition which provides a measure of gene disruption efficacy. ABO-101 was designed to specifically inactivate the *HAO1* gene in liver hepatocytes and provide a durable reduction of oxalate levels through a single course of treatment. ABO-101 is comprised of a lipid nanoparticle (LNP) which encapsulates our proprietary engineered variant of Cas12i2 (ABR-001) and a guide RNA (gRNA), selected to share 100% sequence complementarity to the cynomolgus macaque NHP *HAO1* gene. In NHPs treated with ABO-101, we achieved *HAO1* indels of approximately 60% in whole liver tissue, which correlates to a greater than 2x increase in serum glycolate, all with a well-tolerated safety profile. We also observed a dose-dependent increase in indel levels with a concomitant increase in serum glycolate levels and further reduction of GO protein and enzyme activity levels. In addition to both rodent and primate pharmacology data, we also describe our approach optimizing our lead gRNA, ratios of components inside the LNP, and the mRNA encoding the gene editing machinery; these optimizations resulted in our lead therapeutic candidate ABO-101. Taken together, these results provide *in vivo* proof of pharmacology for a gene editing approach and support further advancement of ABO-101 as a potential treatment for PH1.

166. Ubiquitous Promoter in AAV9 GAMT Gene Therapy Yields Lower Levels of Guanidinoacetic Acid in the Brain

Puja Patel[1], Ilona Tkachyova[2], Andreas Schulze[2], Gerald S. Lipshutz[1]

[1]Pharmacology & Surgery, UCLA School of Medicine, Los Angeles, CA,[2]Biochemistry & Metabolism, The Hospital for Sick Children, Toronto, ON, Canada

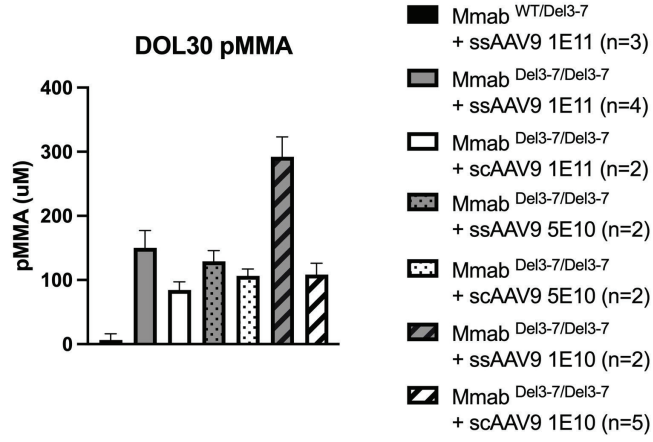
Introduction: Guanidinoacetate methyltransferase (GAMT) deficiency is a disorder of creatine biosynthesis that utilizes guanidinoacetate & S-adenosylmethionine to synthesize creatine, a vital high energy molecule for highly active tissues (i.e., brain, muscle). In people, this disorder is marked by developmental delays, intellectual disability, prominent speech delay, seizures, involuntary movements & decreased muscle tone. Present day therapy with high-dose creatine & ornithine is poor-tasting and commonly incomplete, leaving children at risk for intellectual disability & epilepsy that can be refractory to therapy. A mouse model of the disorder has been described that demonstrated marked guanidinoacetic acid (GAA), a neurotoxin, in the brain along with low levels of guanidinobutyric acid (GBA) which is normally not found in the brain. Previously our group demonstrated that an adeno-associated viral (AAV) GAMT gene therapy could restore the creatine biosynthesis pathway by targeting the liver exclusively; however, the mice had reduced but persistent levels of GAA in the brain. This poses as a problem as its presence may continue to affect brain function. In this study, we aimed to create an array of novel AAV based GAMT vectors & assess their efficacy in not only restoring function of the GAMT enzyme but also reducing the amount of GAA & GBA present in the brain. We hypothesized that by utilizing a ubiquitous promoter there would be increased expression of GAMT in the brain to lower the amount of GAA present & increase the amount of creatine available for brain function. Methods: Four AAVs were developed for this study: 1) a single stranded AAV rh10 serotype containing a TBG promoter; 2) a single stranded AAV8 serotype containing a TBG promoter; 3) a single stranded AAV9 containing a CAG promoter/enhancer; & 4) a self-complementary AAV9 containing a CAG/enhancer promoter. These 4 viruses were injected into two groups of Gamt-deficient mice at one of two doses, $1x10^{13}$ genome copies (GC)/kg or $5x10^{13}$ GC/kg. Both groups were euthanized 60 days post injection followed by transcardiac perfusion with PBS. GAA & creatine levels were analyzed in the plasma, brain, and liver; GBA levels were also analyzed in the

brain. Results: In comparison to the untreated Gamt-deficient mice, all AAV groups presented with restoration of GAMT function in varying amounts as detected by reduction of GAA & increase of creatine in the liver, brain, & plasma. Within the $5x10^{13}$ GC/kg group, the AAV9 with CAG promoter/enhancer proved to be the most effective in lowering GAA levels in the brain & the rh10 with TBG treatment proving to be the most effective in lowering the amount of GAA in the liver. The rh10 with TBG promoter, AAV8 with CAG promoter/enhancer, and AAV9 with CAG promoter/enhancer all were equally effective in lowering the total amount of GAA and increasing the total creatine levels in plasma. In addition, GBA was reduced in the brain in all groups but was completely resolved only with AAV9 with the CAG enhancer/promoter. Conclusion: The clinical signs & symptoms of GAMT deficiency, caused by both buildup of the neurotoxin GAA reduced creatine in the brain, can be effectively treated in a murine model by AAV-based gene therapy. While the liver is the main site for GAA synthesis, it is important that the brain also be transduced to reduce levels further. AAV-based approaches that utilize a serotype that can transduce the brain & a promoter that is ubiquitous can effectively restore function of the GAMT protein in both sites as demonstrated by lower levels of GAA in both the liver &, importantly the brain, along with restoration of creatine levels. The ability of AAV9 with a ubiquitous promoter to correct the biochemical abnormalities in Gamt deficiency may pave the way for a new treatment to replace the current and incomplete dietary standard of care.

167. *In Vitro* and *In Vivo* Efficacy of Single-Strand and Self-Complementary AAV9 Vectors to Treat Isolated Cobalamin B Type Methylmalonic Acidemia

Karenna Choi, Eun-Young Choi, Charles P. Venditti
NHGRI/NIH, Bethesda, MD

Background: Cobalamin B type (*cblB*) methylmalonic acidemia (MMAB) is a rare and severe inborn error of metabolism caused by mutations in the *MMAB* gene. *MMAB* encodes ATP cob(I) alamin adenosyltransferase and catalyzes the synthesis of 5'-deoxyadenosylcobalamin (AdoCbl), the cofactor for methylmalonyl-CoA mutase (MMUT). Patients with MMAB deficiency exhibit elevated levels of plasma methylmalonic acid (pMMA) and fibroblast growth factor 21 (FGF21), and deficiency of MMUT. Clinically, patients present with metabolic instability, and some require elective liver and/or liver-kidney transplantation. Currently, there are no therapies for MMAB deficiency, an indication for the Platform Vector Gene Therapy Program (PaVe-GT). **Methods:** A series of AAV gene therapy vectors designed to widely express MMAB in vivo were designed, tested in human MMAB knock out cells and then in *Mmab* mouse models. Optimized human *MMAB* genes were cloned into AAV backbones under the control of EF1L or EF1S promoters. The plasmids, packaged as either single-stranded or self-complementary vectors, were then pseudoserotyped with an AAV9 capsid (AAV9 EF1L MMAB "SS" or scAAV9 EF1S MMAB "SC", respectively) and tested in an infection study using a HEK293T MMAB knock-out (KO) cell line. Both vectors were assayed for in vivo efficacy using a recently developed MMAB mouse model whereby a severe mutation removing exons 3-7 was engineered into the Mmab locus, generating the $Mmab^{Del3-7}$ allele. **Results:** Both classes of AAV9 vectors produced robust MMAB expression in KO cells, demonstrating AAV9 vector-directed expression of properly processed MMAB. The SC vector showed higher MMAB expression compared to SS in all tested doses ranging from 5E4 GC/cell to 1E6 GC/cell. $Mmab^{Del3-7/Del3-7}$ mice, which display lethality around weaning age and manifest massively increased pMMA (~1500uM, 360x WT), much like patients MMAB deficiency, were treated on DOL1 with either class of vector, systemically delivered at a dose of 1E10, 5E10 or 1E11GC/pup. One month after a single neonatal injection of either 1E11, 5E10 or 1E10 of either vector, there was a drastic reduction of pMMA, approximately 17.7-fold for SC 1E11 compared to untreated mice. 1E10 SC treatment displayed similar level of metabolic correction as 1E11 SS treatment at both one month and two-months post-treatment (Figure 1). Further, a 2-fold reduction in plasma FGF21, as well as robust transgene expression in the liver and kidney, were observed in both SS and SC-treated mice that received 1E10 at DOL1 and sacrificed at DOL120 (Liver: SS 598%, SC 390% of WT; Kidney SS 105%, SC 98% of WT). The DOL30 weight of mutants treated with SC at all tested doses were closer to that of untreated heterozygotes compared to the weight of mutant mice treated with SS. **Conclusions:** We have developed single strand and self-complementary AAV9 vectors that can be used for neonatal gene therapy for MMAB deficiency. Both classes of AAV9 vectors produce high titer AAV and are potent in vitro. After systemic delivery over a wide dose range, correction of survival, reduction of pMMA and FGF21, and robust hepatorenal transgene expression were observed with both classes of vectors, with the metabolic phenotypes improved more in the SC-treated mice than those treated with SS vector at the same dose. Our study enables a new class of vectors and AAV9 gene therapeutics to treat patients with MMAB deficiency that are suitable for immediate human translation.

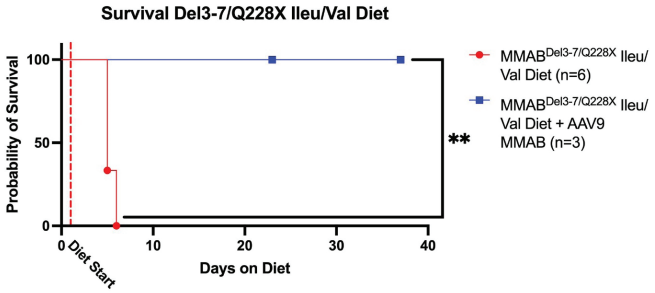


168. Hypomorphic Model of MMAB Deficiency: Resistance to Lethality from Dietary Challenge Conferred by Systemic AAV Gene Therapy

John M. Selser, Eun-Young Choi, Charles P. Venditti
National Human Genome Research Institute, Bethesda, MD

Background: Cobalamin B-type (cblB) methylmalonic acidemia (MMA) or MMAB is a severe ultra-rare autosomal recessive inborn error of metabolism. It is caused by pathogenic variants in the *MMAB* gene that encodes the ATP:cob(I)alamin adenosyltransferase (ATR) enzyme. ATR generates 5'-deoxyadenosyl-cobalamin (AdoCbl),

the obligate cofactor for the methylmalonyl-CoA mutase (MMUT) reaction. Current treatment relies on supportive care including dietary management, carnitine supplementation, and intermittent antibiotics. Elective liver (LT) or combined liver kidney (LKT) transplantation has been offered to severely affected patients as an experimental surgical therapy for the treatment of MMAB. **Methods**: Informed by successful strategies to develop mouse models and AAV gene therapy for the related form of MMA caused by deficiency, we first identified relevant patient mutations in a large cohort of subjects with MMAB deficiency evaluated in a natural history study (NCT00078078). One mutation was selected for further modeling in mice: the orthologue of a nonsense mutation frequently found in patients (Q234X, Q228X in mouse), bred to homozygosity ($Mmab^{Q228X/Q228X}$), and with mice carrying a null allele ($Mmab^{Del3\text{-}7/Q228X}$). These knock-in and compound mutants were characterized and challenged with a diet consisting of a 10-fold increase in isoleucine and valine amino acids while maintaining total caloric intake. In a proof-of-concept study, MMAB mice were then treated with a systemic retro-orbital injection of 1e+13 genome copies per kilogram of ssAAV9 EF1L MMAB and examined for response to challenge. **Results**: The hypomorphic model, $Mmab^{Del3\text{-}7/Q228X}$, displayed similar survival and weight gain compared to wild type controls, while homozygote mutant $Mmab^{Del3\text{-}7/Del3\text{-}7}$ showed neonatal lethality. $Mmab^{Del3\text{-}7/Q228X}$ mice displayed mild methylmalonic acidemia [MMA] (80 +/- 77 uM, ~100x WT) very much like patients with attenuated forms of *cblB* MMA. When treated with isoleucine and valine enriched diet, $Mmab^{Del3\text{-}7/Q228X}$ demonstrated significant weight loss (-6.2 +/- .8g n=6 over 4 days) and 100% lethality within 5 days, while controls fed the same diet thrived and gained weight. Plasma [MMA] levels were drastically increased with Isoleucine/Valine diet (1223 +/- 738 uM, ~1500x WT) and reflective of severe clinical disease. $Mmab^{Q228X/Q228X}$ mice, in contrast, survived an Isoleucine/Valine diet challenge and manifested mild MMAemia (142 +/- 74 uM) when challenged on the Isoleucine/Valine enriched diet compared to compound $Mmab^{Del3\text{-}7/Q228X}$ mice. In a proof-of-concept study, treatment of $Mmab^{Del3\text{-}7/Q228X}$ mice with an AAV9 MMAB vector, systemically delivered at a dose of 1e13 GC/kg on DOL26, approximately two weeks prior of Isoleucine/Valine diet resulted in complete rescue from lethality (p=.0081), and reduced weight loss. **Conclusions**: We have developed a new clinically relevant murine MMAB mutation and a suite of new murine models to enable preclinical gene therapy studies in adult mice. The $Mmab^{Del3\text{-}7/Q228X}$ mice models can be used in conjunction with an Isoleucine/Valine challenge diet to induce a lethal metabolic crisis, and fully rescued from lethality by systemic AAV9 gene therapy. This model shows that AAV9 gene therapy can be used to protect against acute lethal metabolic instability and will allow the facile assessment of vector efficacy and dose finding in the development of new gene therapeutics for patients with MMAB deficiency.



169. *In Vivo* Correction of Metabolic Pathogenic Variants via Base Editing and Prime Editing: Toward the Equitable Treatment of Hepatic Inborn Errors of Metabolism

Dominique Brooks[1], Madelynn Whittaker[1], Ping Qu[1], William Peranteau[2], Mohamad-Gabriel Alameh[2], Rebecca Ahrens-Nicklas[2], Kiran Musunuru[1], Xiao Wang[1]

[1]University of Pennsylvania, Philadelphia, PA,[2]Children's Hospital of Philadelphia, Philadelphia, PA

Phenylketonuria (PKU), an autosomal recessive disorder caused by pathogenic variants in the phenylalanine hydroxylase (*PAH*) gene, is the most common inborn error of metabolism. PKU results in the buildup of blood phenylalanine (Phe) to neurotoxic levels. Current dietary and medical treatments are chronic and reduce, rather than normalize, blood Phe levels. The most frequently occurring *PAH* variant in PKU patients is the c.1222C>T (R408W) variant. Using real-world data from a PKU cohort at an academic medical center, we found that most patients with at least one R408W allele (n = 36) experience chronic, severe Phe elevations and do not meet Phe monitoring guidelines—highlighting the high unmet medical need arising from the challenges of lifelong adherence to dietary and medical therapy. Motivated by these findings, we are developing "one-and-done" CRISPR editing therapeutics for PKU. We have established a pipeline to rapidly screen and optimize base editing or prime editing strategies *in vitro* and deploy them *in vivo* with either lipid nanoparticles (LNPs) or adeno-associated viral (AAV) vectors on a timescale of months. In initial work, we created six humanized PKU mouse models for the six most frequent *PAH* variants and, in parallel, prime-edited variant-bearing hepatocyte cell lines. In homozygous or compound heterozygous PKU mice, we observe complete and long-term durable normalization of blood Phe levels (>90% reduction) as soon as 48 hours after treatment, with whole-liver corrective *PAH* editing as high as >50% with either base editing or prime editing. We have developed therapeutic leads for multiple *PAH* variants to permanently normalize blood Phe levels and definitively treat PKU in these patients and are on track for early-phase clinical trials within a few years. Despite these encouraging results, we are cognizant of the risk of "mutational discrimination" in unduly focusing on more common diseases and on high-prevalence variants skewed towards specific ancestry groups. Accordingly, we have extended our pipeline to develop and validate a corrective therapeutic for *any* variant in *any* patient with a hepatic inborn error of metabolism. We have begun to apply this workflow in real time to patients with devastating, ultra-rare inborn errors of

metabolism, namely the urea cycle disorders and organic acidemias, tackling any variant—even n-of-1 variants—identified via universal newborn screening. The first four patients have (1) citrullinemia type 1, with homozygosity for an *ASS1* transition variant (G>A), (2) argininosuccinic aciduria, with homozygosity for an *ASL* transition variant (A>G), (3) propionic acidemia, with homozygosity for a *PCCA* 1-bp deletion, and (4) CPS deficiency, with compound heterozygosity for a *CPS1* partial gene deletion and a transversion variant (A>C). To address these specific variants in parallel, we have developed (1) a high-throughput lentivirus-based multiple-variant prime editing screening platform in human hepatocytes and (2) a multi-humanized mouse with a string of genomic sequences harboring each of the four variants inserted into the *Rosa26* safe harbor locus, allowing for *in vivo* testing of lead editing solutions for all four variants in a single mouse model, which is now underway. This augmented, scalable pipeline represents a critical step towards our goal of rapidly devising and deploying a personalized editing treatment for any patient in need.

Correction of Genetic Disorders of the Blood and Immune System

170. Highly Specific Non-Viral Gene Editing with P-KLKB1-101 for Hereditary Angioedema

Oscar Alvarez, Lucas Homa, Khalid Hajj, Zhibo Ma, Cindy Negron, Matthew Nitzahn, Mauricio Barajas-Mora, Arturo Barcenas, Alicia Davis, Mona Connerney, Pavan Battiprolu, Michael Bennett, Bonnie Jacques, Joshua Rychak, Brent Warner

Poseida Therapeutics, San Diego, CA

Recent advancements with our gene editing technology hold great promise for addressing debilitating genetic diseases like hereditary angioedema (HAE). HAE is a rare genetic condition characterized by excess plasma kallikrein activity leading to subcutaneous and submucosal edema with the risk of life-threatening swelling of the upper respiratory tract. Current treatments focused on C1 inhibitor restoration or plasma kallikrein antagonism, however, are inadequate due to lack of consistency (emergent attacks even with treatment) or can be refractory to standard-of-care and require continuous lifelong treatment. Here, we present an effective strategy for targeted inactivation of the gene encoding pre-kallikrein, *KLKB1*, using our Cas-CLOVER™ Gene Editing System and our non-viral lipid nanoparticle (LNP) delivery system. Our primary aim is to establish a high-fidelity gene therapy by inactivating the *KLKB1* gene for sustained relief across both Type I and II HAE patients. The Cas-CLOVER gene editing system is comprised of a next generation nuclease with a dual guide RNA (gRNA) targeting system that achieves high on-target editing. A comprehensive screening process identified lead human-specific gRNA pairs for optimal *KLKB1* editing in human hepatoma cell lines. Subsequent evaluations in primary human hepatocytes (PHH) revealed robust KLKB1 editing (>90%) and 85% reduction in KLKB1 protein secretion.Critical to the safety of any gene editing therapeutic, Cas-CLOVER off-target sites were nominated by iGUIDE, with amplicon-seq confirmatory studies revealing minimal off-target editing, at levels near background (<0.2%) in PHHs. Remarkably, at concentrations 10-fold higher than our EC90 LNP concentration, the low level off-target editing remained constant, demonstrating best in-class fidelity compared to other RNA-guided nucleases like Cas9.Activity of our P-KLKB1-101 LNP-encapsulated Cas-CLOVER mRNA with *KLKB1*-targeting gRNAs was then assessed in a TK-NOG mouse model in which livers are humanized by engraftment of PHHs. Liver-humanized mice received a single intravenous injection of P-KLKB1-101, achieving 60% successful editing at the *KLKB1* locus with no off-target edits detected.To further confirm the ability of P-KLKB1-101 to impact biomarkers of HAE *in vivo*, C57BL/6 male mice received increasing doses of a mouse-specific surrogate gRNA pair targeting *Klkb1* (dose range 0.1 to 3 mg/Kg). A single intravenous LNP injection achieved dose-responsive editing and more than 90% reduction in serum pre-kallikrein levels, within a wide therapeutic window, and well within the range predicted to achieve therapeutic benefit in humans. All doses were well-tolerated, with no serious adverse effects; importantly, no editing was detected in testes (no higher than PBS vehicle controls). In summary, P-KLKB1-101, utilizing our Cas-CLOVER gene editing system, demonstrates efficacy and specificity, laying a foundation for a targeted gene therapy for HAE. Our preliminary data offer insights into potential clinical applications, advancing targeted gene therapies for genetic disorders.

171. Base Editing of BCL11A Cis-Regulatory Regions to Treat Beta-Hemoglobinopathies

Letizia Fontana, Panagiotis Antoniou, Pierre Martinucci, Simone Amistadi, Annarita Miccio

Imagine Institute, Paris, France

Beta-hemoglobinopathies (e.g., sickle cell disease, SCD) result from mutations affecting β-globin chain production. The clinical severity is reduced by mutations causing elevated fetal hemoglobin (HbF) production, a condition termed hereditary persistence of HbF (HPFH). Transplantation of autologous, genetically corrected hematopoietic stem/progenitor cells (HSPC) is a promising therapy. Notably, a CRISPR/Cas9 approach to down-regulate BCL11A, a major HbF repressor (by targeting the erythroid-specific *BCL11A* enhancer) has been recently approved for the treatment of β-hemoglobinopathies. However, genotoxicity concerns arise from CRISPR/Cas9-induced double-strand breaks (DSBs). Therefore, novel therapeutic strategies need to be developed. Here, to down-regulate BCL11A, we targeted transcriptional activator binding sites (BS) within *BCL11A* erythroid-specific enhancers with base editors (BE) to precisely introduce point mutations (i.e., A>G or C>T) without generating DSBs. In particular, we targeted the GATA1 and ATF4 BSs in the +58-kb and +55-kb *BCL11A* enhancer regions, respectively. Single guide RNAs (sgRNA) were tested in HSPCs from SCD patients in combination with adenine or cytosine BEs (ABE or CBE), generating different nucleotide changes within the BS motifs with an overall editing efficiency up to 90%, with no or little DSB-induced Indels. HbF reactivation was observed in all edited samples as measured by cation-exchange HPLC. However, the HbF levels were not sufficient to achieve the full rescue of the sickling phenotype in erythroid cells derived from edited SCD HSPCs. Hence, we decided to simultaneously target the GATA1 and ATF4 BS with the aim to further increase HbF levels in edited cells. Furthermore, we obtained new editing profiles by combining selected sgRNAs

with newly developed dual base editor (TadDE) to simultaneously induce A>G and C>T conversions in the activator BSs. Erythroid cells differentiated from SCD HSPCs edited at both +58 and +55 enhancers showed an increased HbF expression compared to cells receiving individual sgRNAs. In particular, in cells edited at both enhancers, HbF represented up to around 34% of total hemoglobin, exceeding the levels achieved using the recently approved CRISPR/Cas9 nuclease-based strategy. Simultaneous targeting of *BCL11A* enhancers did not impact erythroid differentiation, as measured by flow cytometry analysis of erythroid markers and enucleation rates. Finally, HbF reactivation was sufficient to allow a substantial rescue of the sickling phenotype, as measured by microscopy under hypoxic conditions decreasing the proportion of sickling cells up to 16.4%. To assess DNA and RNA off-target activity, we used GUIDE-seq coupled with targeted sequencing, whole exome sequencing and RNA-seq, while on-target unwanted events were evaluated by long-read sequencing. Finally, to evaluate the BE efficacy in repopulating bona fide hematopoietic stem cells (HSCs), we transplanted edited HSPCs in immunodeficient mice. In conclusion, we have provided ample evidence of the effectiveness of simultaneous base editing of γ-globin *trans*-regulatory elements for reactivating HbF. This proof-of-concept work will enable the pre-clinical and clinical development of base-edited HSCs for the therapy of β-hemoglobinopathies.

172. A Novel Model of X-Linked Sideroblastic Anemia (*Alas2-KO*, XLSA) with Ring Sideroblasts, Severe Anemia, and Iron Overload is Rescued by Lentiviral Gene Transfer

Carlo Castruccio Castracani[1], Amaliris Guerra[1], Laura Breda[1], Tyler Papp[2], Enrico Radaelli[2], Charles-Antoine Assenmacher[2], Giovanni Finesso[2], Yink Tam[3], Barbara Mui[3], Simona Fontana[4], Chiara Riganti[4], Veronica Fiorito[4], Sara Petrillo[4], Emanuela Tolosano[4], Hamideh Parhiz[5], Stefano Rivella[1,2]

[1]Children's Hospital of Philadelphia, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, Philadelphia, PA,[3]Acuitas Therapeutics, Vancouver, BC, Canada,[4]University of Turin, Turin, Italy,[5]University of Pennsylvania, Philadelphia, PA

X-linked sideroblastic anemia (XLSA) is the most common form of congenital sideroblastic anemia caused by mutations in *Alas2*, a gene responsible for heme synthesis in erythroid cells. Most patients carry hypomorphic mutations, with variable phenotypic manifestations. Treatment options include pyridoxine supplements, transfusions, and allogeneic bone marrow (BM) transplantation. To study an inducible severe manifestation of XLSA, we engineered a conditional *Alas2*-KO mouse model using two approaches: tamoxifen administration and treatment with lipid nanoparticles (LNP) carrying Cre-mRNA and conjugated to the anti-CD117 antibody. *Alas2*-KO-BM animals displayed fetal anemia characterized by ineffective erythropoiesis (IE) and ring sideroblasts, the first model showing this phenotype. Erythropoiesis in these animals showed expansion of polychromatic erythroid cells, decreased activity in the electron transport chain and mitochondria's function, and reduced activity of crucial Tricarboxylic Acid (TCA) cycle enzymes. The IE was associated with marked splenomegaly and increased ERFE, the erythroid suppressor of hepcidin. Since hepcidin is responsible for limiting dietary iron absorption, decreased hepcidin levels were associated with increased serum iron, transferrin saturation levels, and iron overload. To investigate a gene therapy approach for XLSA, we developed a lentiviral vector (X-ALAS2-LV) that exploits a globin promoter and enhancers to direct human *ALAS2* expression in erythroid cells. We also included an insulator in the 3' self-inactivating long terminal repeat (SIN-LTR) to enhance safety. Infusion of BM cells with 0.6-1.4 copies of the X-ALAS2-LV in *Alas2*-KO-BM mice rescued these mice by improving erythropoiesis and tissue iron accumulation. While the hemoglobin and RBC levels were improved or corrected in the LV-treated group, the non-treated group showed fatal anemia, with low RBC levels. Accordingly, the untreated group showed increased erythropoietin levels and reticulocyte counts because of IE and severe anemia. The ineffective erythropoiesis was characterized by extra-medullary erythropoiesis and severe splenomegaly, while these parameters were improved or normalized in the X-ALAS2-LV-treated group. These data show that the fatal anemia in *Alas2*-KO animals can be improved or corrected by X-ALAS2-LV. Notably, animals rescued by X-ALAS2-LV showed vector copy numbers (VCN) in the range of 0.6-1.4, indicating that a small number of integrations were sufficient to rescue *Alas2*-KO animals. As XLSA patients still exhibit hypomorphic or residual expression of the ALAS2 gene (as the complete absence is incompatible with survival), we hypothesize minimal gene transfer or mini-transplants with HSCs treated with XALAS2-LV could be curative in this disease. Moreover, since X-ALAS2-LV rescues *Alas2*-KO-BM mice, we are now modifying X-ALAS2-LV with *ALAS2* human mutations to study milder forms of XLAS and develop alternative therapeutic interventions, such as pharmacological treatments and *in vivo* gene editing.

173. Development of Best-in-Class Gene Editing Therapy for β-Hemoglobinopathies Using Innovative Transformer Base Editor (tBE)

Lijie Wang[1], Peixue Li[1], Yichuan Wang[1], Rongrong Liu[2], Yaliang Li[1], Kewei Lan[1], Huiming Zheng[1], Di Zhu[1], Yan Zhang[1], Runni Guo[1], Hongxia Ma[1], Jing He[1], Qing Su[1], Lang Zhang[1], Gaohui Yang[2], Lingling Shi[2], Zhenbin Wei[2], Xuemei Zhou[2], Bei Luo[1], Yeqian Chen[1], Jia Wei[3], Yongrong Lai[2], Li Yang[3], Jia Chen[4], Xiaodun Mou[1], Bingbing Wu[1]

[1]CorrectSequence Therapeutics Co., Ltd, Shanghai, China,[2]The First Affiliated Hospital of Guangxi Medical University, Guangxi, China,[3]Fudan University, Shanghai, China,[4]ShanghaiTech University, Shanghai, China

Background: β-thalassemia and sickle cell disease (SCD) are among the most prevalent monogenic disorders worldwide and both these β-hemoglobinopathies are caused by mutations in the β-globin gene *HBB*. Reactivating the expression of the γ-globin genes (*HBG1/2*) mimicking the naturally occurring hereditary persistence of HbF (HPFH) is expected to be a universal strategy to treat β-thalassemia and SCD by the induction of fetal hemoglobin (HbF). **Methods**: CorrectSequence Therapeutics' first pipeline, CS-101, uses transformer Base Editor (tBE) to precisely edit the binding motif of transcription repressor BCL11A in *HBG1/2* promoter region in the CD34+

hematopoietic stem cells to trigger the reactivation of fetal hemoglobin (HbF) for treatment of β-Hemoglobinopathies. **Results**: Compared with the commonly used gene editing methods such as CRISPR/Cas nucleases or other base editors, tBE is a base editing system that avoids causing DNA double strand breaks or off-target mutations and exhibits more potent globin reactivation. A commercial scale manufacturing process to *ex vivo* edit HSC has been developed and validated with more than 10 batches of commercial scale production, all of which exhibited consistent process performance and product quality. Pre-clinical *in vivo* studies showed that while tBE-mediated editing of HSC induces robust γ-globin expression, tBE did not cause adverse effect on the engraftment or differentiation of the HSC in mice after transplantation. In the investigator-initiated trial study, one patient who suffered from transfusion-dependent β-thalassemia (β^0/β^+ type) had been receiving 4 units of red blood cells (RBC) every two weeks before beginning CS-101 treatment. After receiving CS-101 treatment, the patient successfully achieved hematopoietic reconstruction, with neutrophil engraftment occurring within 20 days, and stopped receiving blood transfusions within 20 days post-transplantation. Up to now, the patient's hemoglobin level has been maintained at or above 130 g/L, and no adverse effects related to CS-101 have been observed. In addition, another patient has been free from transfusion for more than one month after receiving CS-101 treatment. **Conclusions**: CS-101 holds great promise to become the best-in-class gene editing therapy and first-in-class base editing therapy for β-hemoglobinopathies.

174. A Non-Genotoxic Conditioning Regimen Based on a Combination of Monoclonal Antibodies and Hematopoietic Stem and Progenitors Cell Mobilizers Enhances Engraftment Levels and Theraputic Efficacy

Isabel Ojeda Pérez[1,2,3], Omaira Alberquilla-Fernandéz[1,2,3], Aida García-Torralba[1,2,3], Rebeca Sánchez-Domínguez[1,2,3], Jose-Carlos Segovia[1,2,3]

[1]CIEMAT, Madrid, Spain,[2]Centro de Investigación Biomédica en Red de Enfermedades Raras, Madrid, Spain,[3]Unidad Mixta de Terapias Avanzadas. Instituto de Investigación Sanitaria Fundación Jiménez Díaz (IIS-FJD, UAM), Madrid, Spain

Hematopoietic gene therapy holds significant promise as a therapeutic alternative for various hematological disorders including Piruvate Kinase Deficiency (PKD), a rare genetic condition causing chronic hemolytic anemia by affecting red blood cell function. However, current genotoxic conditioning treatments, necessary for the successful engraftment of corrected hematopoietic stem cells (HSC) are associated with high toxicity, leading to substantial adverse effects in patients. To establish gene therapy as a primary treatment for hematopoietic inherited disorders, such as PKD, it is necessary to develop less toxic conditioning regimens that specifically target HSCs without adversely affecting other tissues. Here, we have explored a synergistic approach that combines monoclonal antibody (mAb) treatment to eliminate diseased HSCs, with HSC mobilizers to enhance the accessibility of mAbs to these cells. Our novel combination approach, which consists of a combination of 6 monoclonal antibodies (anti-CD117, anti-CD47, anti-CD40/CD40L, anti-CD122, anti-CD4 and anti-CD8) and Plerixafor, a CXCR4 antogonist, has demonstrated specific depletion of endogenous HSCs in both wild-type (WT) and PKD mice. The results revealed a remarkable three-fold increase in exogenous engraftment in WT mice. Moreover, the combination reduced the mortality of PKD mice by 60% in comparison with PKD mice treated with the combination of MoAbs alone. This new non-genotoxic conditioning regime has demonstrated therapeutic efficacy when PKD mice were transplanted with congenic wild type cells. Our non-genotoxic approach showed that the percentage of healthy cells required to reverse the disease phenotype was half of that needed with conventional total body irradiation genotoxic conditioning. This finding suggests a significantly reduced toxicity associated with our approach. These results constitute preclinical proof of concept for a non-genotoxic conditioning regimen that enables the establishment of a therapeutic hematopoietic graft. This represents a crucial step toward establishing gene therapy in the forefront for the treatment of genetic hematological disorders.

175. Hematopoietic Stem Cell Gene Therapy for the Treatment of X-Linked Agammaglobulinemia

Christopher Luthers[1], Aurelien Colomartino[2], Annika Mittlehauser[2], Zulema Romero[2], Donald B. Kohn[2]

[1]Molecular Biology, University of California, Los Angeles, Los Angeles, CA,[2]MIMG, University of California, Los Angeles, Los Angeles, CA

Introduction Bruton's Tyrosine kinase (BTK) is expressed primarily in B cells and is critical to their development and function. Absence of *BTK* gene function is the genetic cause of the primary immune deficiency X-linked agammaglobulinemia (XLA). Males with XLA lack mature B cells and have negligible antibody levels, leaving them susceptible to recurrent infections and various infectious complications. Autologous hematopoietic stem cell gene therapy (HSC-GT) with gene-corrected HSC serves as a promising therapeutic strategy for the treatment of XLA; this therapy would improve their quality of life, provide a one-time cure, and could reduce life-long costs versus chronic immunoglobulin replacement therapy. HSC-GT of XLA will require precisely regulated expression of the *BTK* gene to support optimal B cell development and humoral immune function. To achieve this, we have developed methods for site-specific insertion of a normal *BTK* gene into the 5' end of the endogenous *BTK* gene locus, using CRIPSR-Cas9 dsDNA break followed by adeno-associated virus (AAV) mediated delivery of a wild type *BTK* gene. **Methods** To study the effectiveness of this therapy, we edited murine hematopoietic stem and progenitor cells using the CRISPR-Cas9/rAAV6 method and transplanted them in a murine gene knock-out model of human XLA. Following transplant, tissue editing efficiency and engraftment of donor cells was measured via droplet digital PCR. Immunophenotypic analysis of B, T, NK, and myeloid cell frequencies in the spleen, bone marrow, and blood and analysis of mature B cell frequencies from the spleen as well as frequencies of B-progenitors from the bone marrow were performed via FACS analysis. ELISA analysis was performed on serum isolated from peripheral blood to characterize levels of total immunoglobulins as well as responses to immunization via NP-CGG foreign antigen. **Results** Upon transplantation of edited cells into myeloablated XLA mice, high levels of engraftment as well as mature cells from the spleen, blood, and bone marrow with detectable levels of Btk gene integration were detected, being highest in the spleen. Upon

immunophenotypic analysis, there were no significant increases in the frequencies of non-B immune cells (T, NK, Myeloid), confirming that our site-specific editing approach did not result in proliferation of non-target cell types. Edited mice showed significant increases in their B cell levels above that of the XLA mouse model that received no transplant, and not different than XLA mice that received a transplant of WT cells. ELISA analysis showed increased total antibody production of IgG, IgD, IgA, and IgE in edited mice relative to the unedited XLA mouse. The B cell deficiency in XLA is caused by a loss of the progenitor development of early Pre-Pro B cells to immature and mature B cells. Using FACS on bone marrow cells of edited mice, we were able to detect a significant increase in the proportion of immature/mature B cells relative to early pre/pro B cells, elucidating a mechanism for the increases in mature B cells detected in secondary lymphoid tissues. Edited mice displayed antigen-specific primary and secondary humoral responses as evidenced by increases in NP-specific antibodies. **Conclusions** Collectively, we have modeled a gene therapy strategy in a disease model of XLA and thoroughly validated our site-specific genome editing approach. Through extensive peripheral blood, spleen, and bone marrow analysis of edited XLA mouse cells, we have characterized the promising potential of our therapeutic approach. The findings from this study will guide further FDA interactions as we work towards a phase I clinical trial, and serve as further validation for similar monogenic diseases in which site-specific genome editing could be a feasible approach, advancing the field as a whole.

176. Lipid Nanoparticle Delivery of Circular mRNA Improves Hematopoietic Function in Fanconi Anemia HSPCs

Sarah Adams[1], Omar Banda[2], Linah Omer[1], Seul Jung[1], Stefano Rivella[3], Mohamad-Gabriel Alameh[2], Peter Kurre[4]

[1]Comprehensive Bone Marrow Failure, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Department of Medicine, University of Pennsylvania, Philadelphia, PA,[3]Department of Pediatrics, Children's Hospital of Philadelphia; Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[4]Comprehensive Bone Marrow Failure, Children's Hospital of Philadelphia; Perelman School of Medicine; University of Pennsylvania, Philadelphia, PA

Fanconi Anemia (FA) is a recessive multisystem disorder, characterized by defect in DNA repair and symptomatic failure of hematopoiesis early in life. Lentiviral gene therapy has shown promise based on the curative outcomes of allogeneic stem cell transplantation. Still, depleted stem cell pools, poor mobilization, conditioning toxicity, and oligoclonal repopulation continue to limit this approach. Lipid nanoparticle (LNP) delivery of mRNA for protein replacement has successfully been applied to monogenic metabolic disorders. Therefore, we set out to adapt this approach using modified Fancc mRNA lipid nanoparticle (LNP^{Fancc}) delivery to improve function in Fancc -/- hematopoietic stem and progenitor cells (HSPC). Using 5' Clean Cap, 101 poly-A tail methyl-pseudouridine modified linear mRNA constructs, we first performed intravenous and intrafemoral injections in C57B/6 animals and showed mCherry mRNA expression in both hematopoietic and non-hematopoietic cells, that peaked between 6 and 24 hours, remaining detectable for at least 72 hours. To improve durability of expression, we next investigated a circularized mRNA (luciferase, for enhanced sensitivity). Luminescence was detectable for up to 4 days after HSPCs were treated in vitro with linear luciferase mRNA, compared with 7 days after circularized luciferase mRNA. For functional testing we tested circularized Fancc mRNA constructs as LNP cargo, LNP^{Fancc}. To directly investigate FA HSPC function we tested LNP performance in an ex vivo long-term expansion system with LNP dosing on days 7-, 10- and 14. At baseline, we first noted an 18.7% deficit in cell doubling (HSC+MPP1-4) between days 7 and 10 in Fancc-/- HSPC, compared to wild-type (WT). By contrast, when Fancc-/- HSPCs were treated with LNP^{Fancc} we observed an 11.5% increase in doublings between days 7 and 10 from baseline. Importantly, flow-cytometric immunophenotyping of ex vivo expanded cells on day 15 revealed 8.5% fewer Fancc-/- long-term-hematopoietic stem cells (LT-HSC) than WT, a gap that narrowed to 1% after LNP^{Fancc}. Moreover, LNP treatment of FA HSPC in vivo conferred improved progenitor colony formation. FA hematopoietic cells are sensitive to inflammatory cytokines (IFNg, TNFa) and toll like rector (TLR)-8 engagement. Alongside understanding the innate immunogenicity of LNPs as some studies have reported, we wanted to explore if LNP^{Fancc} ameliorated the FA innate immune response upon treatment. Using a commercial PCR array to screen for common immune response targets, we exposed ex vivo expanded cells WT and Fancc-/- HSPC (HSC+MPP1-4) to LNP^{Fancc}. At 6- and 24 hours, we harvested cells, extracted RNA and performed RT-PCR. There was modest upregulation of innate immune targets due to LNP administration in general. More selectively, candidate targets relevant to FA, IL-6, IL-1β, GM-CSF and TLR-8, were substantially downregulated after administration of LNP^{Fancc}. These results were further confirmed by target specific RT-PCR. In conclusion, the data indicate that LNP^{Fancc} delivery is a promising protein replacement approach for temporary functional restoration of FA HSPC function. mRNA delivery provides a potential bridging approach for BM HSPC mobilization (defective in FA patients and mice), and for minimizing losses during ex vivo manipulation in the context of conventional gene therapy.

Ophthalmic and Auditory: Disease Focus

177. Gene Therapy for Clarin-2, a New Deafness Gene in Humans and Mice

Clara Mendia[1], Thibault Peineau[2], Chloé Felgerolle[1], Nawal Yahiaoui[1], Carlos Aguilar[3], Michael R Bowl[3], Didier Dulon[2], Aziz El-Amraoui[1], Sandrine Vitry[4]

[1]Institut de l'Audition / Institut Pasteur, Paris, France,[2]Université de Bordeaux, Bordeaux, France,[3]UCL Ear Institute, London, United Kingdom,[4]Institut Pasteur, Paris, France

Hearing impairment is one of the most prevalent sensory disorders worldwide, affecting social interaction, cognition, education and employment, and increasing the risk of dementia. Current treatments are limited to cochlear implants, unable to restore quality hearing function. Adeno-associated virus (AAV)-mediated gene therapy has emerged as a promising alternative, though some key hurdles remain. We used Clarin-2 deficient mice, a transgenic model of progressive hearing loss, to address some of these challenges, namely

the effective window for intervention and the long-term efficacy of AAV-mediated gene supplementation. We delivered AAV9-PHP.eB coding for human or murine *Clarin-2* locally into the inner ear of *Clrn2*$^{-/-}$ mice. Injections were done at postnatal day 1, 5, 10 or 14 and beneficial outcomes assessed up to 6 months after treatment. Gene transduction efficiency was evaluated by qRTPCR, RNAscope and confocal microscopy. Scanning electron microscopy was used to monitor the architecture of hearing sensory hair cells. Auditory brainstem responses (ABR), distortion product otoacoustic emissions (DPOAE), along with mechano-electrical transduction (MET) and capacitance measurements were used to assess hearing sensitivity, hair cell MET and synaptic activity. Our AAV injections lead to robust transgene expression in inner and outer hair cells, and prevent all the defects described in the *Clrn2*$^{-/-}$ inner ear. The structure of sensory hair bundles is preserved, as is the function of their MET channels and synapses. The ABR and DPOAE thresholds of treated mice remain normal over time. We found that early neonatal injections allowed for the best therapeutic outcomes, with effectiveness decreasing with intervention age. To confirm the pathogenicity of the c.494C>A *CLRN2* mutation detected in deaf patients, we demonstrated that, unlike the wild-type human allele, its variants T165K and 146fs*26 were unable to rescue hearing in *Clrn2*$^{-/-}$ mice. Our work shows that AAV-mediated gene supplementation substantially and durably preserves normal hearing when applied before symptom onset, underscoring the promising potential of gene therapy for auditory restoration. *This study was recently accepted for publication in Molecular Therapy (DOI: 10.1016/j.ymthe.2024.01.021).*

178. *In-Vitro* and *In-Vivo* Studies of HG202 as a CRISPR/Cas13 RNA Targeting Therapy for Macular Degeneration

Weiwei Wu, Qi Yang, Sheng He, Mengke Zhu, Chenhua Yang, Shaoran Wang, Xing Wang, Xuan Yao, Hui Yang, Alvin Luk, Linyu Shi

HuidaGene Therapeutics Co., Ltd., Shanghai, China

Background: The current gold-standard treatment for neovascular age-related macular degeneration (nAMD) is frequent intravitreal injections of anti-VEGF agents, leading to suboptimal patient adherence and elevated risk of complications. Moreover, a considerable proportion of nAMD patients have a poor response to standard anti-VEGF therapy. To address these issues, we have developed a novel RNA-targeting therapy, HG202, which is a single adeno-associated virus (AAV) vector incorporating high-fidelity CRISPR/Cas13 RNA targeting technology to specifically and effectively suppress the expression of VEGFA in the retina for nAMD treatment. **Methods:** Choroidal neovascularization (CNV) was induced in mouse eyes through laser photocoagulation. Seven days after injury, fundus fluorescein angiography (FFA) and optical coherence tomography (OCT) were performed, followed by immune-stained choroidal flatmounts. We evaluated the *in vitro* and *in vivo* editing efficiency of HG202, quantified the suppression of *Vegfa* mRNA expression in the retina, and measured the reduction in laser-induced CNV area. We compared the efficacy of HG202 with AAV-anti-VEGF and Aflibercept and conducted a toxicity study in non-human primates (NHPs) to evaluate the safety of HG202. **Results:** After injecting HG202, over 40% of *Vegfa* mRNA expression in the retina of mice (n=8) was suppressed. Mice treated with HG202 showed an 87% reduction ($P < 0.0001$, $n = 14$) in the area of laser-induced CNV. In comparison, AAV-anti-VEGF achieved a 70% reduction ($P < 0.01$, $n = 14$) when compared to the vehicle control, while Aflibercept showed a 47% reduction ($P < 0.01$, $n = 18$) when compared to HG202. In the mouse model, a single injection of HG202 resulted in a durable therapeutic effect for at least 13 weeks. No systemic toxicity and immunotoxicity were observed in NHPs. Only the high-dose group (Figure 1) showed moderate fluorescence leakage and retinal structure changes. There were no obvious abnormalities in retinal function observed in any of the tested animals. **Conclusion:** By successfully blocking VEGFA signaling, HG202 demonstrates long-term transgene expression and RNA editing after a single injection, thus eliminating the need for monthly injections and providing enduring therapeutic benefits. Therefore, HG202 has the potential to provide an effective and long-lasting treatment option for patients with nAMD who do not respond well or are resistant to anti-VEGF therapy. HG202 as a CRISPR RNA-targeting strategy is currently being investigated in humans.

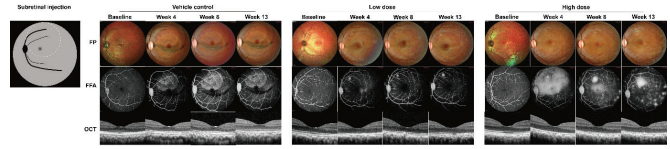


Figure1 Ophthalmological examinations after subretinal delivery of HG202 at different doses in NHPs.

179. AAV-Mediated RhoA Knockdown as a Potential Gene Therapy for Glaucoma

Tapan Sharma[1,2], Meng Gao[1,3], Bo Tian[1,3], Haijiang Lin[1,3], Jun Xie[1,2,4], Guangping Gao[1,2], Phillip Wl Tai[1,2]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA,[3]Department of Neurobiology, UMass Chan Medical School, Worcester, MA,[4]Viral Vector Core, UMass Chan Medical School, Worcester, MA

Glaucoma is one of the leading causes of adult blindness and affects about 80 million individuals globally. In primary open angle glaucoma (POAG), one of the high-risk factors that aggravates blindness is the accumulation of aqueous humor (AH), which results in increased intraocular pressure (IOP) and causes damage to the optic nerve. The cells of the ciliary body synthesize AH, while natural drainage happens through Schlemm's canal and layers of the trabecular meshwork (TM). All current glaucoma treatments aim to decrease the secretion and increase the outflow of AH to reduce IOP. Rho kinase (ROCK) inhibitors are a new class of glaucoma drugs, which act on the trabecular tissue to increase AH outflow. RhoA is a ubiquitously expressed GTPase that is known to regulate actin polymerization/depolymerization. Activated GTP-bound RhoA can associate with serine/threonine kinases of the ROCK1/2 family and can assist with phosphorylation of a variety of substates, such as myosin light chain (MLC) and myosin phosphatase target subunit (MYPT-1), which contribute to increased actin polymerization, contraction of the TM, and resistance to AH outflow. Using artificial microRNAs (amiRs), we aimed to target RhoA expression. We screened eleven seed sequences targeting the coding sequence (CDS) of RhoA and its 3'-UTR in human trabecular meshwork (hTM1) cells. Three amiR candidate designs displayed reduced expression of the target gene at the protein level (60-80% reduction). Evaluation of off-target effects

using small-RNA sequencing showed that endogenous microRNAs were largely unaffected. We also found that transduction of TM cells via intracameral injection by AAV2 outperformed those of AAV3b, AAV6, and AAV8; thus, the delivery of *amiR-RhoA-10* by AAV2 was chosen as the final vector strategy. We demonstrate that when scAAV2-*amiR-RhoA-10* is administered intracamerally to wildtype mice (7.33E8 vg/eye), mean IOP was dramatically reduced within three weeks of dosing (from 16.5 mmHg to 14.5-15.0 mmHg) and remained so for over a period of twelve weeks. Furthermore, the observed reduction was also dose dependent.In summary, our proof-of-concept study demonstrates that AAV-mediated knockdown of RhoA using amiRs can reduce intraocular pressure in mice, showing significant promise as a gene therapy for treating primary open angle glaucoma. J.X., G.G. and P.W.L.T. are corresponding authors

180. AAV-Mediated Delivery of a Novel CasX-Editor Molecule Achieves Allele-Specific and Potent Editing of P23H Rhodopsin in a Mouse Model of Retinitis Pigmentosa

Cécile Fortuny[1], Lewis E. Fry[2], Michelle E. McClements[2], Sean Higgins[1], Isabel Colin[1], Angelica Castaneda[1], Ross White[1], Maroof M. Adil[1], Imran H. Yusuf[2], Sarah K. Denny[1], Brett T. Staahl[1], Robert E. MacLaren[2], Benjamin Oakes[1]

[1]Scribe Therapeutics Inc, Alameda, CA,[2]Oxford University, Oxford, United Kingdom

Inherited retinal dystrophies are a heterogeneous group of progressive blinding genetic diseases caused by mutations in over 200 genes, resulting in a strong phenotypic convergence towards the gradual loss of photoreceptors. Autosomal dominant retinitis pigmentosa (adRP) is a genetic disorder that results in progressive vision loss and blindness and is commonly induced by mutations in the RHO gene, with the P23H gain-of-function mutation being a highly prevalent causative mutation. To date, effective therapy for RHO-based adRP remains a significant unmet need. One strategy to address P23H. RHO-related adRP is the selective removal of the mutant Rho protein while preserving its wild-type (WT) counterpart, rescuing affected rod photoreceptors, and ultimately preventing vision loss. Here, we demonstrated for the first time a novel gene editing strategy using a highly engineered CasX and gRNA molecule called the X-Editor (XE) to allele-specifically edit the mutant RHO locus in rod photoreceptors, converting the P23H missense into a null variant without affecting the WT RHO allele both in vitro and in vivo. We first showed that XE, delivered by all-in-one AAV vectors, was able to edit efficiently a reporter locus in rod photoreceptors selectively when administered subretinally at a therapeutically relevant dose. We next examined the effects of XE-mediated allele-specific editing of the mutant P23H. RHO allele on retinal structure and function in RHOP23H/+ mice. These results demonstrated the first AAV-mediated delivery of an engineered XE in vivo to achieve allele-specific editing of the P23H. RHO locus resulting in therapeutic benefit. Further engineering of XE subsequently enhanced therapeutic potency. Our study illustrates the potential for XE to treat P23H.RHO-related adRP, and more broadly, other forms of autosomal dominant disorders in an allele-specific manner.

181. LX103 a Novel Recombinant AAV Gene Therapy for Intravitreal Treatment of X Linked Juvenile Retinoschisis

Zhuoyu Ni[1], Fei Wu[1], Junran Sun[2], Ping Gan[1], Ying Zhu[1], Yang Zhou[1], Linhao Lu[1], Yang Liu[1], Xiaodong Sun[2], Fenghua Wang[1]

[1]Innostellar Biotherapeutics, Shanghai, China,[2]Shanghai General Hospital, Shanghai, China

X-linked juvenile retinoschisis (XLRS) is an early onset retinal degenerative disease involving abnormal schisis through the retinal neurosensory layers that leads to vision impairment. It is a rare X-linked monogenic disease caused by mutations in the RS1 gene which is normally expressed in photoreceptors and bipolar cells and secreted to intercellular space as a mediator for cell-cell adhesion. LX103 is a novel recombinant adeno-associated virus (rAAV) vector carrying normal human retinoschisin RS1 gene. Its vector is a novel Capstellar capsid RC-C08 engineered from wild type AAV2. RC-C08 capsid possesses much better ILM (inner limiting membrane) penetration and retinal cell transduction capability and lower immunogenicity. Via intravitreal (IVT) administration, LX103 can effectively deliver and express RS1 gene in expected retinal cells in both rodent and large animal models. When rs1-/- knockout mice were treated with LX103, correct RS1 protein expression and retina structural improvement were observed in a dose-dependent manner. To evaluate the safety and efficacy of LX103 as a potential therapeutic clinical intervention, a cohort of XLRS patients (n=6) was enrolled in a single-arm, open-label investigator-initiated trial (IIT). Fundus examinations, visual acuity, visual fields, electroretinography (ERG), and optical coherence tomography (OCT) were employed to monitor both safety and efficacy outcomes. Following intravitreal injection of LX103 (1 month to a year), no serious ocular or systemic AEs were encountered, and no patient experienced vision decrease. 3-month follow-up analysis (n=5) demonstrated a substantial enlargement in dynamic visual field and an increase in ETDRS BCVA in all subjects, and different levels CST decrease and structural ameliorations in the macular EZ intensity. Overall, LX103 was developed with a novel engineered AAV capsid that can efficiently deliver RS1 gene via intravitreal injection. Both the animal studies and the IIT clinical study highlight the promising potential of LX103 as a novel therapeutic approach for XLRS. Further research and clinical studies are warranted to verify these initial results.

182. AAV Vectorized Antibody Fragment-Mediated Dual Complement Pathway Inhibition for the Treatment of AMD

Suzanne B. R. Jacobs[1], Jennifer Sullivan[1], Amy Frederick[1], Ronald Gorham[2], Michael Charewycz[2], Matthew A. Adamowicz[1], Soumya Nair[2], Ken Simmons[2], Anna Park[3], Hendrik Wesseling[4], Ingeborg Langohr[5], James E. Morris[1], Michael Storek[2], Vaishnavi Rajagopal[2], Christian Mueller[1], Catherine O'Riordan[1]

[1]Genomic Medicine Unit, Sanofi, Waltham, MA,[2]Immunology and Inflammation Research, Sanofi, Cambridge, MA,[3]Large Molecules Research, Sanofi, Cambridge, MA,[4]Precision Medicine and Computational Biology, Sanofi, Cambridge, MA,[5]Translational Models Research Platform, Sanofi, Cambridge, MA

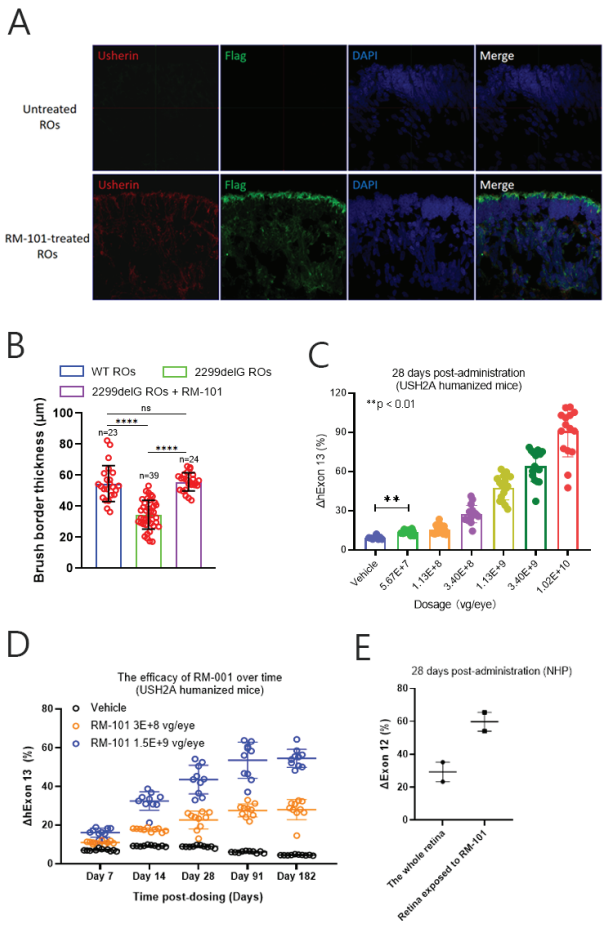
Age-related macular degeneration (AMD) is a common, complex disease that is the leading cause of central vision loss in individuals 60 years of age and older. In geographic atrophy (GA), an advanced form of AMD, metabolic and inflammatory stresses in the retina lead to the death of photoreceptors and retinal pigment epithelial cells, the appearance of atrophic lesions, and eventual blindness. Human genetic variants in complement pathway genes are among the most common and strongest genetic risk factors associated with AMD and implicate roles for both the classical and alternative pathways in disease pathogenesis. Here, we describe an AAV-mediated gene therapy approach to inhibit proximal steps in both the classical and alternative complement pathways through sustained expression of vectorized antibody fragments targeting active C1s (aC1s) and Factor Bb in the eye. In addition to its benefit as a potential one-time treatment for GA, this bifunctional strategy has potential to show improved efficacy compared to approaches that target downstream components in the complement pathway because it will broadly inhibit both proximal and terminal mediators of inflammation, phagocytosis, and membrane attack complex-mediated cell lysis. To inhibit both the classical and alternative pathways, we designed a novel AAV vector AAV-aC1s$_{scFab}$-Bb$_{scFab}$ that co-expresses anti-aC1s and anti-Bb single-chain antibody fragments (scFabs). The vector derived scFabs demonstrate target engagement and inhibitory activity for their respective targets and pathways *in vitro*. AAV-aC1s$_{scFab}$-Bb$_{scFab}$ was evaluated *in vivo* following intravitreal (IVT) administration in wild-type C57BL/6 mice and cynomolgus monkeys. At multiple dose levels and timepoints following IVT delivery, the AAV-aC1s$_{scFab}$-Bb$_{scFab}$ vector transduces the retina and expresses transcripts encoding both scFabs. Vector-derived scFabs secreted from transduced cells are detectable in the aqueous and vitreous humors, maintain target engagement capacity, and improve clinical indicators in an NHP model of ocular inflammation. Overall, these data demonstrate that dual complement pathway inhibition through IVT delivery of vectorized antibody fragments has potential as a one-time treatment for GA secondary to AMD.

183. Pre-Clinical Study of RM-101, a Novel AAV-Based Gene Therapy for *USH2A*-Related Retinitis Pigmentosa

Jiayu Ou[1], Jiaqi Tu[1], Hong Zhang[1], Fazhou Zhang[1], Kun Wu[1], Yan Li[1], Hui Xu[1], Zhendong Xiao[2], Junjiu Huang[3], Junbin Liang[1]

[1]Reforgene Medicine, Guangzhou, China,[2]Biotherapy Center, The Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China,[3]School of Life Sciences, Sun Yat-sen University, Guangzhou, China

Introduction: Mutations in the *USH2A* gene are the leading cause of autosomal recessive retinitis pigmentosa and Usher syndrome, with exon 13 mutations being the most common. Previous studies have demonstrated that the removal of exon 13 from the *USH2A* gene can lead to the production of a functional protein, suggesting that exon skipping could be a potential treatment for retinal degeneration associated with *USH2A* mutations. Here, we reported RM-101, a novel strategy based on adeno-associated virus (AAV) for exon skipping, aimed at treating retinal degeneration caused by *USH2A* exon 13 mutations. **Methods:** We developed a U7 SmOPT snRNA that specifically targets *USH2A* exon 13 and encapsulated it into an AAV delivery system (RM-101). The safety and efficacy of RM-101 were evaluated in retinal organoids (ROs), *USH2A* humanized mice, and non-human primates. The ROs used in our study contained a biallelic c.2299delG mutation in the *USH2A* gene, along with a flag tag inserted before the stop codon. The *USH2A* humanized mouse model was created by replacing the mouse *Ush2a* exon 12 and its flanking sequences with a human *USH2A* exon 13 carrying the c.2802T>G mutation and the flanking sequences. **Results:** In ROs treated with RM-101, the skipping of exon 13 in *USH2A* transcripts was 52.8% ± 12.5%, while no exon 13 skipping was observed in control samples. Immunohistochemistry demonstrated that the expression of Usherin protein was restored in RM-101-treated ROs with correct subcellular localization (Fig. 1A), and the deficiency of brush border was rescued (Fig. 1B). In *USH2A* humanized mice treated with RM-101, *USH2A* exon 13 skipping was significantly increased in a dose-dependent manner (Fig. 1C) and efficacy was sustained over time (Fig. 1D). A single subretinal administration of RM-101 (5E+10 vg/eye) in cynomolgus monkeys resulted in 24.3% exon 12 skipping detected in *USH2A* transcripts from cells throughout the retina, and that was 59.8% in cells of the retinal region directly exposed to RM-101 (Fig. 1E). In a GLP toxicity study in 30 rhesus monkeys, no RM-101-related abnormalities were observed during the 13-week observation period. RNA-Seq analysis of retinal organoids and monkey retina and choroid samples confirmed that RM-101 specifically induced skipping of the target exon and no significant off-target splicing was found. **Conclusions:** Our results demonstrated that RM-101 can effectively induce *USH2A* exon 13 skipping both *in vitro* and *in vivo*, and it has an acceptable safety profile. These preclinical studies provide support for RM-101 as a potential gene therapy for the treatment of *USH2A*-related retinal degeneration.



CAR T-cell Therapies

184. Innovative Applications of CAR T-Cell Therapy in Organ Transplant Patients

Yan Luo[1,2], Tanya Hundal[1], Rose Mary Attieh[3], Shuhua Li[1], Yaqing Qie[1], Martha Gadd[1], Mao Shennen[3], Burcin C. Taner[3], Mohamed A. Kharfan-Dabaja[4,5], Tambi Jarmi[3], Hong Qin[1,2,4,6]

[1]Regenerative Immunotherapy and CAR-T Translational Research Program, Mayo Clinic Jacksonville, Jacksonville, FL,[2]Department of Cancer Biology, Mayo Clinic, Jacksonville, FL,[3]Department of Transplantation, Mayo Clinic Jacksonville, Jacksonville, FL,[4]Department of Internal Medicine, Mayo Clinic Jacksonville, Jacksonville, FL,[5]Blood and Marrow Transplantation and Cellular Therapy Program, Mayo Clinic, Jacksonville, FL,[6]Department of Immunology, Mayo Clinic, Jacksonville, FL

Anti-HLA donor specific antibodies have been extensively documented for their critical role in organ transplant rejection and resulting adverse outcomes. Several approaches have been employed to desensitize these patients; however, none of these explored therapeutic approaches has exhibited enduring clinical benefits. In this proof-of-principle study, we explore a novel strategy of utilizing chimeric antigen receptor T cells (CAR T-cells) to target B cells in sensitized organ transplant recipients. Specially, we assessed our innovative MC10029 CAR T-cells, which are designed to target the B cell activating factor receptor (BAFF-R), for their cytotoxicity against patient autologous B cells. Ten sensitized patients were enrolled in the study, with their B cells confirmed for BAFF-R expression. Employing our optimized protocol for CAR T-cell generation from minimal blood samples, we successfully produced patient-specific MC10029 CAR T-cells for all participants. Among the seven patients with adequate autologous B cells, consistent antigen-specific cytotoxicity of their CAR T-cells against autologous B cells was observed, accompanied by cytotoxic granule release. For the remaining three patients with insufficient autologous B cell availability, we demonstrated the antigen-specific cytotoxicity of their CAR T-cells using a set of BAFF-R-positive and BAFF-R-negative target cells. The proof-of-principle data from this study pave the way for the pioneering the first-ever application of CAR T-cell therapy in sensitized patients waiting for life-saving organ transplants.

185. Discovering Novel Transcriptional Regulators of T Cell Exhaustion for Epigenetic Reversal of T Cell Dysfunction

Christian McRoberts Amador[1], Aretha Gao[2], Sean McCutcheon[3], Charles A. Gersbach[3]

[1]Pharmacology and Cancer Biology, Duke University, Durham, NC,[2]Biology, Duke University, Durham, NC,[3]Biomedical Engineering, Duke University, Durham, NC

T cells are part of the immune system's adaptive defense; they specifically target and kill both virally infected and cancerous cells through antigen recognition. Unfortunately, cancer can exploit intrinsic T cell mechanisms to survive attack by the immune system. T cell exhaustion arises from chronic antigen stimulation, which shifts a portion of the T cell population to the exhausted state (T_{EX}), resulting in diminished T cell proliferation and tumor/viral clearance. Modern genome engineering technologies have the potential to dramatically advance T cell therapy by programming T_{EX} cells into desirable phenotypic states. The transcription factor (TF) TOX drives and maintains the T_{EX} state through epigenetic regulation of exhaustion-associated programs. To recapitulate T cell exhaustion *in vitro*, we repeatedly stimulated primary human T cells with anti-CD3/CD28 beads to mimic chronic stimulation and confirmed induction of the exhaustion program via RNA- and ATAC-seq. Specifically, multiple genomic regions within the TOX locus became differentially accessible with *in vitro* chronic stimulation. Through a CRISPR interference (CRISPRi) screen tiling the TOX locus, we confirmed modulation of TOX expression by targeting these accessible non-coding regions, emphasizing the highly regulated epigenetic control of TOX downstream of chronic stimulation. To understand what other TF regulators might be driving or preventing T cell exhaustion upstream of TOX, we designed two screens using a CRISPR activation (CRISPRa) approach targeting all genes encoding TFs in the human genome or an Open Reading Frame (ORF) library encoding all the TFs in the human genome with TOX protein expression as a readout. CD8+ T cells from three distinct donors were used in an acute stimulation setting (one TCR stimulation with anti-CD3/CD28 beads) and a chronic stimulation setting (two additional stimulations). Comparing screen results in the

context of acute versus chronic stimulation facilitated identification of factors specific to the TEX state. The ORF screen yielded more TF hits that were modulating TOX expression than the CRISPRa screen (3 gRNA hits versus >100 ORF hits). Over 100 TF ORFs were enriched in TOX-high and TOX-low bins, including both known and novel regulators of TOX. Individual validations of top enriched TFs unique to chronic stimulation confirmed modulation of TOX expression in the expected direction at the protein and transcript level. FACS staining of exhaustion (LAG3, PD-1, and TIGIT) and memory (IL7R) surface markers revealed significant differences in expression levels, suggesting potential phenotypic changes in T cell function. Ongoing work is focused on validating the effects of these TFs on T cell function (T cell killing of cancer cells and cytokine expression assays), transcriptome (RNA-seq), and epigenome (ATAC-seq). In addition, we are testing these candidate TFs to reprogram tumor-infiltrating lymphocytes from primary lung tumor resections. Through epigenetic reprogramming, we hope to achieve clinically relevant T cell phenotypes to improve current immunotherapies in both viral infection and cancer contexts.

186. T-Cell Epigenetic Reprogramming with TGIF2LX-Overexpression Enhances Adoptive Cell Therapy

Tomer Rotstein[1], Mike Brown[2], Sean McCutcheon[3], Charles A. Gersbach[4]

[1]Biomedical Engineering, Duke University, Durham, NC,[2]Neurosurgery, Duke University, Durham, NC,[3]Duke University, Apex, NC,[4]Duke University, Durham, NC

Memory T-cells exhibit distinct functional characteristics depending on their location within the body. Recent advancements have unveiled a significant population of memory T-cells residing in non-lymphoid tissues. These resident memory T-cells are critical for combating infections and their abundance has a positive correlation with improved survival rates across many solid tumor types. Motivated by this observation, we aimed to harness the potential of resident memory T-cells for adoptive cell therapy. However, isolating these cells from donor-derived tissues is impractical. Therefore, we focused on epigenetic reprogramming of blood-derived T-cells towards a tissue resident state. We conducted two cDNA overexpression screens, using open reading frames (ORFs) encoding all known transcription factors, in human primary CD8 T-cells to identify genetic regulators of *CD103* (a residency marker) and *IL7R* (a memory marker). The intersection of these screens identified the transcription factor *TGIF2LX*, capable of upregulating both markers. Interestingly, this transcription factor is only expressed in male reproductive tissues, although other TGIF family members have been implicated in T-cell function. Bulk RNA-sequencing revealed that *TGIF2LX* overexpression in T-cells leads to widespread transcriptional reprogramming, including activation of genes associated with increased tissue residency programs (e.g. *ITGAE*, *CD69*, *CXCR6*) and decreases in genes associated with circulation programs (e.g. *KLF2*, *S1PR1*). We further characterized *TGIF2LX*-overexpressing T-cells using a multimodal single cell readout, pairing scRNA-seq with a CITE-seq library of 134 unique cell surface markers. This demonstrated a distinct cell state with *TGIFL2X*-overexpressing T-cells existing in clusters unique from those containing unenhanced cells. Of note, these cells also clustered separately from T-cells overexpressing RUNX3, a transcription factor previously identified to enhance tissue residency and improve tumor control, suggesting a novel axis for controlling tissue residency. We tested the effect of TGIF2LX overexpression in modulating CAR-T efficacy in a human breast cancer xenograft model. HCC1954 breast cancer cells were implanted orthotopically into the mammary fat of NSG mice. After 10 days, HER2-targeting CAR-T cells were administered intravenously at a subcurative dose (5x10^5 cells). TGIF2LX-overexpression dramatically improved tumor control by CAR-T cells relative to unmodified control and RUNX3-overexpressing CAR-Ts. These data provide proof-of-concept of a novel strategy for enhancing adoptive cell therapies for solid tumors.

187. Identification of Exhaustion-Associated Genes by Non Viral Editing of Human CD8+ T Cells

Brandon W. Simone[1], Leonel Torres[1], Junwei Shi[2], E. John Wherry[3], Saar Gill[1]

[1]Center for Cellular Immunotherapies, University of Pennsylvania, Philadelphia, PA,[2]Epigenetics Institute, University of Pennsylvania, Philadelphia, PA,[3]Institute for Immunology and Immune Health, University of Pennsylvania, Philadelphia, PA

T cell exhaustion is a hypofunctional state caused by chronic antigen exposure, characterized by decreases in cytotoxicity, proliferation, and cytokine production. Immunotherapies such as Chimeric Antigen Receptor (CAR-T) cells and bispecific T-cell engagers (BiTEs) have been transformative in the treatment of B-cell malignancies, resulting in durable remission in approximately 50% of patients. A prevailing hypothesis explaining CAR-T and other immunotherapy failures implicates T cell exhaustion. Current approaches thought to mitigate T cell exhaustion include monoclonal antibody-based blockade of inhibitory receptor (IR) signaling, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) or the programmed death-1 (PD-1)/PD-L1 axis. It was recently estimated that approximately 40% of US cancer patients may be eligible for immune checkpoint inhibitors (ICI), but that the overall response rate is approximately 10%. Thus, while ICI therapy has been transformational and possibly curative for some patients, ultimately only a minority of patients benefit. It is now thought that ICI largely act to prevent the transition from T_{pex} to T_{ex}, and even genetic interventions such as transcription factor deletion (e.g. *TOX*) do not appear to reverse truly exhausted cells. Thus, genes with a role in reversing established exhaustion are yet to be described. To overcome the mechanistic and therapeutic gaps in T cell exhaustion, we developed an *in vitro* T cell exhaustion assay coupled with non-viral cell-penetrating peptides to deliver CRISPR reagents to already-exhausted cells. Our system enables the screening and identification of the underlying genetic effectors and mechanisms of T cell exhaustion and facilitates the identification of targets capable of reversing T cell exhaustion. In this work we demonstrate that a blinatumomab-based *in vitro* exhaustion assay results in phenotypic (increased PD1, CTLA-4, TOX expression) and functional (decreased cytokine secretion and proliferation in response to antigen) alterations consistent with T cell exhaustion. We show that *in vitro* exhausted cells are amenable to non-viral gene editing using cell penetrating peptides combined with CRISPR nucleases, enabling the identification of genes associated with reversal (rather than prevention) of T cell exhaustion. We used the amphiphilic peptide A5K coupled with Cas9 protein to

efficiently knockout exhaustion-associated genes such as *PRDM1* in phenotypically exhausted T cells. In addition to targeted gene disruption, we show that our system is compatible with off-the-shelf CRISPR/Cas9 sgRNA libraries and enables forward genetic screening for identifying exhaustion-associated genes. Finally, we demonstrate an orthogonal approach using cell-penetrating Cas12a to demonstrate efficient gene editing in exhausted T cells. Taken together, this work enables editing of already-exhausted T cells by leveraging non-viral CRISPR effectors to identify genes associated with reversal, rather than prevention, of T cell exhaustion by targeted or pooled gene editing.

188. CRISPR Engineering of Armoured CAR T Cells Enables Tumour-Restricted Payload Delivery with Enhanced Safety and Efficacy

Amanda X. Y. Chen, Kah Min Yap, Phillip K. Darcy, Imran G. House, Paul A. Beavis

Cancer Immunology Program, The Peter MacCallum Cancer Centre, Melbourne, Australia

INTRODUCTION: Chimeric antigen receptor (CAR) T cell therapy, while highly efficacious for the treatment of certain haematological malignancies, remains ineffective in solid cancers. This can be attributed to various factors, including immunosuppression in the tumour microenvironment, tumour antigen heterogeneity and limited trafficking of CAR T cells into the tumour. "Armouring" CAR T cells to express anti-tumour factors such as proinflammatory cytokines is a promising strategy to address their limitations in the solid tumour setting and engage endogenous anti-tumour immunity, which we have demonstrated to be critical for the treatment of heterogeneous solid cancers[1]. However, conventional armoured CAR T cells utilise a synthetic promoter to drive cytokine expression, which leads to unrestricted cytokine production and can cause severe clinical toxicities[2]. We aimed to use CRISPR/Cas9-mediated homology directed repair (CRISPR-HDR) to achieve expression of proinflammatory cytokines under the control of endogenous tumour-restricted promoters, and hypothesised that this would enhance the safety and efficacy of armoured CAR T cells. **METHODS:** Genome-wide RNA sequencing was performed in both a syngeneic murine CAR T cell model and a xenogeneic human CAR T cell model to identify genes that are specifically upregulated in CAR T cells within the tumour. Tumour specificity of candidate target genes was assessed *in vivo* through HDR-mediated insertion of GFP into each locus followed by comparison of GFP expression between CAR T cells within the tumour and peripheral sites. Following identification of ideal target genes, a panel of proinflammatory cytokines was inserted into these loci, and the safety, therapeutic efficacy and mechanism of the engineered T cells was evaluated in 2 immunocompetent murine tumour models and 1 xenograft human tumour model. **RESULTS:** Through assessment of 27 potential target genes, *NR4A2* was identified as the top candidate for enabling stringent tumour-restricted payload expression. Strikingly, expression of IL-12 via the endogenous *NR4A2* promoter mediated robust therapeutic responses in the absence of toxicities, in contrast to promoters with higher peripheral expression such as that of *PD-1*. Additionally, NR4A2/IL-12-engineered CAR T cells exhibited enhanced cytotoxic and proliferative capacity as well as the ability to stimulate endogenous anti-tumour immunity. We further demonstrate that our approach enables superior tumor-restricted expression of payloads compared to currently utilised synthetic systems such as the NFAT-inducible promoter system. **CONCLUSION:** Tumour-localised delivery of potent payloads is a significant challenge in developing safe and effective cancer therapies. By leveraging endogenous tumour-restricted promoters to drive transgene expression, we have engineered armoured CAR T cells to deliver proinflammatory payloads directly to the tumour site, enabling robust anti-tumour effects while minimising systemic toxicities. **REFERENCES:** 1. Lai J, Mardiana S. et al. Nat Immunol. 2020 Aug;21(8):914-926. doi:10.1038/s41590-020-0676-7. 2. Zhang L. et al. Clin Cancer Res. 2015 May 15;21(10):2278-88. doi: 10.1158/1078-0432.CCR-14-2085.

189. Preclinical Specificity and Potency Evaluation of a Novel CD19-Specific KIR-CAR T Cell Therapy (SynKIR-310)

Nora Yucel[1], Selene Nunez-Cruz[2,3], John Leferovich[2,3], Gayathri Gulendran[2,3], Megan Blair[1], Chune Zhang[2,3], William Stanley[1], Jun Xu[1], Laura Johnson[1], Don Siegel[2,3], Michael C. Milone[2,3]

[1]Verismo Therapeutics, Philadelphia, PA,[2]Department of Pathology and Laboratory Medicine, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[3]Center for Cellular Immunotherapies, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

Single-chain CD3-based chimeric antigen receptors (CARs) targeting CD19 with FMC63-derived single-chain variable fragment (scFv) show remarkable clinical responses in patients (pts) with B-cell malignancies. Despite high response rates in diffuse large B cell lymphoma, about a third of pts do not respond, and of those who do, around a third relapse. Therapies that improve CAR T cell function are thus critical. Previously we developed a multi-chain mesothelin-specific killer immunoglobulin-like receptor (KIR)-CAR T cell therapy, SynKIR-110, with enhanced anti-tumor function and durability in a murine xenograft model of human mesothelioma [1, 2]. SynKIR-110 is currently being investigated in a Phase I trial, STAR-101 (NCT05568680) for pts with mesothelin-positive solid tumors. Using our KIR-CAR platform we next developed a novel CD19-specific KIR-based CAR T cell therapy for CD19-positive hematologic malignancies. To identify an optimal anti-huCD19 scFv for KIR-CAR we screened a highly diverse 40-billion member canine scFv phage display library. 19 human CD19-specific scFvs were identified that competed with the clinically validated FMC63 scFv. KIR-CARs were constructed with candidate scFvs, and used to transduce primary T cells. We compared in vitro and in vivo potency of these unique KIR-CAR T cells with FMC63-KIR-CAR T cells. In traditional in vitro potency assays such as antigen (ag)-specific tumor lysis and cytokine secretion, we saw no differences across KIR-CAR T cells. However while other binders demonstrated less effective or durable tumor control in a NSG xenograft mouse relapse model of B-cell leukemia NALM6, the selected DS191 had comparable in vivo anti-tumor efficacy to FMC63. Thus SynKIR-310 was generated in human T cells with a lentiviral vector to express DS191 in the KIR-CAR platform for further characterization. Ag sensitivity of SynKIR-310 was compared to FMC63-KIR-CAR T in vitro using target cells with a 5 log-fold range of CD19 expression. SynKIR-310 and FMC63-KIR-CAR T cells showed comparable dose-dependent, ag-specific activation, as well as specificity and sensitivity. Anti-tumor efficacy was next assessed in vivo. Using increasing doses of cells, both SynKIR-310 and FMC63-

KIR-CAR T cells showed similar dose-dependent anti-tumor efficacy. In a membrane proteome array of 5,220 unique human proteins and 243 heterocomplexes, DS191 showed no off-target binding, supporting CD19 specificity. Co-culture of SynKIR-310 with a panel of CD19-negative primary cell types from normal tissues showed no evidence of off-target cytotoxicity, supporting minimal cross-reactivity. Together, these data demonstrate high CD19 ag sensitivity, specificity, and potency of DS191 scFv, that combined with the prolonged functionality of KIR-CAR, supports SynKIR-310 as a promising T cell therapy against CD19-positive malignancies. Finally, these results suggest that current in vitro assays do not sufficiently correlate to in vivo potency, highlighting the need for improved in vitro potency assays for CAR T preclinical development. 1.Wang, E., et al., Generation of Potent T-cell Immunotherapy for Cancer Using DAP12-Based, Multichain, Chimeric Immunoreceptors. Cancer Immunol Res, 2015. 3(7): p. 815-26.2.Xu, J., et al., 321 Preclinical potency assessment of SynKIR-110, a mesothelin-specific KIR-CAR T cell therapy for mesothelioma. Journal for ImmunoTherapy of Cancer, 2023. 11(Suppl 1): p. A365-A365.

190. *In Vivo* Generation of αCD19-CAR T Cells Using a Novel LV-based Platform Successfully Clears Advanced NALM-6 Tumor without Noticeable Toxicity

Karina Krotova[1], Gopal Naik Nenavath[1], Nandakumar Packiriswamy[1], Yumeng McDaniel[1], Rianna Vandergaast[1], Chia-Hsuan Chin[1], Kyle Gromer[1], Luke Schnebec[1], Zachary Larson[1], Kah-Whye Peng[2], Stephen J. Russell[2]

[1]Imanis Life Sciences, Rochester, MN,[2]Vyriad, Rochester, MN

In the past few years, there has been a surge of interest in generating CAR-T cells directly in patients through a straightforward injection of vector containing the CAR transgene. This "in vivo" approach has the potential to streamline the treatment procedure and decrease its overall cost. Imanis Life Sciences and Vyriad recently developed a new platform for efficient in vivo T cell-specific delivery. It is based on lentiviral vectors pseudotyped with LDLR detargeted VSV-G glycoproteins incorporating a CD3-specific single-chain antibody fused at the N-terminus (G-CD3). Targeting T cells by G-CD3-LV was first confirmed in ex vivo experiments with human peripheral blood mononuclear cell cultures and then with fresh human whole blood. T cells were efficiently and exclusively infected in these heterogeneous cell populations without the need for prior activation or the presence of any cytokines. For in vivo evaluation, G-CD3 LV encoding αCD19-CAR/GFP (G-CD3/LV-CAR) was used to treat advanced NALM6 -Luc tumors in NSG mice engrafted with human PBMC. Intraperitoneal administration of G-CD3/LV-CAR led to complete tumor eradication without recurrence by the 60 days post-injection study end point. The peak level of circulating CAR-T cells in these animals was 57.5±10.7% of human T cells on day 15 post injection, at which point integration analysis revealed a copy number of 1.2±0.6 integration sites per transduced human T cell. At later time points, after tumor clearance, the number of circulating CAR-T cells was greatly reduced and their phenotype shifted from mostly effector/effector memory toward central memory/stem-like cells. In general, mice tolerated the treatment well, and analysis of cytokines associated with CAR-T cell cytotoxicity confirmed the absence of severe cytokine storm. Interestingly IL-6 was undetectable at all time points analyzed and IFN-gamma was the only cytokine which significantly increased during the expansion period of CAR-T cells, while IL-2, IL-10, and TNF-alpha exhibited only a minor increase. Aside from the possibility of a CAR-T driven cytokine storm, we also investigated whether the infused CD3-targeted vector particles might activate T cells sufficiently to provoke an immediate "infusional" cytokine storm. Exposing human PBMC to G-CD3-LV led to the rapid induction of T cell activation markers CD69 and CD25 and to the release of IFN gamma and TNF alpha measured 6 hours after infection in the culture medium. To evaluate CD3 scFv -induced toxicity in vivo, fully immunocompetent mice transgenic for the γ, δ, and ε subunits of human CD3 ("GDE mice") were used. After infusing high doses of the G-CD3 LV into GDE mice, CD69 was rapidly upregulated on the T cells after 2-6 hours. However, despite this evidence for vector-mediated activation of the T cells (presumably by delivering signal 1), there was no detectable increase in serum IL-2, IL-4, IFNγ, TNFα or IL-6, neither at early (2-6 hours) nor later (1-4 days) time points. Also, the animals did not lose weight and did not appear unwell after vector infusion. These data suggest that though G-CD3 LV activates T cells, it does not induce an infusion-related cytokine storm in vivo. In summary we demonstrate novel CD3-targeted lentiviral vectors that efficiently and accurately deliver their CAR-T genetic payload to human T cells in fresh whole blood and humanized tumor-bearing mice, leading to in vivo CAR-T cell generation and potent antitumor activity without severe cytotoxicity.

Targeted Gene Insertion

191. Efficient and Versatile Programmable Large-Gene Integration by Evolved Recombinases and Prime Editing

Xin D. Gao[1,2,3], Smriti Pandey[1,2,3], Nicholas A. Krasnow[1,2,3], Amber McElroy[4], Jordyn E. Duby[1,2,3], Sarah Pierce[1,2,3], Jakub Tolar[4], Mark J. Osborn[4], David R. Liu[1,2,3]

[1]Merkin Institute of Transformative Technologies in Healthcare, Broad Institute of MIT and Harvard, Cambridge, MA,[2]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA,[3]Howard Hughes Medical Institute, Harvard University, Cambridge, MA,[4]Department of Pediatrics, University of Minnesota Medical School, Minneapolis, MN

Current methods to achieve targeted integration of multi-kilobase DNA sequences into mammalian genomes suffer from low efficiencies, specificities, or programmability, limiting their usage in synthetic biology, biotechnology, and cell and gene therapies. To overcome these challenges, we developed a phage-assisted continuous evolution (PACE) selection system for recombinases, then applied the system to substantially improve the activity of large serine recombinase (LSR) for prime editing-assisted site-specific integrase gene editing (PASSIGE), an approach that couples the programmability of prime editing with the specificity of recombinases to precisely integrate large DNA cargoes into the mammalian genome. PACE-evolved LSR variants mediate up to 60% donor integration in human cell lines with pre-installed recombinase landing sites. When used in tandem with prime editing,

the evolved recombinase variants can consistently achieve single-transfection targeted gene integration efficiencies of ≥30% (up to 50% without any selection) at therapeutically relevant sites in human cells. PASSIGE with the evolved and engineered Bxb1 variant substantially outperforms previously reported methods for programmable large gene integration—for example, outperforming PASTE and original PASSIGE system by an average of 16-fold and 4.2-fold, respectively across three human and mouse cell types and 12 genomic loci. To our knowledge, the evolved and engineered Bxb1 variants generated in this study enable the most effective targeted gene integration in mammalian cells reported to date. We anticipate that these recombinase variants may also be applied to modify DNA for various applications that will advance both biological research and therapeutic development. Finally, the rapid continuous evolution system developed in this study can also be used to improve the activity of other recently discovered integrase systems to enhance their activity. X.D.G. and S.P. contributed equally to this work.

192. Directed Evolution of Bxb1 for the Development of Modular Integrases (MInts)

Sebastian Arangundy Franklin[1], Friederich Fauser[1], Luis Rodriguez[1], Nicol Schmidt[1], Nicholas A. Scarlott[1], Adeline Chen[1], Rakshaa Mureli[1], Bhakti N. Kadam[1], Jessica E. Davis[1], Lifeng Liu[1], Danny F. Xia[1], Mohammad Qasim[1], Vishvesha Vaidya[1], Sarah J. Hinkley[1], Emily C. Tait[1], Bryan Bourgeois[1], Nga Nguyen[1], Stephen Lam[1], Andrew Nguyen[1], David Paschon[1], Gregory Davis[2], Jeffrey Miller[1]

[1]Sangamo Therapeutics, Richmond, CA,[2]Sangamo Therapeutics, Inc., Richmond, CA

Serine integrases have recently emerged as promising reagents for the site-specific integration of large DNA cargo into genomes. Their ability to insert large DNA payloads with high precision at pre-defined sites without causing double-strand breaks or nicks and without reliance on a host's DNA repair machinery makes them attractive tools for genome engineering for therapeutic applications and synthetic biology more generally. The recent discovery of thousands of serine integrases and their putative attB and attP sites has significantly expanded the toolbox of integrases available for synthetic biology. Nevertheless, such novel integrases are constrained by their natural DNA sequence preference, and their use has been limited to integration at sites pre-inserted into host genomes using complex prime editing reagents. A much more desirable alternative may be a set of bespoke engineered integrases able to target genomes in the absence of other gene editing tools with high precision and efficiency. We sought to provide such an alternative by the directed evolution of the serine integrase from the Bxb1 phage. While structures of a distantly related serine recombinase from the *Listeria inoccua* prophage have shed light on the mechanism of recombination, the low homology to other well-characterized serine integrases and extensive DNA contact surface have posed a significant challenge to the engineering of the large serine integrases. Based on the published integrase structures as well as structural predictions made by RoseTTAfold, we hypothesized that serine integrases may recognize their DNA targets through distinct and structurally independent submotifs (Modules), and as such, allow the systematic reprogramming of integrases towards divergent attachment sites. Using site-directed mutagenesis and plasmid recombination assays, we mapped protein submotifs crucial for the protein-DNA contacts between Bxb1 and its attB site. We then developed an ultra-high throughput bacterial selection system capable of assaying billions of Bxb1 variants, and carried out hundreds of selections against artificial attB and attP sites, with nearly 70% of selections yielding clear consensus sequences at the selected peptide submotif. We characterized the molecular specificity of hundreds of selected submotifs and found that their sequence preference has been profoundly changed from that of their natural counterpart. Importantly, we discovered that such novel Bxb1 variants can be assembled and give rise to fully synthetic, Modular Integrases (MInts), with attB sequences diverging significantly from the natural Bxb1 attB site. Moreover, such MInts can be used for the targeted integration of large DNA cargo in human cells (See Poster by Fauser et al) . We estimate that our current bank of pre-characterized MInts may allow us to target the human genome every 10kb, with the potential to target the first intron of most disease-related human alleles in cases where other gene editing approaches are insufficient as a means of correcting multiple mutations and to address multiple patient populations at once.

193. Large Serine Integrase Off-Target Discovery and Validation for Therapeutic Human Genome Editing

Dane Z. Hazelbaker, Japan B. Mehta, Didac Santesmasses, Connor McGinnis, Thomas Biondi, Anne M. Bara, Xiaoyu Liang, Brett Estes, Jenny Xie, Kaivalya Molugu, Ravindra Amunugama, Chong Luo, Jesse Cochrane, Sandeep Kumar, Matthew H. Bakalar, Jie Wang, Daniel J. O'Connell, Jonathan D. Finn

Tome Biosciences, Watertown, MA

While numerous technologies for the characterization of potential off-target editing by CRISPR/Cas9 have been described, the development of new technologies and analytical methods for off-target recombination by Large Serine Integrases (LSIs) are required to advance the application of LSIs for therapeutic gene integration. Here we present the development of two complementary off-target recombination discovery technologies and a hybrid capture validation technology as a comprehensive framework for off-target characterization of LSIs. HIDE-Seq (High-throughput Integrase-mediated DNA Event Sequencing) is a PCR-free unbiased genome-wide biochemical assay capable of discovering sites with LSI-mediated DNA double-strand breaks and off-target recombination events. Cryptic-Seq is a PCR-based unbiased genome-wide biochemical or cellular-based assay that is more sensitive than HIDE-Seq but is limited to the discovery of sites with off-target recombination. Potential off-target loci discovered by HIDE-Seq and Cryptic-Seq can then be validated with our hybrid capture NGS sequencing of genomic DNA from cells after LSI-mediated gene insertion that has been qualified to 0.1% sensitivity. Hybrid capture sequencing of >450 discovered off-target sites led to the validation of 90 detectable off-target recombination sites with less than 1% frequency in cells. Furthermore, the lack of indels at these sites suggests that Bxb1 integrase does not generate significant double strand breaks, consistent with our HIDE-seq data. To demonstrate the sensitivity of our framework to discover bona fide sites of off-target recombination, we performed unbiased whole genome sequencing

(WGS) on LSI-edited samples and found WGS detected only 2 of the 90 off-target sites discovered by our discovery and validation framework, yielding a 45-fold increase in sensitivity. We expect the dissemination of these sensitive technologies will advance the application of LSIs in therapeutic genome editing by establishing benchmarks for sensitivity of off-target detection.

194. *In Vivo* Expansion of Gene-Targeted Hepatocytes through Transient Inhibition of an Essential Gene

Marco De Giorgi[1], So Hyun Park[2], Adam Castoreno[3], Mingming Cao[2], Ayrea Hurley[1], Lavanya Saxena[2], Marcel A. Chuecos[1], Christopher J. Walkey[1], Alexandria M. Doerfler[1], Mia N. Furgurson[1], M. Cecilia Ljungberg[1], Kalyani R. Patel[4], Sarah Hyde[3], Tyler Chickering[3], Stephanie Lefebvre[3], Kelly Wassarman[3], Patrick Miller[3], June Qin[3], Mark K. Schlegel[3], Ivan Zlatev[3], Rich Gang Li[1], Jong Kim[1], James F. Martin[1], Karl-Dimiter Bissig[5], Vasant Jadhav[3], Gang Bao[2], William R. Lagor[1]

[1]Baylor College of Medicine, Houston, TX,[2]Rice University, Houston, TX,[3]Alnylam Pharmaceuticals Inc, Cambridge, MA,[4]Texas Children's Hospital, Houston, TX,[5]Duke University, Durham, NC

Homology Directed Repair (HDR)-based genome editing is an approach that could permanently correct a broad range of genetic diseases. However, its utility is limited by inefficient and imprecise DNA repair mechanisms in terminally differentiated tissues. For example, HDR requires cell division, generally limiting targeting of adult liver to ~1% of hepatocytes. The goal of this work is to develop a system for selective expansion of gene-targeted hepatocytes using essential genes, which we call "Repair Drive". Repair Drive consists of: (1) transient conditioning of the liver by knocking down an essential gene - Fumarylacetoacetate hydrolase (Fah), which is important for tyrosine catabolism, and (2) delivery of an untargetable version of the essential gene in *cis* with the therapeutic transgene. To test this approach, we used AAV vectors to insert *FAH* in tandem with a fluorescent marker (TdTomato) into a highly expressed locus in the liver (*Apoa1*). Mice were injected with AAV8 vectors encoding CRISPR/Cas9 (AAV-CRISPR) and a Donor template (AAV-Donor) with *FAH* and a TdTomato transgene. Monthly injections of N-acetylgalactosamine (GalNac)-modified siRNA targeting murine *Fah* were used as a conditioning agent to deplete untargeted hepatocytes and drive expansion of correctly repaired cells. Three months later, mice receiving the conditioning siRNA - Repair Drive mice - showed a dramatic increase in the number of TdTomato positive hepatocytes, versus those receiving the viral vectors alone - Unselected mice (~10% vs 0.5%). Selective expansion could be further improved to >25% of hepatocytes by manipulation of tyrosine catabolism with a high protein diet. To evaluate the translatability and long-term safety of this approach, we inserted a human Factor IX (*FIX*) transgene into the *Apoa1* locus. Following genome editing, the mice were injected monthly with the conditioning siRNA for three months, and then followed for 1 year. Repair Drive mice showed sustained FIX expression over 1 year with a ~5-fold increase as compared to Unselected mice. Liver conditioning caused only a transient elevation of ALT at early time points (4 to 8 weeks), which fully resolved over time. Histology revealed no evidence of liver toxicity and full reconstitution of Fah expression. Importantly, the expansion protocol did not increase the risk of tumorigenesis. SMRT-seq analysis at the *Apoa1* locus revealed 20- to 50-fold increase of correct HDR-, 5'-HDR/3'-NHEJ- or partial integration of the Donor cassette in Repair Drive mice. NGS data showed a dramatic reduction in the relative frequency of off-target to on-target editing in Repair Drive mice. Overall, these data show a novel strategy for selectively expanding targeted hepatocytes for liver gene therapy.

195. Programmable Genomic Integration in Induced Pluripotent Stem Cells and Hematopoietic Stem and Progenitor Cells

Minggang Fang, Ravindra Amunugama, Kaivalya Molugu, Marie-Joe Kimaz, Lauren Herchenroder, Adam Zieba, Jie Wang, Wei Wang, Kangni Zheng, Jason Zhang, Devin Harrison, Sandeep Kumar, Daniel O'Connell, Chong Luo, Jonathan Finn

Tome Biosciences, Watertown, MA

CRISPR/Cas9-based technologies have revolutionized basic and applied biomedical research in the past decade. Current targeted gene insertion approaches require DNA double-strand breaks (DSBs), risking translocations, and rely on DNA repair pathways (e.g., homology-directed repair, HDR) that are inactive in quiescent or terminally differentiated cells. Programmable and multiplexed genome integration of multi-kilobase (kb) DNA cargo independent of HDR is still challenging. Here, we present a proof-of-concept (PoC) evaluation of Tome Biosciences' proprietary technology, integrase-mediated programmable genomic integration (I-PGI), on induced pluripotent stem cells (iPSCs) and CD34+ hematopoietic stem cells (HSCs). I-PGI combines CRISPR-mediated genome editing and site-specific integrases, enabling efficient and targeted genomic integration without DSBs. Two unique features of iPSCs, unlimited expansion and the ability to differentiate into almost all cell types, provide an unprecedented opportunity for cell therapy. The current FDA-approved autologous cell therapies are costly, labor-intensive, time-consuming to manufacture, and carry a risk of oncogenic transformation due to random integration using lentivirus. Allogeneic iPSC-derived cell products, which can be manufactured on a large scale and used "off-the-shelf", hold promise to overcome these limitations. First, we leveraged attachment site-containing guide RNAs (atgRNAs), Cas9 nickase, and reverse transcriptase to place integrase-recognizing landing sites (beacons) at the B2M locus and achieved over 90% beacon placement (BP) efficiency. We followed by integrating a DNA cargo of 5.2kb into the placed beacon and achieved up to 46% efficiency. We then tested a template of 31kb and successfully reached 6% integration. To assess multiplex integration, we simultaneously placed orthogonal beacons at four different genetic loci and integrated 4 corresponding templates into these beacons. We observed integration of 1.4%, 6.8%, 8.1% and 10.2% respectively with DNA templates ranging in size from 4 to 6kb. HSC transplantation has been a curative therapy for many hematological disorders and malignancies in the last few decades, and the field is further energized by the recent approval of Casgevy. However, durable gene replacement requires editing of the quiescent long-term (LT)-HSC population, which is limited by HDR-mediated approaches. In donor-derived CD34+ HSCs, we achieved BP efficiencies of over 80% at the B2M locus and over 30% I-PGI efficiencies. To determine if I-PGI can

engineer LT-HSCs, we isolated the primitive HSC sub-population and demonstrated that PGI efficiencies correlated with the bulk engineered HSC population. We are currently evaluating if the I-PGI engineered HSC successfully engraft and persist *in vivo.* In summary, our PoC study demonstrated efficient, DSB-free engineering of iPSCs and HSPCs by I-PGI technology. This novel approach gives us the advantage of speed, precision, and specificity for next generation cell therapies.

196. Internal Base Modifications Enable Highly Efficient Gene Insertion in Primary Cells Using Single Stranded DNA

Sriram Vaidyanathan[1], Karen Kanke[1], Cormet-Boyaka Estelle[2], Mark Hester[3], Rachael E. Rayner[2], Ma Suu[2]

[1]Nationwide Children's Hospital, Columbus, OH,[2]The Ohio State University, Columbus, OH,[3]The Research Institute at Nationwide Childrens Hospital, Columbus, OH

In vivo genome editing using CRISPR/Cas9 has the potential to durably treat chronic disorders including monogenic diseases such as cystic fibrosis (CF). The development of lipid nanoparticles (LNP) has enabled the delivery of Cas9 mRNA and sgRNA without eliciting significant immune responses. Although this enables therapies that rely on knocking out genes, the ability to insert genes in vivo can further improve our ability to durably treat diseases. Gene insertion relies on homologous recombination (HR) which requires a template carrying the sequence of interest. Adeno-associated viruses result in efficient gene insertion but can trigger innate and adaptive immune responses. Single-stranded DNA (ssDNA) templates may enable high efficiency gene insertion using LNPs packaged with Cas9 mRNA/sgRNA and ssDNA templates. However, ssDNA templates suffer from low efficiency. Previous studies have used chemical modifications (e.g. phosphorothioate) at the ends of ssDNA templates to improve efficiency. We reasoned that the chemical modification of internal bases may further protect ssDNA templates and improve gene insertion further. However, it was unclear if the base modifications that prevent nuclease activity may also prevent efficient HR. We tested the use of ssDNA with 10% - 33% modified bases to improve gene insertion in airway basal stem cells (ABCs), hematopoietic stem cells, T-cells, and vascular endothelial cells *in vitro* by delivery using electroporation. We used 200 bp templates targeting the *CFTR*, *HBB* and *CCR5* loci involved in cystic fibrosis, hemoglobinopathies and HIV infections respectively. Our results show that ssDNA with chemical modifications in 10-17% of internal bases (eDNA) is compatible with the homologous recombination machinery. In the *CFTR* locus in airway basal stem cells (ABCs), eDNA templates showed gene insertion in 41 ± 15% of alleles compared to 24 ± 6% for end-modified and 15 ± 5% for unmodified ssDNA (Figure 1). Compared to end-modified templates, improved gene insertion by eDNA templates was also observed in the *HBB* (51 ± 9% vs 22 ± 4%) and *CCR5* (41 ± 5% vs 17 ± 1%) loci in ABCs. Similar improvements in gene insertion were also observed in hematopoietic stem and progenitor cells, T-cells and endothelial cells. We further tested the use of this approach to correct the F508del mutation in the *CFTR* gene using CF patient-derived airway cells. We achieved correction in 49 ± 15% alleles in CF ABCs. Upon differentiation, the corrected cells showed CFTR function that was 30-60% relative to non-CF controls. This level of correction is therapeutically relevant and is comparable to adeno-associated virus-based templates. Further studies are needed to determine if eDNA templates show similar improvements in gene insertion when delivered using LNP formulations *in vivo*.

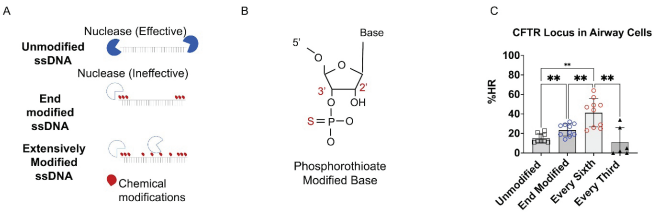


Figure 1: A) Graphic showing the chemical modifications of ssDNA and predicted nuclease degradation activity and inactivity. B) Graphic showing the chemical structure of modified bases. C) In airway cells at the CFTR locus, ssDNA templates with every sixth base modified show significantly higher HR than end-modified ssDNA and unmodified ssDNA (15 ± 5%). Modifying every third base results in the least gene insertion (11 ± 15%). Statistical comparison was made using one-way ANOVA and Tukey-test. ****, ***, ** and * represent p <0.0001, p < 0.001, p< 0.01 and p < 0.05 respectively for all panels.

197. Targeted Gene Insertion of Vectorized Monoclonal Antibodies in Non-Human Primates Overcomes AAV Genome Silencing in the Liver and Supports High, Sustained *In Vivo* Expression of Functional Antibodies

Rachel Sattler, Anthony LoMastro, Juanita Ezemba, Tara Sterlacci, Ellen-Marie Koehler-Stec, Asma Parveen, Elena Ivanova, Kristen Pascal, Leah Sabin, Christos Kyratsous

Regeneron Pharmaceuticals, Inc., Tarrytown, NY

As more liver-directed, adeno-associated virus (AAV)-based therapies move to human clinical trials, a strong disconnect in the efficiency of the strategy is observed when transitioning from mouse to monkey to human. These conventional AAV gene therapies rely on formation of extrachromosomal DNA episomes in the nucleus of host cells and a promoter to drive transgene expression. In our work, and work reported by others, we observe that conventional AAV episomes support robust liver expression in mouse models but achieve significantly lower levels of mRNA and protein expression in livers of both non-human primates (NHPs) and mice with human-engrafted hepatocytes. This phenomenon persists across multiple transgenes, promoters, and capsids. It poses a major limitation for clinical translatability of liver-directed, conventional AAV therapy. The exact mechanism underlying the species-specific differences in expression is under active investigation. Reported data suggests multiple causes, including epigenetic regulation of the episomal DNA and interactions between host immune factors and the AAV capsid. We have developed an alternative, CRISPR-based approach for gene therapy in the liver. Using a dual lipid nanoparticle (LNP) and AAV delivery system, this turnkey platform enables targeted insertion of a promoterless expression cassette into the *Albumin* locus of liver hepatocytes. In this study, we investigated if the targeted gene insertion of a monoclonal antibody (mAb) cassette would overcome the limitations of conventional AAV episomes in NHPs. Vectorized antibody treatment is an attractive

alternative to traditional antibody-based drugs for chronic conditions requiring continuous dosing, and for use as a vaccine alternative or prophylaxis treatment in special populations. We delivered the same transgene, encoding an anti-SARS-CoV2 antibody, to both mice and NHPs using either conventional AAV episomes or the CRISPR-based targeted insertion approach. As expected, when conventional AAV episomes were used, significantly lower levels of circulating mAb expression were measured in NHPs compared to mice. Conversely, when CRISPR-based targeted insertion was used, therapeutically relevant levels of circulating mAb were measured as early as day 7 post-dosing in both mice and NHPs. Further, peak mAb levels of greater than 100 ug/ml were measured in both species and levels were sustained through the study completion at week 19. Of note, vector DNA levels in the NHP livers were similar regardless of the platform used, but mRNA and protein levels measured more than a log higher in the livers of NHPs that received the targeted gene insertion. In summary, durable, therapeutic levels of mAb expression were achieved in NHPs using liver-directed, CRISPR-based, targeted gene insertion. This approach overcomes the increasingly reported disconnect between mouse and NHP studies that use conventional AAV gene delivery for liver expression of therapeutic proteins. Since the insertion platform is a "plug and play" approach, the demonstration here of high levels of mAb expression via insertion suggests that the platform will enable robust protein expression in other therapeutic contexts as well. Finally, the expression of therapeutic mAbs was long-lasting, providing a path forward for truly "one-and-done" liver-directed gene therapies.

Emerging Viral Vectors

198. Lentiviral Gene Therapy to the Liver for Homozygous Familial Hypercholesterolemia (FH): Assessing the Best Vector Design

Cesare Canepari[1], Michela Milani[1], Francesco Starinieri[1], Anna Fabiano[1], Fabio Russo[1], Mauro Biffi[1], Rossana Norata[1], Martina Rocchi[2], Francesca Sanvito[2], Alessio Cantore[1,3]

[1]San Raffaele Telethon Institute for Gene Therapy, Milan, Italy,[2]IRCCS San Raffaele Scientific Institute, Milan, Italy,[3]Vita Salute San Raffaele University, Milan, Italy

Homozygous FH (HoFH) is a severe rare early-onset inherited disease, mostly due to mutations in low-density lipoprotein (LDL) receptor (LDLR)-encoding gene. The disease is characterized by high circulating LDL cholesterol (LDL-c), due to impairment in its clearance by LDLR. Patients rapidly develop atherosclerosis. Available treatments do not normalize LDL-c. *In vivo* gene transfer to hepatocytes may rescue LDLR expression upon a single intravenous (i.v.) administration. Lentiviral vectors (LV) may be used for this purpose since they integrate in the genome of target cells and thus are maintained upon cell proliferation in liver growth. For this reason, LV may be applied to pediatric patients. LV are typically pseudotyped with vesicular stomatitis virus glycoprotein (VSV.G). However, VSV.G-LV have been shown to interact with LDLR and LDLR family members to access target cells. Indeed, when producing VSV.G-LV expressing LDLR transgene, we obtained very low infectious titers (>10-fold lower than infectious titers of other LV), likely due to interaction between VSV.G and LDLR overexpressed by producer cells and subsequent re-infection during LV production. We overcame this issue by avoiding LDLR expression during production. Specifically, we placed the LDLR sequence and its hepatocyte specific promoter in reverse orientation with respect to LV genome transcription. Alternatively, we added a LDLR negative modulator during LV production. These improved LV designs showed complete rescue of infectious titer. We then tested if hepatic gene transfer was feasible in mice Ldlr-/-, mouse model of HoFH. We injected them with LV expressing a reporter coagulation factor IX (FIX) transgene and observed higher transgene output in mice Ldlr-/- compared to controls at two different LV doses, possibly due to efficient LV uptake by LDLR family members expressed by hepatocytes. We then moved on to evaluate therapeutic efficacy: we produced LV expressing LDLR under the control of a hepatocyte-specific cassette based on a strong enhanced transthyretin (ET) promoter. We injected ET.LDLR-LV into juvenile Ldlr-/- mice and observed normalization of blood LDL-c. We stressed the system by feeding the mice with Western Diet (21% fat, 0.2% cholesterol) for three months. No atherosclerosis was present in treated mice (N=6), while it was marked in Ldlr-/- untreated controls (N=5). Histopathology analysis showed abnormalities in the liver of LV-treated mice. Moreover, liver lipid vacuolation was higher in those showing lower corrected liver area, suggesting that few LDLR-overexpressing cells experienced excessive lipid intake. To improve this outcome, we tested a weaker alpha-1-antitrypsin (HAAT) promoter. At matched LV doses, HAAT was ~20-fold less potent than ET in the human Huh7 hepatoma cell line and around 7-fold lower *in vivo*. Interestingly, when tested in Ldlr-/- mice, we found similar therapeutic efficacy of LV equipped with the different promoters at similar doses (N=5). HAAT-LV treated mice are currently being challenged with Western diet. Histopathological analysis of the liver and comparisons between the LV with the two different promoters will be presented. Here, we show, for the first time, to our knowledge, full normalization of LDL-c and atherosclerosis prevention, by liver-directed LV gene therapy in a mouse model of HoFH. These data provide the basis for further development towards clinical translation.

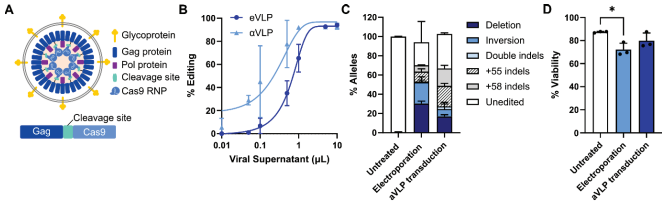
199. Alpha-Retrovirus-Based Virus-Like Particles for Efficient Gene Editor Delivery into Hematopoietic Stem Cells

Denise Klatt[1], Josephine Mayer[1], Jing Zeng[1], Chi-Yuan Zhang[2], Nola Neri[1], Sébastien Levesque[1], Axel Schambach[1,3], Els Verhoeyen[4,5], Daniel E. Bauer[1], David A. Williams[1], Christian Brendel[1]

[1]Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, Boston, MA,[2]Dana-Farber Cancer Institute, Boston, MA,[3]Hannover Medical School, Hannover, Germany,[4]INSERM/CIRI/EVIR, Lyon, France,[5]Université Côte d'Azur, Nice, France

Electroporation of ribonucleoprotein (RNP) complexes or mRNA is currently the method of choice for delivery of CRISPR-Cas9-based genome editors into hematopoietic stem cells (HSCs). While efficient, these methods affect cell viability and physiology, require specific equipment, and are limited to *ex vivo* applications. Cas9-delivery by virus-like particles (VLP) is a promising alternative, such as the γ-retrovirus-based Nanoblade or eVLP systems. These VLP systems consist of a designer nuclease fused as a RNP complex to structural

components of the virus shell, which is liberated into target cells by the viral protease. Here, we engineered α-retrovirus-based VLPs (αVLPs) for genome editor delivery (Figure A). This approach was motivated by the favorable genome organization of α-retroviruses, where the gag-Cas9 fusion resides entirely within the gag-gene reading frame, which is >10-fold more efficiently translated than the downstream pol-genes, where the Cas9 fusion is placed in the eVLP system. We showed that the αVLPs are 5-fold more efficient than the described eVLP system for Cas9 delivery using VSVg-pseudotyped particles as assessed by indel formation in various cell lines and human CD34+ HSCs (Figure B). We identified optimal configurations through sequential testing of four different gag-Cas9 fusions, multiple viral protease sites and tuning the component ratios during αVLP-production. Optimal editing was achieved by the fusion of Cas9 downstream of the nucleocapsid coding region separated by the naturally present protease cleavage site. Physical characterization revealed a larger size of αVLPs compared to eVLPs (118 nm vs 108 nm) but no significant difference in the amount of Cas9 molecules per editing particle. Over 90% indel rates were observed targeting multiple different loci, such as B2M, SBDS or BCL11A, with <1 µL viral supernatant in cell lines (1.1-9.8x10^8 editing VLPs/mL) and over 70% indel formation in HSCs using a MOI 100 while maintaining viability equivalent to untransduced cells. We additionally explored αVLP for multiplex editing, simultaneous delivery of an integrating payload, and flexible pseudotyping for editing quiescent HSCs. Using a hybrid envelope consisting of VSVg and BaEVRLess, we were able to edit over 50% of alleles in freshly thawed, quiescent CD34+ HSCs using a MOI 100 during a two-hour transduction period. As a therapeutic target to treat sickle cell disease, we deleted the erythroid enhancer of BCL11A in CD34+ HSCs and compared αVLPs to electroporation. Both methods achieved over 60% editing of the BCL11A locus and about 50% HbF induction upon erythroid differentiation (Figure C). In contrast to electroporation, αVLP mediated editing was less cytotoxic to HSC, indicated by a viability of 80% compared to 72%, which resulted in higher human chimerism in transplanted NBSGW mice (Figure D). Next, we explored the delivery of prime editors via αVLPs to avoid DNA double-strand breaks and improve safety. Initial results demonstrated over 60% prime editing in HEK293T cells targeting the B2M locus. In summary, αVLPs are an efficient and versatile tool for the delivery of CRISPR-Cas9-based genome editors and have potential use for *in vivo* gene editing.



200. Structure Derived Insights into the Similarities and Differences in Coagulation Factor Binding to Species C and Species D Adenoviruses

Michael A. Barry[1], Haley Mudrick[1], Shao-Chia Lu[1], Janarjan Bhandari[2], Mary E. Barry[1], Olivia Ma[2], Vijay Reddy[3]

[1]Mayo Clinic, Rochester, MN,[2]University of Minnesota, Austin, MN,[3]Hormel Institute, Austin, MN

Adenoviral (Ad) vectors are potent vectors for *in vivo* applications. However, Ads suffer from strong innate and adaptive immune responses that can start by the binding of host proteins to the surfaces of their virions in a serotype-specific fashion. For example, human species C human Ads bind vitamin K-dependent blood clotting factors FII and FX whereas most other serotypes do not. Conversely, all Ad serotypes can be bound by natural antibodies or neutralizing antibodies that can trigger complement activation, innate immune responses, and their destruction by macrophages and Kupffer cells. This problem of host protein binding to Ad virions was made more apparently by observations of vaccine-induced thrombotic thrombocytopenia (VITT) in Ad COVID-19 vaccines. It has been proposed that binding of another blood protein, platelet factor 4 (PF4), different adenoviruses surfaces can induce antibodies against PF4 to induce VITT. While VITT could theoretically occur with any Ad, the particular serotype of each Ad may influence the risk of this side effect because different proteins bind to different Ad serotype's hexon proteins. For example, blood clotting factors FII and FX bind human species C Ads, Ad5, Ad6 and Ad57 with affinities that are up to a 1000-fold higher than the affinity PF4 these viruses. In addition, the concentration of FX and FII in human blood is 1,000- and 10,000-fold higher than PF4, respectively. While FII and FX bind species C Ads, they do not bind other adenoviruses including species D Ad26 that has been used as HIV and COVID-19 vaccine. Given these variations in host protein binding to different Ad serotypes, we examined the interactions of PF4, FII, and FX to species D and species C Ads by cryo-EM. The Gla-domains of both FII and FX were observed binding to the exterior cavities of Ad5, Ad6, and Ad657 hexons primarily by interactions with hypervariable loops 5 and 7. Only the Gla-domain of FX could be observed at high (~3.7Å) resolution. In contrast, both the Gla and KR1 domains of FII was observed bound to all 3 viruses. Both the Gla its proximal EGF domain of FX was visible at lower resolution (10Å) and showed three equivalent binding modes that were consistent with the 3-fold symmetry of the hexon trimer. Docking of AlphaFold2 models of FX and FII into EM-densities suggests that both of their Gla-domains bind similarly in the hexon cavities. However, the different c-terminal regions of FII and FX modify the degree to which the two proteins shield the surface of hexon trimers. FII appeared to bind all three viruses equally well, while FX bound Ad5 and Ad657 viruses significantly better than Ad6. While FII and FX were readily observed by cryo-EM, PF4 binding was not observed on any adenovirus including Ad26 by this method. These data suggest that there are unique binding modes to how different highly abundant host blood proteins bind to the surfaces of different adenovirus serotypes. Understanding these natural interactions will allow these viruses to be engineered to increase the efficacy and decrease side effects of different Ad serotypes as vaccines, as gene therapies, or as oncolytic viruses.

201. Rational Design of a Detargeted Vesiculovirus Fusogen to Enable Targeted *In Vivo* Gene Delivery

Ronnie M. Russell, Debasish Boral, James I. Andorko, Bruce C. Schnepp, Timothy D. Culp, Philip R. Johnson
Interius BioTherapeutics, Philadelphia, PA

Current ex vivo autologous and allogenic CAR T cell approaches utilize wild type (WT) VSV-G pseudotyped lentiviral vectors to achieve high efficiency transduction of patient or donor cells. In vivo generation of CAR T cells is meant to circumvent complications with ex vivo approaches, such as limited accessibility and lengthy manipulation processes that can negatively affect the outcome of therapy; however, WT VSV-G cannot be used for in vivo approaches due to a broad tropism that is not limited to T cells. Our goal was to rationally design a fusogenic molecule to enable targeted in vivo delivery of a gene of interest. Previous work has shown that binding and fusion mediated by VSV-G are uncoupled processes[1]; thus, we reasoned the tropism of VSV-G pseudotyped vectors could be redefined by reducing the affinity of VSV-G for its native receptor (LDL-R) while simultaneously redirecting vector binding to a target molecule expressed by cells of interest. Using the available crystal structure of VSV-G bound to LDL-R[1], we visually scanned for residues in VSV-G whose side chains protrude toward the negatively charged contact residues in LDL-R. We then introduced negatively charged side chains at the identified sites in VSV-G to destabilize critical ionic interactions with its receptor. Substitution of isoleucine at position 182 with either glutamic acid or aspartic acid was sufficient to abolish the transduction by vector pseudotyped with the mutant glycoproteins. Providing a membrane-anchored scFv redirecting vector to target cells restored titers to within a log of WT VSV-G, indicating these mutations only impacted binding to LDL-R, but not fusogenic activity. Importantly, substitution of alanine at residue 182 did not have this effect, suggesting the mechanism is dependent on charge as designed. Cells transfected with the I182E mutant were not labeled by a soluble form of LDL-R (sLDL-R), confirming the reduced affinity to the native receptor. As a concern with using VSV-G based vectors is the reported sensitivity to complement, we also incorporated substitutions in VSV-G that have been shown to improve stability in human serum[2]. Reflecting the iterative nature of this fusogen's design, we dubbed this version Generation 2.1 or "Gen 2.1" for short. To further demonstrate specific retargeting, lentiviral vectors pseudotyped with the Gen 2.1 fusogen were produced and used to transduce CD7+ cells. As expected, vector pseudotyped with the Gen 2.1 fusogen alone did not effectively mediate transduction. When a plasmid encoding a binder specific to CD7 was included during vector production, infectivity titers were restored on CD7+ SupT1 cells, but not on the same cells in which CD7 was knocked out or a panel of CD7- B cell lines. Additionally, vectors pseudotyped with the Gen 2.1 fusogen were resistant to neutralization by sLDL-R, confirming the retargeting via the CD7 binder. These data suggest that the Gen 2.1 fusogen has potential to be used as a platform for targeted *in vivo* delivery. Leveraging industry standard manufacturing processes for WT VSV-G based lentivirus production, we have successfully manufactured Phase 1 clinical supplies of a CAR20 vector product (INT2104) made with Gen 2.1 fusogen and a CD7 binder to support a FIH study in oncology starting in 4Q2024. [1]Nikolic, J., et al. "Structural basis for the recognition of LDL-receptor family members by VSV glycoprotein." *Nature Communications* 9.1 (2018): 1029. [2]Hwang, B-Y., and D. V. Schaffer. "Engineering a serum-resistant and thermostable vesicular stomatitis virus G glycoprotein for pseudotyping retroviral and lentiviral vectors." *Gene Therapy* 20.8 (2013): 807-815.

202. Engineering Single-Cycle Measles Vector for CRISPR/Cas9 Gene Editing

Naga Sai Ramya Rallabandi[1], Brenna Sharp[2], Spencer Majerus[2], Sarriana (Ria) Hoffer[2], Ikeda Mia[2], Patricia Devaux[1,2]
[1]Mayo Graduate School, Rochester, MN,[2]Mayo Clinic, Rochester, MN

Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated 9 (CRISPR/Cas9) mediated gene editing has vast applications in basic and clinical research and is a promising therapeutic for several disorders. Our lab previously developed a non-integrating RNA virus, measles virus (MeV), as a single-cycle vector by replacing the viral attachment protein with the reprogramming factors, OCT4, SOX2, KLF4, and c-MYC for induced pluripotent stem cell generation (Rallabandi et al. 2022 and unpublished). Encouraged by the MeV reprogramming vector efficiency, in this study, we develop a single-cycle MeV vector to deliver gRNAs targeting specific human genes and Cas9 nuclease for efficient gene editing. We showed that the expression of both gRNA and Cas9 from a single MeV genome did not affect the propagation of the viral vector compared to a control MeV vector. We showed that the MeV editing vector achieved efficient on-target knock-out gene editing of a reporter (*mCherry*) and endogenous genes (*HBB* and *FANCD1*) in human cells via non-homology end-joining repair. Analysis of off-target sites for the gRNAs used to target *HBB* and *FANCD1* genes showed limited to no off-target in all samples tested. Finally, the MeV vector achieved up to 13% precise knock-in via homology-directed repair (HDR) using a single-stranded oligodeoxynucleotide (ssODN) donor. In conclusion, for the first time, we present a novel single-cycle MeV vector platform as a new and flexible platform for gene knock-out and knock-in modifications in human cells, capable of incorporating new editing technologies as they are developed.

203. Anellovectors, a Gene Delivery Platform Based on Commensal Human Anelloviruses, Have the Potential to Evade the Immune System and Deliver DNA Payloads to a Broad Range of Tissues in a Redosable Manner

George S. Bounoutas, Riana Pozsgai, Isabella Gold, Alexander Auld, Parmi Thakker, Hiral Gujar, Erin Ozturk, Maitri Trivedi, Tuyen Ong, Ashley Mackey, Chris Wright
Ring Therapeutics, Cambridge, MA

Anelloviruses are a highly diverse family of commensal ssDNA viruses that are a principal constituent of the human virome. These viruses have been found in many vertebrates and demonstrate broad tropism to multiple organs and tissues in humans. They are capable of evading induction of the humoral immune response, persist for many years, and do not appear to cause any pathology in humans. Though the mechanism of immune evasion is currently under investigation,

these properties make anellovectors (vectorized anelloviruses) an attractive candidate as a potentially redosable vector platform for gene therapy. An anellovirus originally discovered in a human subject was vectorized as a wild-type vector or to include the CMV.eGFP payload and has demonstrated broad tropism to multiple tissues in mice. Here, we compare the redosability of this anellovector (ANV.X) to AAV2, which has been shown to elicit an immune response in mice and patients. Mice were initially pre-dosed intravenously (IV) with either vehicle, ANV.X-WT, or AAV2-mCherry (dose-matched to ANV.X). After three weeks, mice were then re-dosed IV with ANV.X-eGFP or AAV2-eGFP (also dose-matched to ANV.X). Mice pre-dosed with AAV2-mCherry demonstrated fewer AAV2-eGFP DNA copies in liver, heart, lung, and kidney in comparison to mice pre-dosed with vehicle. This data suggests that the initial dose of AAV2 triggered an immune response which generated neutralizing antibodies against AAV2. In contrast, mice that were pre-dosed with ANV.X-WT demonstrated similar or greater ANV.X-eGFP DNA copies in all assayed organs as compared to mice pre-dosed with vehicle suggesting no neutralizing antibodies were raised against ANV.X. Current viral gene therapies cannot be re-administered if the initial dose was subtherapeutic due to immunogenicity. In addition, cell turnover dilutes therapeutic genes over time, causing waning clinical efficacy. A redosable gene therapy platform that evades the immune system, such as the Anellovector, has the potential to ensure sustained efficacy for patients suffering from a broad range of genetically-driven diseases.

204. Novel Self-Replicating RNA Vectors Broaden Therapeutic Window and Expand Use Outside of Vaccines

Darina Spasova, Shigeki Miyake-Stoner, Christian Maine, Melanie Olesiuk, Christine Domingo, Gaelle Picarda, Hunter Little, Emily Brand, Annie Chou, Jessica Sparks, Jacqueline Posy, Terrina Bayone, Thomas Goodman, Andrew Geall, Nathaniel Wang, Parinaz Aliahmad

Replicate Bioscience, Inc, San Diego, CA

Clinical applications using RNA technology have thus far been limited to vaccines for infectious disease and oncology. This constraint is due to the narrow therapeutic window for RNA technology as doses needed to achieve bioactivity are often nearing the maximum tolerated dose (MTD). Self-replicating RNA (srRNA) technology is a fully synthetic platform that allows for lower dosing due to the co-encoding of a viral replicase machinery that self-amplifies *in situ*. Using novel proprietary srRNA vectors, we have demonstrated enhanced bioactivity and safety, thus broadening the therapeutic window for applications outside of simple infectious disease vaccines.Higher bioactivity from these next generation vectors was exemplified by achieving protective antibody titers using picogram doses of vaccines in preclinical mouse studies using influenza as a model. Immunization with two doses as low as 1 picogram led to neutralizing antibody titers above the WHO protective threshold as tested by hemagglutination inhibition assay. In addition, a comprehensive cellular immune response, inclusive of polyfunctional CD8 and CD4 T cells, was also observed. Importantly, these ultra-low doses led to minimal reactogenicity *in vivo* demonstrating the broadening of the therapeutic window from optimized srRNA vectors. Implications of a broader therapeutic window is the ability to expand the utility of RNA technology outside of simple infectious disease vaccines. Specifically, bioactivity at lower doses allows us to express multiplex targets for complex vaccines in infectious disease or oncology. In addition, an increased therapeutic window, which translates into higher clinical doses and/or more frequent dosing without nearing a MTD, allows expanded use of RNA in protein replacement therapies currently inaccessible with traditional mRNA. Our current srRNA vectors have shown superior pharmacokinetic profiles to mRNA and circular RNA amenable to expression of proteins at biotherapeutic levels.

Liver Genetic Diseases

205. Correction of Ornithine Transcarbamylase Deficiency Using a Mutation-Independent CRISPR-Cas9-Mediated Targeted Integration Approach with Restoration of Liver-Wide Metabolic Zonation

Samantha L. Ginn[1], Fatemeh Doroudian[1], Caitlin W. Lucas[1], Erhua Zhu[1], Sile F. Yang[1], Beena Devanapalli[2], Anne H. Klein[3], Sharon C. Cunningham[1], Sophia H. Y. Liao[1], Joseph Vitale[1], Marti Cabanes-Creus[4], Leszek Lisowski[4], Ian E. Alexander[5]

[1]Gene Therapy Research Unit, Children's Medical Research Institute, Westmead, Australia,[2]NSW Biochemical Genetics Service, The Children's Hospital at Westmead, Westmead, Australia,[3]Australian e-Health Research Centre, Commonwealth Scientific and Industrial Research Organisation (CSIRO), Sydney, Australia,[4]Translational Vectorology Research Unit, Children's Medical Research Institute, Westmead, Australia,[5]Gene Therapy Research Unit, Sydney Children's Hospitals Network, Westmead, Australia

Ornithine transcarbamlyase deficiency (OTC) is an X-linked urea cycle disorder that can lead to episodes hyperammonemia resulting in coma and death without therapeutic intervention. For urea cycle disorders, genome editing technologies represent a promising approach for permanent disease correction. This is of particular importance in the context of the growing pediatric liver where benefit may be lost in concert with liver growth when using vector systems such as recombinant adeno-associated virus (rAAV) vectors that are maintained predominantly as episomes. Major challenges associated with the use of genome editing technologies include achieving therapeutically effective levels of editing, particularly *in vivo*, avoidance of unwanted on- and off-target events and the inadvertent inactivation of hypomorphic target alleles. In addition, implementing universally configured, mutation agnostic, reagents circumvent the need to re-design new vectors for each individual patient mutation. As validation of our editing approach, we are targeting *OTC* as model locus with a high therapeutic threshold. Here we report efficient, deep-intronic, targeted integration into the *OTC* locus in both the murine liver and patient-derived primary human hepatocytes xenografted into the FRG mouse liver *in vivo*. This was achieved by using dual rAAV vectors to deliver both the CRISPR-SaCas9 editing machinery and a promoterless donor template for site-specific integration. Notably, in both wild-type and Otc-deficient mice, liver-wide metabolic zonation

was observed, consistent with on-target integration events that captured the native promoter to drive gene expression. Using optimally configured vector doses, we observed expression in greater than 80% of hepatocytes *in vivo*. Importantly, in treated Otc-deficient mice, the appearance of donor-derived transcripts, increased enzymatic activity and correction of their metabolic phenotype (elevated urinary orotic acid) was observed. In patient-derived OTC-deficient primary human hepatocytes engrafted into the FRG mouse, again we observed high levels of targeted integration, appearance of donor-derived transcripts and restoration of OTC expression. Collectively, these data demonstrate that targeted integration can be achieved in both the murine liver and primary human hepatocytes *in vivo* at levels that would provide benefit for even the most therapeutically challenging liver disorders.

206. Safe and Effective Liver Directed AAV-Mediated Homology Independent Targeted Integration in Mouse Models of Inherited Diseases

Federica Esposito[1], Fabio Dell'Aquila[1], Manuel Rhiel[2], Stefano Auricchio[1], Kay Ole Chmielewski[3], Geoffroy Andrieux[3], Rita Ferla[4], Paula Sureda Horrach[5], Arjun Padmanabhan[6], Roberto Di Cunto[1], Simone Notaro[1], Manel Llado Santaeularia[1], Melanie Boerries[7], Margherita Dell'Anno[1], Edoardo Nusco[1], Tatjana Cornu[8], Agnese Padula[9], Pasquale Piccolo[6], Ivana Trapani[5], Toni Cathomen[10], Alberto Auricchio[11]

[1]TIGEM, Pozzuoli, Italy,[2]University Medical Center Freiburg, Freiburg, Germany,[3]University of Freiburg, Freiburg, Germany,[4]AAVantgarde Bio, Milan, Italy,[5]Tigem, Pozzuoli, Italy,[6]Fondazione Telethon, Pozzuoli, Italy,[7]Faculty of Medicine, University of Freiburg, Freiburg, Germany,[8]Medical Center – University of Freiburg, Freiburg, Germany,[9]Fondazione Telethon, Pozzuoli (Naples), Italy,[10]Medical Center - University of Freiburg, Freiburg, Germany,[11]Fondazione Telethon, Roma, Italy

Liver-directed gene transfer using adeno-associated viral (AAV) vectors holds promise for treating liver and systemic diseases. However, the non-integrative nature of AAV vectors limits their application to newborn livers which is necessary for treating conditions like early-onset inborn errors of metabolism.To overcome this shortcoming, we developed CRISPR/Cas9 AAV-mediated homology-independent targeted integration (HITI) of therapeutic transgenes at the 3' end of mouse Albumin locus which is highly expressed in hepatocytes.We show the effectiveness of AAV-HITI in two different mouse models of inherited diseases, Haemophilia A (HemA) and Mucopolysaccharidosis type VI (MPS VI) in which we achieve stable therapeutic levels of systemic proteins, even at low AAV doses. Moreover, we extensively characterised the landscape of HITI donor DNA integrations at the on-target, demonstrating partial AAV genome integrations as well as full length HITI donor DNA which is preferentially cleaved from the flanking ITRs as expected based on its design.In addition, within the 1-year follow-up no gross chromosomal rearrangements and negligible insertions/deletions events were found at off-target sites. In line with this, no evidence of hepatocellular carcinoma was observed. Finally, we show that AAV-HITI is effective also in adult MPS VI or HemA livers, at vector doses considered safe if translated to humans. Overall, our data support AAV-HITI as a potential therapeutic option for the treatment of those conditions which involve the liver as target tissue.

207. Correlation of Antigen Expression with Epigenetic Modifications After rAAV Delivery of a Hyperactive Human Factor IX Variant in Mice and Rhesus Macaques

Katja Pekrun[1], Calvin Stephens[1], Adriana Gonzalez-Sandoval[1], Aranyak Goswami[2], Feijie Zhang[1], Alice F. Tarantal[3], Grant Blouse[4], Mark A. Kay[1]

[1]Departments of Pediatrics and Genetics, Stanford School of Medicine, Stanford, CA,[2]Stanford School of Medicine, Stanford, CA,[3]University of California, Davis, Davis, CA,[4]Catalyst Biosciences, South San Francisco, CA

We sought to investigate long-term human coagulation Factor IX expression of a novel high-potency variant when delivered into mice and rhesus macaques and comparing transduction efficiencies using two AAV capsids. Different human FIX variant rAAV vectors packaged with the KP1 capsid were injected into hemophilic mice. As expected, the group of mice that had received vector expressing the hyperactive FIX variant showed ten- and two-fold enhanced specific FIX activity plasma levels when compared to wild-type and Padua huFIX injected mice, respectively. We next infused CpG-depleted human codon-optimized rAAV vectors packaged into the LK03 and the KP1 capsids into rhesus macaques and found that AAV-LK03 outperformed AAV-KP1 in terms of antigen expression and liver transduction. Two animals from each group showed sustained low-level huFIX expression at 3 months post-administration, while one animal from each group lost huFIX mRNA and protein expression. Nevertheless, the two macaques that had lost expression contained vector copy numbers in liver comparable to those from the other animals in the same group. We investigated if epigenetic differences of the vector episomes could explain the loss of transcription. We did not find any indications that CpG methylation within the rAAV promoter region had contributed to the repressed state of transcription. However, Cut&Tag experiments revealed that rAAV genome-associated histones in the two animals that had lost expression contained lower levels of activating histone marks in liver. When comparing rAAV genome-associated histone modifications in rhesus macaques to those in mice administered the same vector, the activating histone marks were starkly reduced for macaque-derived episomes. Differential epigenetic marking of AAV genomes may explain differential expression profiles in mice and rhesus macaques as well as the wide dose response variation observed in primates in both preclinical and human clinical trials.

208. Knock-Out, Tissue Specific Transgenic, and Knock-In Mouse Models of PCCB Deficiency Recapitulate of the Clinical Spectrum of Propionic Acidemia and Enable the First Successful Proof-of-Principial Gene Therapy Studies

Charles P. Venditti[1], Randy J. Chandler[2]

[1]NIH, Bethesda, MD,[2]National Institutes of Health, Bethesda, MD

Propionic acidemia (PA) is rare autosomal recessive metabolic disorder caused by defects in the mitochondrial localized enzyme propionyl-CoA carboxylase (PCC). The PCC enzyme is composed of six nuclear encoded α- and six β-subunits with causative variants in either of the *PCCA* or *PCCB* genes found at equal frequencies in the patients. Individuals with PA can suffer from poor growth, potentially lethal metabolic decompensations and cardiomyopathy despite current medical management, which has led to the pursuit of gene therapy as a new treatment option for patients. While murine models and genomic therapies have been published for PA caused by PCCA deficiency, there are currently no animal models of PCCB deficiency. To explore the pathophysiology of PA and generate a platform to test new therapies for PA, we have developed 5 new murine models of *PCCB* deficiency. CRISPR-Cas9 gene editing of the murine *Pccb* gene was used to create loss of function alleles: a 4 bp deletion in the 7th exon, and an 8 bp deletion in the 14th exon. Homozygous are designated $Pccb^{E14_4bpdel/E14_4bpdel}$ and $Pccb^{E7_8bpdel/E7_8bpdel}$. Both these alleles result in a frameshift and premature stop codon in *Pccb* gene and homozygotes manifest an immediate neonatal lethal phenotype accompanied by massive elevations of plasma 2-methycitrate and 3-hydroxypropionate (Table 1). Due to the severe lethality displayed by the homozygous Pccb knock-out mice, we designed a germline transgene to express the murine *Pccb* cDNA under the control of a muscle specific promoter ($Tg^{MCK\text{-}Pccb}$) to rescue the $Pccb^{E14_4bpdel/E14_4bpdel}$ mice. The resultant $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{MCK\text{-}Pccb}$ animals are rescued from neonatal lethality but perish by 2 months of age. $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{MCK\text{-}Pccb}$ mice have milder biochemical perturbations of plasma methylcitrate and 3-hydroxypropionate in comparison to the $Pccb^{E7_8bpdel/E7_8bpdel}$ mice but are severely growth retarded, very much like patients with PA, and only achieve ~ 50% of the weight of unaffected littermates at weaning. To generate mice with a late onset form of PA, we used CRISPR-Cas9 gene editing to knock-in a missense mutation noted in mildly affected patients, designated $Pccb^{MS}$, into the murine *Pccb* gene, and studied $Pccb^{MS/MS}$ homozygous and compound $Pccb^{MS/E7_8bpdel}$ mice. Both $Pccb^{MS/MS}$ and $Pccb^{MS/E7_8bpdel}$ mice display attenuated metabolic changes and less impaired growth than $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{MCK\text{-}Pccb}$ animals but experience lethal metabolic decompensation when fed an increased protein diet. To demonstrate the utility of these models to test new therapies, $Pccb^{E14_4bpdel/E14_4bpdel}$ were treated with AAV9.CAG.PCCB at a dose of 1e11 vg/pup at birth, which resulted in a significant increase in survival in comparison to untreated mutant controls ($p<0.01$). We also treated $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{MCK\text{-}Pccb}$ mice at weaning with a dose of 1e14 vg/kg with either AAV9.CAG.PCCB or AAV9.EF1a.PCCB vectors, which likewise resulted in a significant increase in survival and growth in comparison to untreated $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{MCK\text{-}Pccb}$ controls . These new murine models replicate the clinical and biochemical features of PA over wide clinical spectrum, spanning neonatal, juvenile, and late onset disease presentations, and can be used to test the effects of new genomic and small molecule therapies for PCCB deficiency.

209. *In Vivo* Lentiviral Gene Therapy for Arc Syndrome

Andrei Claudiu Cozmescu[1,2], Loukia Touramanidou[1], Sonam Gurung[1], Dany Perocheau[1], Yi-Ting Hu[1], John R. Counsell[3], Rajvinder Karda[4], Simon Waddington[4], Julien Baruteau[1,2,5], Giandomenico Turchiano[1], Paul Gissen[1,2,5]

[1]Great Ormond Street Institute of Child Health, University College London, London, United Kingdom,[2]NIHR Great Ormond Street Hospital Biomedical Research Centre, London, United Kingdom,[3]Research Department of Targeted Intervention, Division of Surgery and Interventional Science, University College London, London, United Kingdom,[4]EGA Institute for Women's Health, University College London, London, United Kingdom,[5]Metabolic Medicine, Great Ormond Street Hospital for Children NHS Foundation Trust, London, United Kingdom

Arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome is a severe autosomal recessive disorder caused by mutations in the *vacuolar protein sorting 33 homolog B* (*VPS33B*) gene. Patients with ARC syndrome exhibit characteristic symptoms such as congenital joint contractures, renal tubular acidosis, and neonatal cholestatic jaundice. The genetic anomaly in the hepatocytes impairs the secretion of various bile components, leading to liver fibrosis, cirrhosis, and terminal liver disease. While liver-directed AAV gene therapy is effective in treating adults with haemophilia, sustaining long-term AAV expression in young children's livers may be challenging due to the episomal nature of the vector. Recent studies on animal models suggest lentiviral vectors might be safe for *in vivo* use and can promote persistent transgene expression. Our group has previously presented the safety and efficacy of a liver-specific LV.*coVPS33B* lentiviral vector in treating ARC syndrome's liver phenotype when administered to neonate *Vps33b*(fl/fl)xAlfp-Cre mice. Here, we aimed to improve the therapeutic impact of the LV.*coVPS33B* lentiviral vector. The results have shown that the effects of the treatment have been enhanced by transient depletion of Kupffer cells with clodronate-encapsulated liposomes before LV.*coVPS33B* administration and prolonged selective pressure imposed on treated hepatocytes by administering a 0.25% cholic acid diet for 8 weeks to *Vps33b*(fl/fl)xAlfp-Cre mice. This regimen has normalised blood parameters of liver toxicity close to wild-type values. Given ARC syndrome is a systemic disorder, we have also developed an LV.Ubq.*coVPS33B* vector that can express the therapeutic gene in multiple tissues, by the use of a ubiquitous (Ubq) promoter. Assessing the safety of this vector in 9 months old *Vps33b*(fl/-)xAlfp-Cre mice treated as neonates with the LV.Ubq.*coVPS33B*, we have observed an increased incidence of hepatocellular carcinoma (3:10) compared to (0:10) observed in the safety analysis of the LV.*coVPS33B* vector. These malignancies are currently under investigation to better understand the causative mechanism. While the novel results build a stronger case for the efficacy of the liver-specific LV.*coVPS33B* therapeutic approach, they also emphasize the importance of safety screening for newly developed lentiviral vectors.

210. Sustained FVIII Expression with a Tolerable, Titratable, Fully Non-Viral Gene Therapy for Hemophilia A

Brian Truong, Khalid Hajj, Johannes Schwerk, Dongyang Zhang, Johnnatan Tamayo, Hua Wang, Alicia Davis, Lijun Huang, Gopi Vemuri, Kevin Johnson, Sang Jun Lee, Bernard Kok, Mu Li, Pranavanand Nyshadham, Fernando L. Espinoza, James Yen, Min Tong, Joseph Lucas, Bonnie Jacques, Joshua Rychak, Blair B. Madison

Poseida Therapeutics, San Diego, CA

Primary lines of treatment for Hemophilia A (HemA) involve clotting factor replacement or bi-specific antibodies, necessitating lifelong infusions with continued risk of breakthrough bleeds and chronic joint damage. Viral-based gene therapy faces multiple challenges with prevalent pre-existing immunity, liver toxicity, and waning expression over time. Thus, there remains a high unmet need for safe, effective, and durable therapies for HemA patients. Employing our piggyBac® DNA insertion system, we highlight our novel hepatocyte-targeted lipid nanoparticle (LNP) technology. With this technology we achieve robust durability and safety. For potential individualized correction in HemA patients we also demonstrate the ability to achieve near full correction (96%) in HemA mice through repeat dosing. Overcoming historical challenges in DNA delivery, our non-viral platform efficiently delivers DNA and mRNA, combining the super piggyBac (SPB) transposase mRNA with plasmid human FVIII (hFVIII) transposon DNA (P-FVIII-101). A single dose in adult HemA mice showed sustained hFVIII expression for over 12 months, unlike non-integrated controls that declined steadily, underscoring the need for integration for lasting expression. Additional delivery enhancements, through novel formulation excipients, provided additional benefits to DNA delivery. Such enhancements are critical for FVIII delivery, which has been hindered by large transgene size and DNA toxicity at high doses. This optimization led to a 10-fold increase in hFVIII expression, addressing both efficacy and safety concerns. Typical LNPs function through passive mechanisms that result in uptake by immune cells, resulting in an immune response and toxicity. To augment hepatocyte specificity, we added N-Acetylgalactosamine (GalNAc) targeting ligands to our co-encapsulated LNP formulation and demonstrated a 2 to 3-log reduction in pro-inflammatory serum cytokines (IL-6, IFNγ). We maintained high hFVIII expression and no elevation in transaminases (ALT, AST) after single and repeat dosing in immunocompetent animals. To further demonstrate the translatability of these stacked improvements to our non-viral gene integration platform for FVIII, we tested them in rat. Adult Sprague-Dawley rats and C57Bl6 mice were administered the same LNPs. Delivery to rats resulted in 50-75% hFVIII Ag expression, which translates to anticipated therapeutic correction. This validation of Poseida's non-viral platform components—including novel ionizable lipids, targeting ligands, and excipients—highlights the capabilities of the piggyBac DNA insertion system and our non-viral approach for stable FVIII transgene expression, with potential for re-dosing, providing proof-of-principle for an effective and durable therapy for Hemophilia A.

211. Pursuing Optimal Therapeutic Conditions through *In Vivo* Lentiviral Vector Gene Therapy for Primary Hyperoxaluria Type 1

Andrea Molinos Vicente[1,2], Aida García Torralba[1,2], Virginia Nieto Romero[1,2], Alessio Cantore[3,4], Michael Tadros[5], Eduardo Salido[6], Jose Carlos Segovia[1,2], María García Bravo[1,2]

[1]Biomedical Innovation Unit, Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) and Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain,[2]Unidad Mixta de Terapias Avanzadas. Instituto de Investigación Sanitaria Fundación Jiménez. (IIS-FJD, UAM), Madrid, Spain,[3]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Milan, Italy,[4]"Vita-Salute San Raffaele" University, Milan, Italy,[5]DanausGT Biotech, Madrid, Spain,[6]Pathology Department, Hospital Universitario de Canarias, Universidad La Laguna. Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), La Laguna, Spain

Primary hyperoxaluria type 1 (PH1) is a rare autosomal recessive metabolic disorder caused by a deficiency in the liver enzyme alanine glyoxylate aminotransferase, encoded by the *AGXT* gene. PH1 patients suffer from oxalate overproduction, which can lead to end-stage renal disease and life-threatening oxalosis. Thirty percent patients show infantile onset. The only permanent curative treatment is double liver and kidney transplantation. *In vivo* lentiviral vector (LV) gene therapy has emerged as a promising strategy for liver monogenic diseases, especially for pediatric patients, taking advantage of its integrative capacity. We have previously reported that *in vivo* injection of a hepatocyte-specific LV encoding an enhanced AGT protein into *Agxt1* KO mice ($2x10^{10}$ TU/kg) showed a partial pathological phenotype reversion with an estimated transduction efficiency of 5% of hepatocytes. No further improvements were obtained with increasing doses. *In vivo* LV administration induces an IFN-alfa-mediated immune response that may interfere with LV hepatocyte transduction. The addition of a peri-injection immunosuppressive treatment to the *in vivo* LV protocol tripled the basal transduction efficiency to 15% in adult wild-type mice. Therefore, adult PH1 mice were intravenously injected with the LV expressing the enhanced AGT protein in combination with dexamethasone. These mice showed an increase in *AGXT* expression levels compared to mice treated with the LV alone, consistent with an improvement in transduction efficiency. Treated male *Agxt1* KO mice showed a 43% reduction in urinary oxalate production compared to untreated mice, reaching wild-type-like levels and a reduced number of animals developed renal damage in response to ethylene glycol treatment in both sexes. Due to the important infantile onset in PH1 together with the integrative capacity of LV, we tested whether this strategy could be efficiently implemented in juvenile mice toward application to pediatric context. *In vivo* LV injection in 3-week-old wild-type mice showed a significant improvement in hepatocyte transduction to 10%. Therefore, 3-week-old *Agxt1* KO mice were intravenously injected with the therapeutic LV and sacrificed six months later. We observed maintenance of LV integrations and detectable transgene expression, suggesting that transduced cells were maintained throughout the growth of the animals. In addition, we observed a partial pathological phenotype reversion with a 29% reduction in urinary oxalate levels in LV-treated animals compared to untreated animals together with a reduced

number of animals developing renal damage in response to ethylene glycol treatment. Immunosuppressive treatment in young animals is underway. In conclusion, we have demonstrated the therapeutic efficacy of an improved *in vivo* LV gene therapy protocol in the context of PH1, achieving a clinically relevant reversion of the pathological phenotype. Furthermore, the proposed *in vivo* LV treatment could potentially be implemented in pediatric patients. These results reinforce *in vivo* LV gene therapy as a viable option for PH1 patients.

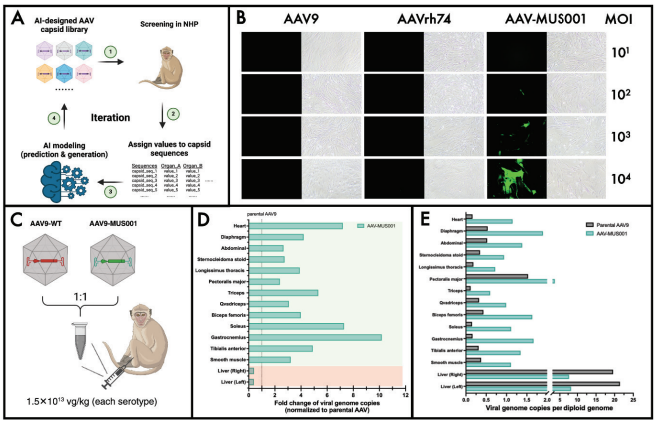
Developing the Next Generation of Muscle-Targeted Gene Delivery Vehicles via AAV Capsid Engineering

212. An AI-Generated Novel AAV Variant Targeting Pan-Muscle and Sparing from the Liver in NHP

Heng Zhang[1], Qingkai Song[2], Hui Chen[1], Zhike Lu[1], Lijia Ma[2]

[1]Westlake Genetech, Hangzhou, China,[2]School of Life Sciences, Westlake University, Hangzhou, China

Background: As of the end of June 2023, the FDA has approved seven gene therapy drugs using adeno-associated virus (AAV) as a vector. While a single injection can cure severe genetic diseases, controversy arises due to the serious side effects caused by the high-dose and price. As a gene therapy vector, AAV typically requires a dose of more than 1×10^{14} vg/kg for intravenous administration to compensate for its insufficient tropism to the therapeutic targeting organs, significantly increasing the risks and production costs. Therefore, altering the in vivo tropism of AAV vectors is one of the most crucial bottlenecks in the current gene therapy development. **Results:** We developed an AI-assisted technology platform to evolve AAV variants from a wild-type parental serotype (AAV9) with altered tropism in non-human primates (NHP) **(Fig. A)**. Data from the original screening was used to train AI models to generate the input sequences for the next iteration of NHP screening. Capsids preferentially targeting various organs emerged from the AI-generated library, including muscle, heart, CNS, et al. We validated the superior transduction efficiency of the leading capsid with muscle tropism, AAV-MUS001, in human primary muscle in vitro with the comparison to the wild-type AAV9 and AAVrh74 **(Fig. B)**. The in vivo transduction efficiency and bio-distribution of AAV-MUS001 were further validated in NHP animals with 1.5×10^{13} vg/kg dose of intravenous injection with a head-to-head comparison to the wild-type AAV9 **(Fig. C)**. The data from this experiment showed an increase in pan-muscle transduction of AAV-MUS001 compared to the wildtype AAV9. This was coupled with decreased liver tropism **(Fig. D)**. Additionally, the overall bio-distribution of AAV-MUS001 shifted from the liver to the muscles, as indicated by the viral genome copies per diploid genome **(Fig. E)**. A set of cell surface receptors was identified from CRISPR screening, which was further validated to be responsible for the high transduction efficiency of AAV-MUS001 to muscles. **Conclusion:** In this study, we demonstrated the acceleration contribution of artificial intelligence in evolving capsids. The AI-generated leading capsid AAV-MUS001 exhibited promising clinical potential to develop gene therapy drugs, which must be effectively delivered to muscle and heart with a lowered livered transduction. This platform technology, together with the in vitro and in vivo validation data, shed light on the capsid evolution and effective, safe, and accessible gene therapies drug development.



213. An Integrin-Targeting AAV Developed by a Novel Computational Rational Design Methodology Presents an Improved Targeting to the Skeletal Muscle and Reduced Tropism to the Liver

Ai Vu Hong, Laurence Suel, Isabelle Richard

GENETHON, Evry, France

Adeno-associated virus (AAV) has shown potential as a gene therapy vector due to its efficiency in delivering transgenes and low toxicity. However, its effectiveness can be limited by non-optimal tissue targeting and accumulation in the liver. High doses are often required to achieve therapeutic efficacy, particularly in the treatment of muscular diseases where the target tissue represents half the body mass. Such doses can lead to severe adverse effects, including hepatotoxicity as seen in recent clinical trials. With the primary aim to lower treatment doses, we present here a novel methodology for AAV computational rational design targeting skeletal muscle, resulting in a new myotropic AAV variant. First, integrin alpha V beta 6 (AVB6) was selected from screening of potential receptors with high enrichment in skeletal muscle tissue compared to other organs. A liver-detargeting AAV capsid backbone, a hybrid between AAV-9 and -rh74 (AAV9rh74), was engineered to bind specifically to AVB6. Inspired by recent successes of various protein design studies, we modified the entire sequence and structure of variable region IV loop from its predicted 3D structure, in such a way that the designed capsids acquire the RGDLxxL/I motif structure with low estimated energy. It allows a high-affinity binding to AVB6 but remains stable, thus better AAV production. Five AAV variants from *in silico* design were experimentally tested. Compared to AAV9rh74, all designed AAVs showed better productivity, better binding to AVB6, and greater infectivity towards human differentiated myotubes and murine skeletal muscles in vivo while entering poorly in the liver. One notable variant, LICA1, greatly increases transgene expression at 16.6/25.0-fold greater in human myotubes, and murine skeletal muscle at 13.7/129.3-fold greater than AAV9 and AAV9rh74, respectively. We further examined its efficacy, in comparison with

AAV9, in delivering therapeutic transgenes in mouse models of Duchenne muscular dystrophy and limb-girdle muscular dystrophy R3 at a low dose of 5E12 vg/kg. At this dose, AAV9 is suboptimal where it can only infect 22.1-58.1% of total muscle fibers. In contrast, LICA1 effectively delivered and expressed transgenes in 74.8% myofibers in severely affected diaphragm, or at almost 100% in other tested muscles. Consequently, LICA1 corrected pathohistology by lowering centronucleation index and fibrosis, and restored global transcriptomic dysregulation. Compared to AAV9, the anti-capsid antibody in LICA1 one-month treatment in DMD mice halves and lower titers of neutralizing antibodies were observed in a small human serum cohort indicating a lower immunogenic profile. Furthermore, compared to other published state-of-the-art myotropic AAVs, LICA1 is better at production, and targets skeletal more specifically since accumulates significantly less in the liver. These results suggest the potential of our design method for AAV engineering and demonstrated the interest of the novel AAV variant for the gene therapy treatment of muscular diseases.

214. Directed Evolution of Novel MyoAAV Capsid Variants Enabling Effective Systemic Muscle Transduction While De-Targeting the Liver in Non-Human Primates

Saurav Seshadri, Simon Ye, Chelsey Tiger, Danielle Velez, Caroline Dang, Harvir Wetzel, Tamera Toland, Brian Ferguson, Mark Fielden, Nate Jones, Sharif Tabebordbar

Kate Therapeutics, San Diego, CA

Liver toxicity has been reported following systemic administration of multiple AAV gene therapies in clinical trials. Liver safety is particularly important for muscle-directed gene therapy, where high doses are required when using naturally occurring AAV capsids.We previously reported directed evolution of the MyoAAV class of capsids, which enables highly potent systemic gene delivery to skeletal muscles and heart across species. While the first generation of MyoAAV capsids were highly effective in transducing skeletal muscle and heart and thus lowering the efficacious dose, they resulted in similar number of vector genomes per diploid genome in the liver of non-human primates (NHPs) at equivalent doses of naturally occurring capsids (e.g., AAV8 and AAV9). To develop liver de-targeted capsid variants that also transduce muscles effectively, we used the DELIVER (Directed Evolution of AAV capsids Leveraging In Vivo Expression of transgene RNA) platform to simultaneously co-evolve liver de-targeting and muscle-targeting properties. Using this strategy, we successfully evolved capsid variants that functionally transduce skeletal muscles up to 20-fold and cardiac muscle up to 11-fold more effectively than AAV9, while also having 6-10-fold lower vector genomes in the liver when tested in a pooled experiment compared to AAV9 in NHPs. The top liver de-targeted muscle-tropic capsid variants from the pooled benchmarking experiment in NHPs outperformed external engineered capsid variants used in various muscle gene therapy candidates, as well as the first generation MyoAAV capsids. Manufacturability analysis also showed that these new capsids can be produced with similar yields and can be purified by affinity chromatography with similar efficiency as AAV9. We will also present a head-to-head comparison of one of our top liver de-targeted muscle tropic capsid from this directed evolution experiment compared to AAV9 in NHPs.

215. Identification of Natural Human-Derived AAV2 Variants with Muscle Tropic Properties in Non-Human Primate Screens

Fang Zhang[1], Mengtian Cui[1], Jackson McGowan[1], Dan Wang[1,2], Jialing Yang Liang[1], Guangping Gao[1,3], Phillip Tai[1,3]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]RNA Therapeutics Institute, UMass Chan Medical School, Worcester, MA,[3]Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA

Muscle-targeting AAV gene therapy is hampered by high-dose-associated toxicity, immunogenicity, and nonspecific targeting to other organs. While methods like directed evolution and iterative rounds of selection from a complex library have recently produced several promising muscle-tropic capsids, the screening process has several inherent limitations, such as the potential for cross-packaging, capsid mosaicism, and collateral transduction. Furthermore, promising capsids discovered in this manner may have poor production yields. In this study, we sought to evaluate the ability of AAV capsid variants isolated from human tissues to robustly transduce skeletal muscles of non-human primates (NHPs) following direct muscle injections. The rationale for pursuing this route of delivery was to employ the muscle as a continuous and reversible therapeutic bio-factory, distributing secreted transgene products to diseased cells via the bloodstream or the affected muscle itself. One highlighted application is the use of rAAV to deliver genes encoding broadly neutralizing antibodies against deadly viruses causing pandemic infections. Despite being efficacious in rodent and non-human primate studies, anti-antibody responses in NHPs (and humans) present a significant hurdle, potentially hindering initial protection. To advance this strategy, we have screened our library of natural capsid for their potential to robustly transduce muscle tissues following direct intramuscular (IM) injection.Taking advantage of a relatively low complexity library, we have been employing a single variant to single barcoded non-coding RNA transgene production scheme to identify AAV capsids with favored tropism profiles. In the current study, we injected into cynomolgus macaques (n =3) a pool of ~100 AAV2 variants via intramuscular administration (vastus lateralis). After one month, tissues at the site of injection and livers were collected, and subjected to targeted amplicon-sequencing and analyses. Barcode counts among vector DNA and transgene transcripts revealed three variants that displayed strong transduction in muscle, resulting in over 400x higher levels than conferred by AAV2. We then evaluated their transduction performances in mice. The three variants showed over 12x better transduction than achieved by AAV2, and comparable to those of AAV1. This data suggests that the variants' remarkable transduction of muscle is exclusive to NHPs.In conclusion, our research has identified three AAV2 variants with enhanced transduction of non-human primate skeletal muscle. Subsequent investigations will focus on a detailed analysis, comparing the amino acid sequences of these high-performance variants, and delving into the mechanisms that

permit improved tropism in muscle. This effort aims to advance our understanding of AAV capsid biology and ultimately improve muscle targeting for gene therapy. G.G. and P.T. are corresponding authors.

216. Novel Neurotropic and Myotropic AAV Capsids for Translational Gene Therapy Applications

Irvin T. Garza[1], Vijayashree Ramesh[1], Hye Ri Kang[1], Erik A. Lykken[1], Mehmet Takar[1], Matthew C. Rioux[1], Ashwani Kumar[1], Peter Kunach[1], Ruhar Singh[1], Daniel J. Kieffer[1], Cynthia B. Trygg[2], Chao Xing[1], Lukasz Joachimiak[1], Stephen Braun[2], Steven J. Gray[1]

[1]UT Southwestern Medical Center, Dallas, TX,[2]Tulane National Primate Research Center, Covington, LA

Adeno-associated viral (AAV) vectors are generally safe and effective gene delivery vehicles. Hundreds of capsids have been identified and engineered through various means, but AAV9 remains a dominant vector with superior properties to treat neurological, neuromuscular, and muscular diseases, as demonstrated with Zolgensma, one of the first FDA approved gene therapies. While clinical trials continue to show success utilizing AAV capsids, efficiency and specificity of AAVs continue to pose a major caveat of their successful use. Manufacturing costs remain a major barrier to the translational use of AAV, and high doses of AAV particles or unfavorable biodistribution can also result in liver and DRG toxicity. These issues can be addressed with more efficient and better targeted capsid technology. We generated a novel AAV9-based library using random mutagenesis targeted to multiple specific regions across the entire capsid gene. We administered our libraries systemically (IV) and directly CNS-targeted by lumbar intrathecal (IT) administration, to mice and non-human primates (NHPs). To efficiently identify capsids with improved transduction to the CNS, we used single molecule real-time (SMRT) sequencing of capsid amplicons derived out of isolated nuclei from several tissues of the CNS and peripheral organs. To guide our hit selection for downstream validation and characterization, we developed a custom bioinformatics pipeline to analyze our large datasets and utilized computational protein modeling tools. Our lead capsid variant harbors two amino acid alterations in respect to AAV9 that are located in two distinct variable regions of the CAP gene, which was enriched following injection in both mice and NHPs. Our characterization of this lead capsid, as well as other variants, is ongoing. The lead candidate has significantly higher production yields compared to AAV9. Initial in vitro characterization, compared to AAV9, shows that in Lec2 cells, it has reduced cell surface binding kinetics, but greater overall binding capacity. It shows superior transduction, up to 6-fold of all immortalized mouse and human cell lines and iPSC-derived primary human cells including SH-SY5Y, Neuro-N2A, HEK293, HELA, induced atrial myocytes, rat ventricular myocytes, mature human cardiomyocytes, and mature human cortical neurons. We also highlight the significance of biophysical and biochemical alterations to the capsid surface in regards to viral stability (pH 7- pH 4) and zeta potential, which suggest mechanisms for increased manufacturability and decreased propensity for particle aggregation. In vivo we demonstrate notably and significantly improved transduction to the CNS and several muscle tissues in mice by either IT and IV administration. The transduction pattern was mapped in detail using two-photon tomography of the mouse brain from both IT and IV injected mice compared to AAV9. We leveraged machine learning methodologies to aide in further characterizing the molecular mechanism of action. Finally, to verify structural differences, we resolved the structure using Cryo Electron Microscopy. In summary, our novel lead candidate has enhanced manufacturability, and improved transduction in vitro and in vivo while retaining AAV9's ability to spread throughout the CNS. As we continue to collect data on this lead candidate as well as other novel capsid variants, our results highlight the advantages of whole capsid engineering and how we can utilize basic AAV biological findings to further generate novel AAVs. While our initial library screen in mice and NHPs suggests the enhanced performance of these variants across species, studies are ongoing to address this specific question.

217. An AAV Capsid Programmed to Bind Human and Macaque Receptors Mediates Enhanced Gene Delivery to the CNS and Skeletal Muscle *In Vivo*

Jason Wu, Nuria R. Botticello-Romero, Ken Y. Chan, Chin-Yen Lin, Qin Huang, Thomas Beddow, Pamela Brauer, Yujia A. Chan, Benjamin E. Deverman

Broad Institute of MIT and Harvard, Cambridge, MA

Gene therapies for multisystem disorders require efficient gene delivery to many organs. However, natural adeno-associated viruses (AAVs) have limited extrahepatic tissue tropisms, and capsid engineering has largely focused on targeting specific organs such as the central nervous system (CNS), liver, retina, or muscle. To target multiple organs, we used our recently described receptor-targeting platform to target peptide-modified AAV9 capsids to a human receptor that is broadly expressed across diverse cell types, including the CNS and muscle. These receptor-targeting capsids exhibited enhanced binding and transduction of cells in transgenic mice that expressed the human receptor. Two of the capsids, BI-TBA1 and BI-TBA2, efficiently transduced the CNS to an extent comparable to AAV-PHP.eB, a mouse CNS-targeting capsid, when intravenously administered to mice overexpressing the human receptor in the brain microvasculature (Fig. 1A). This enhanced tropism was absent in transgenic mice treated with wild type AAV9 or in mice not expressing the human receptor. When BI-TBA1 was intravenously administered to transgenic mice ubiquitously expressing the human receptor, it achieved substantially enhanced CNS and muscle transduction compared to wild type mice or transgenic mice injected with AAV9 (Fig. 1B). BI-TBA1 was also dramatically de-targeted from the liver compared to AAV9, even in animals where the human receptor was ubiquitously expressed (Fig. 1B). Notably, we demonstrated that BI-TBA1 can more efficiently transduce cells that express human or macaque, but not mouse, orthologs of the targeted receptor (Fig. 1C). Our findings show that BI-TBA1 has the potential to be a cross-species receptor-targeting AAV that can mediate enhanced in vivo gene delivery in a receptor-dependent manner. Based on the broad expression of the receptor in humans, BI-TBA1 and similar capsids may mediate enhanced gene delivery to multiple organs after systemic administration.

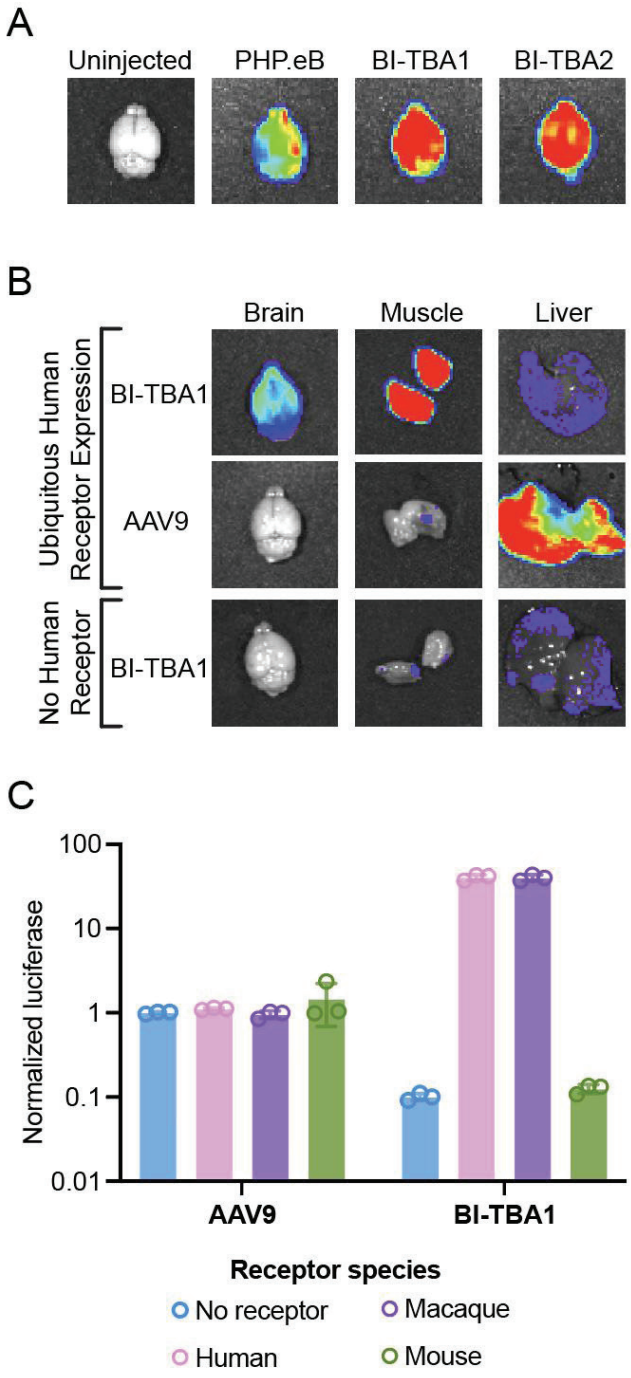


Figure 1. Human receptor-targeted AAVs efficiently transduced the brain and muscle but were de-targeted from the liver in transgenic mice. (A) Representative images show the luciferase signal in brain tissue from transgenic mice expressing the targeted human receptor in the brain vasculature injected with ssAAV-CAG-NLS-mScarlet-P2A-Luciferase-SV40pA packaged into human receptor-targeting capsids (BI-TBA1 or BI-TBA2) or AAV-PHP.eB. (B) Representative images show the luciferase signal in brain, muscle, and liver tissue from wild type mice or transgenic mice ubiquitously expressing the human receptor that were injected with BI-TBA1 or AAV9. (C) BI-TBA1 transduced CHO cells stably expressing the human or macaque orthologs of the targeted human receptor more efficiently than AAV9.

218. Retargeting of AAV Using Bispecific Antibodies

Chun Wai Yip[1], Tri Nguyen[2], Valerie Le Rouzic[3], Naveen Khan[3], Laith Zaki Alyass[1], Michael Golabek[1], Iryna Labinskyy[1], Kristen Tramaglini[4], Michael Stec[3], Yang Shen[2], Christos Kyratsous[1], Sven H. Moller-Tank[1]

[1]Regeneron Genetic Medicines, Regeneron, Tarrytown, NY,[2]Bispecifics, Regeneron, Tarrytown, NY,[3]Muscle & Metabolism, Regeneron, Tarrytown, NY,[4]Research Program Management, Regeneron, Tarrytown, NY

Adeno-associated virus (AAV)-based gene therapies offer potential treatments for a wide range of genetic diseases through the delivery of therapeutic cargo that correct or replace defective genes. However, effective delivery of AAV to peripheral tissues with natural serotypes remains a challenge and often requires very high doses that carry risks of cardiac and liver toxicity. To address this, Regeneron has developed platforms to target AAV to specific tissues using antibodies. Antibodies can bridge AAVs to target receptors specifically expressed on cells and tissues of interest, thereby enhancing AAV binding and uptake. When combined with capsid mutations to ablate glycan binding, significant detargeting from liver can be achieved, thereby improving the safety profile. Targeting receptors for which expression is conserved across species also preserves translatability of this approach from small animal models to NHPs and humans. Previously, we demonstrated AAV targeting to CNS and muscle with our covalent antibody-retargeting platform using Transferrin receptor 1 (TfR1) and calcium voltage-gated channel auxiliary subunit gamma 1 (CACNG1) as target receptors. We have validated these receptors for AAV targeting in humanized mice, diseased mice, and non-human primates. In parallel to the successful engineering of our covalent antibody-AAV platform, we have developed a platform to target these receptors with anti-AAVxCACNG1 and anti-AAVxTfR1 bispecific antibodies. As of today, there has been very limited use of bispecifics to target therapeutics *in vivo*. With intensive screening of antibodies, development of novel antibody formats, and identification of robust targeting receptors, we have engineered bispecifics capable of effective AAV targeting to CNS and skeletal muscle in mice. By incubating our bispecifics with AAV prior to administration, AAV:antibody complexes can achieve greater than 10-fold increased on-target transduction as compared to AAV alone. Enabled by our broad serotype binding anti-AAV arms, the same bispecific can similarly improve transduction of multiple different serotypes. This represents a significant milestone for the bispecific targeting approach. Importantly, this platform has the unique property of being "plug and play" and can be readily combined with existing AAV programs to improve on-target transduction efficacy.

Exosomes, Virus like Particles, and LNPs

219. Durable DNA-Based Gene Therapy Using the Non-Viral Fusogenix Proteolipid Vehicle Platform

Douglas Brown[1], Arun Raturi[1], Ping Wee[1], Prakash Bhandari[2], Amirali Bukhari[2], Maryam Hejazi[1], Jitendra Kumar[2], Roy Duncan[3], John Lewis[2]

[1]Entos Pharmaceuticals, Edmonton, AB, Canada,[2]Entos Pharmaceuticals, San Diego, CA,[3]Dalhousie University, Halifax, NS, Canada

Genetic medicines hold great promise to treat a wide array of diseases, yet success in the clinic has been hindered by limitations in the tolerability, biodistribution, scalability, and immunogenicity of current delivery platforms. We developed a novel delivery platform that overcomes these limitations by combining aspects from viral and non-viral platforms. The Fusogenix™ proteolipid vehicle (PLV™) incorporates fusion-associated small transmembrane (FAST) proteins isolated from fusogenic orthoreoviruses into a well-tolerated lipid formulation using a scalable microfluidic mixing approach. Incorporation of FAST protein into an optimized lipid formulation was found to significantly increase the expression of plasmid DNA (pDNA) *in vitro* and *in vivo*, without accompanying increases in toxicity. Utilizing the FAST-PLV platform for systemic delivery of pDNA, we confirmed successful pDNA delivery to numerous extra-hepatic tissues, such as muscle, lungs, and kidneys, in both murine and non-human primate models. This led to significant gene expression, as confirmed via histological examination and PCR analysis. To establish a suitable benchmark, we compared the systemic delivery of pDNA to a clinically approved lipid nanoparticle (LNP) formulation. The FAST-PLV platform displayed exceptional tolerability, without displaying any signs of liver toxicity or elevated cytokine levels that were observed with the LNP formulation - all without sacrificing pDNA expression. Importantly, the FAST-PLV platform displayed low immunogenicity and sustained efficacy, establishing it as an ideal candidate for durable gene therapy approaches. Here we report two gene therapies whereby pDNA vectors encoding follistatin (pDNA-FST) and leptin (pDNA-Lep) are encapsulated and delivered systemically using the FAST-PLV platform. A single intravenous injection of wild-type mice with pDNA-FST increased circulating follistatin levels, leading to significant enhancements in hindlimb grip strength and body weight three months post-injection. Eight months after pDNA-FST administration, post-mortem analysis confirmed notable gains in muscle mass compared to control animals. Similarly, intravenous injection of leptin-deficient mice with pDNA-Lep resulted in a sustained increase in circulating leptin levels, extending beyond a three-month duration. This was associated with significant improvements in body weight and metabolic function relative to control animals. The favorable activity and safety profile of the FAST-PLV platform defines a new standard for redosable gene therapies and genetic medicines.

220. Engineered Virus-Like Particles for Transient Delivery of Prime Editor Ribonucleoprotein Complexes *In Vivo*

Meirui An[1,2,3], Aditya Raguram[1,2,3], Samuel W. Du[4,5], Samagya Banskota[1,2,3], Jessie R. Davis[1,2,3], Gregory A. Newby[1,2,3], Paul Z. Chen[1,2,3], Krzysztof Palczewski[4,5,6,7], David R. Liu[1,2,3]

[1]Merkin Institute of Transformative Technologies in Healthcare, Broad Institute, Cambridge, MA,[2]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA,[3]Howard Hughes Medical Institute, Harvard University, Cambridge, MA,[4]Gavin Herbert Eye Institute, Center for Translational Vision Research, Department of Ophthalmology, University of California, Irvine, CA,[5]Department of Physiology and Biophysics, University of California, Irvine, CA,[6]Department of Chemistry, University of California, Irvine, MA,[7]Department of Molecular Biology and Biochemistry, University of California, Irvine, CA

Prime editing enables precise installation of genomic substitutions, insertions and deletions in living systems. Efficient in vitro and in vivo delivery of prime editing components, however, remains a challenge. Here we report prime editor engineered virus-like particles (PE-eVLPs) that deliver prime editor proteins, prime editing guide RNAs and nicking single guide RNAs as transient ribonucleoprotein complexes. We systematically engineered v3 and v3b PE-eVLPs with 65- to 170-fold higher editing efficiency in human cells compared to a PE-eVLP construct based on our previously reported base editor eVLP architecture. In two mouse models of genetic blindness, single injections of v3 PE-eVLPs resulted in therapeutically relevant levels of prime editing in the retina, protein expression restoration and partial visual function rescue. Optimized PE-eVLPs support transient in vivo delivery of prime editor ribonucleoproteins, enhancing the potential safety of prime editing by reducing off-target editing and obviating the possibility of oncogenic transgene integration.

221. New Biological Insights into Extracellular Vesicle Associated AAV Help Improve Vector Yield and Provides Rational for Intraluminal AAV Packing

Ming Cheng[1], Demitri de la Cruz[1], Carrie Ng[1], Zachary Elmore[2], Aravind Asokan[2], Casey A. Maguire[1]

[1]The Massachusetts General Hospital, Charlestown, MA,[2]Duke University, Durham, NC

Introduction. Extracellular vesicle-associated adeno-associated virus vectors (EV-AAV) represent a unique population of vectors generated during production in 293 cells, in contrast to conventional non-enveloped AAV capsids. Several studies have provided experimental evidence that EV-AAV provides desirable gene delivery aspects such as greater resistance to antibody neutralization and transduction of organs and tissues *in vivo* in mice. Despite these promising data, there is a great deal of characterization of the EV-AAV system that needs to be performed. Free conventional AAV vectors co-isolating with EV-AAV has hampered the definition of accurate yields and functional activity of EV-AAV. **Results.** Here we use an optimized density gradient to answer some outstanding questions regarding EV-AAV characteristics, as well as using it to assess optimization of the production system.

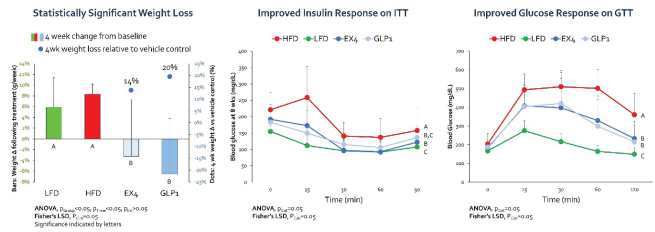
Using a variety of molecular biology and biochemistry methods, we determined that gradient fractions 1-6 contained EV-AAV and fractions 7-10 conventional AAV. First, we quantitated the percentage of AAV genomes on the gradient were in the EV-AAV fractions of AAV serotypes 1, 2, 5, 6, and 9. We found the average value ranged from 2.4% (AAV5) to 11.95% (AAV6). AAV6 and AAV9 had the highest total yields of genomes in the EV-AAV fractions. Next, we assessed whether intraluminal EV-AAV9 could mediate functional transduction of cells (as opposed to AAV9 bound to the surface of EVs, or co-isolating free AAV9 capsids). To do this we performed an immunoprecipitation using an anti-AAV9 antibody conjugated to magnetic beads. Interestingly, we observed that approximately 20% of AAV genomes in the EV-AAV9 pooled fractions were resistant to AAV9-pulldown, suggesting a substantial portion of EV-AAV9 are intraluminal (with no bound AAV on the EV surface). To understand if these membrane-encapsulated vectors could transduce cells and resist antibody neutralization we performed neutralization assays using intravenous immunoglobulin (IVIg) and HeLa cells. We compared transduction in the presence or absence of IVIg with conventional AAV9, purified EV-AAV9, and EV-AAV9 with all surface-bound or free AAV removed by anti-AAV9 pulldown (ultra-pure EV-AAV9, UP-EV-AAV9). Interestingly, we found that in the absence of IVIg, UP-EV-AAV9 gave slightly higher (1.3-fold) transduction efficiency to EV-AAV9 and 2.5-fold higher transduction efficiency than conventional AAV9. UP-EV-AAV9 was similarly resistant to antibody neutralization to EV-AAV9 and much more resistant (6-fold at a 1:500 dilution of IVIg) to conventional AAV9. Finally, we tested whether heterologous expression of membrane associated accessory protein (MAAP) from AAV8 (MAAP8) with an AAV9 Cap (AAV9 MAAP null) production system would alter yields of EV-AAV9. Interestingly, we found that MAAP8/AAV9 Cap increased yields of EV-AAV9 by 3-fold compared to the cis-expression of AAV9 Cap/AAV9 MAAP. **Conclusions.** Gradient purification allowed us to accurately quantitate EV-AAV and conventional AAV yields in producer cell media. Our anti-AAV9 pulldown assay suggests that AAV9 capsids inside EVs are functional at transduction and therefore do not require AAV9 on the EV surface to mediate transgene expression. The antibody neutralization data also suggests that surface bound AAV9 capsids are not required for antibody evasion by EV-AAV as UP-EV-AAV9 was similarly resistant to IVIg as EV-AAV9. Thus, strategies aimed at increasing AAV packing inside EVs are warranted. The experiments with heterologous expression of MAAP8 suggest that yields of EV-AAV9 can be improved using this approach. In sum, these data provide important information for the further development of the EV-AAV vector system.

222. Development of a First-in-Class Adjustable Dose Gene Therapy Platform and its Application to the Treatment of Type 2 Diabetes and Obesity

Alexei Goraltchouk[1], Jared Lourie[2], Judith Hollander[1], Francesco Luppino[1], Alexey Seregin[1], Kai Zou[2]

[1]Remedium Bio, Inc., Needham, MA,[2]University of Massachusetts Boston, Boston, MA

Introduction: Despite enormous therapeutic potential, gene therapy is limited by an inability to adjust the dose following initial administration, high cost, antigenicity, and systemic toxicity. To overcome these challenges, we developed the first dose adjustable gene therapy platform with optimized specificity, localization, biocompatibility, and cost profile. The purpose of the study was to examine the efficacy of the new gene therapy and its application in a mouse model of Type 2 Diabetes (T2D). **Methods:** A novel, lipid nanoparticle (LNP) delivery vector was designed and characterized by dynamic light scattering for size, zeta potential, and polydispersity. System transfection efficiency and cytocompatibility was assayed *in vitro* using primary human adipocytes and preadipocytes. Biodistribution was characterized by in vivo imaging (IVIS), qPCR, and immunohistochemistry in C57BL/6N and B6N-*Tyr*$^{c\text{-}Brd}$/BrdCrCrl albino mice received subcutaneous injections of LNP. Durability, immunogenicity, and dose adjustability were confirmed via bioluminescent reporter transgene quantification. Efficacy was confirmed in a high fat diet (HFD) and low-dose (40mg/g BW) streptozotocin (STZ) induced T2D model by quantifying body weight, food intake, insulin sensitivity, and glucose tolerance following treatment with LNP-DNA-Exenatide or LNP-DNA-GLP1 relative to vehicle controls. **Results:** The optimized system demonstrated high transfection efficiency of primary human adipocytes and pre-adipocytes, reaching 81.3% +/-8.3% without compromising cytocompatibility. The vector was able to efficiently encase and deliver a broad range of genetic cassette sizes (1,871-6,203 bp) without impacting critical physico-chemical parameters including size, zeta potential, and polydispersity. Durable, localized, and adipocyte-specific expression was observed *in vivo* over 6 months, with no detectable transgene in the liver, heart, spleen, or kidney. Transgene expression was adjustable using several physical and pharmacological means, including cryolipolysis, focused ultrasound, and an inducible suicide gene system. A single treatment regimen expressing Exenatide or GLP1 produced pronounced weight loss, improved insulin response, and glycemic control in T2D mice relative to baseline and vehicle control. **Conclusion:** We report, for the first time, the development of a novel, ultra-low-cost, dose adjustable gene therapy platform and its application to the treatment of type 2 diabetes and obesity in a rodent model of disease.



223. Exosome-Mediated Intracellular Delivery of Arginase 1: A Potential Application for the Treatment of Arginase 1 Deficiency

Li-En Hsieh, Mafalda Cacciottolo, Michael LeClaire, Christy Lau, Kristi Elliott, Minghao Sun

Capricor Therapeutics, San Diego, CA

Arginase 1 deficiency (ARG1-D) is a rare genetic disorder caused by the loss of arginase 1 (Arg1), the final enzyme in the urea cycle. Hepatocytes of ARG1-D patients are impaired in converting ammonia into urea, resulting in elevated arginine and ammonia in blood and cerebrospinal fluid, which leads to progressive neurological symptoms. Current therapeutic strategies mainly focus on managing the plasma

arginine and ammonia but the long-term outcomes remain poor. Currently, there is no approved treatment specific for ARG1-D available in the United States. Exosomes, small extracellular vesicles involved in cell communication, are emerging as a powerful therapeutic vehicle. Using Capricor's Stealth-X™ platform, human Arg1 enzyme was engineered for expression on exosome membrane with either intra- or extra-exosomal orientation. The produced Arg1-exos were evaluated for their in vitro functionality as a disease-specific approach for the treatment of ARG1-D. Incorporation of Arg1 protein into the exosomes was confirmed by flow and Jess Western Blot. Regardless its localization on the exosome membrane, Arg1 was biologically active and able to convert arginine into urea. Surprisingly, nanograms of Arg1 delivered by exosomes were as potent as micrograms of human recombinant Arg1 protein. In addition, engineered exosomes carrying intra-exosomal Arg1 were capable of delivering Arg1 protein into 293F and HepG2 cells in a time and dose-dependent manner, contrary to human recombinant Arg1 protein alone. The data suggest that Arg1 exosomes were able to not only catalyze arginine efficiently at a lower dose but also protect the Arg1 protein to be delivered into target cells and retain its enzymatic activity in vitro. Taken together, Stealth-X™ platform engineered Arg1 exosomes have the potential to serve as an enzyme replacement therapy to deliver Arg1 to the target cell, the hepatocytes, and possibly have broader clinical benefits for the ARG1-D patient. The in vivo activities of Arg1 exosomes are currently under evaluation.

224. Engineering Endogenous Loading Methods for Therapeutic RNA Cargo into Extracellular Vesicles

Steven M. Jay

Fischell Department of Bioengineering, University of Maryland, College Park, MD

Extracellular vesicles (EVs) are an exciting platform for the delivery for a variety of therapeutic RNAs, but a major limitation is their low level of native RNA content. Strategies to improve RNA loading into EVs have been heavily investigated, as doing so would improve efficacy and reduce the need for repeated dosing, thus limiting the potential for immunogenicity and increasing the safety profile. Initial investigations of exogenous approaches such as electroporation or sonoporation have been expanded in recent years to include endogenous strategies that involve genetic engineering of EV producer cells. These latter methods are expected to be more scalable and cost-effective, but it has been difficult to match the effectiveness of exogenous loading to date. Our group has pursued development of novel endogenous loading methods across the spectrum of therapeutic RNAs, with successful adaptations for both small RNAs such as microRNAs (miRNAs) as well as for long non-coding RNAs (lncRNAs). With respect to lncRNAs, we have shown that RNA affinity sequences can be effectively adapted to increase EV loading of these large molecules. We have successfully delivered lncRNA HOTAIR and demonstrated increased efficacy in vivo in a wound repair model and we have further refined our approach to improve loading by >200 fold over a simple transfection approach. We have further demonstrated that this benefit can be retained for a wide variety of lncRNAs, including CARINH which we are currently testing in an animal model of colitis. With respect to miRNAs, we have engineered the microRNA biogenesis pathway for enhanced EV loading of miR-146a-5p by manipulating its interactions with the miRNA biogenesis enzymes RNA Polymerase, Drosha, Exportin-5, Dicer, and Ago2. We found that independence from interaction with either Dicer or Drosha (but not both) enabled increased EV localization of miR cargo. We also showed that altering the non-seed nucleotide sequence of miRs to allow for efficient transcription under the U6 promoter preceded by a cytomegalovirus (CMV) enhancer in an expression plasmid increases EV miR loading. Overall, these advances bring endogenous loading of cargo into EVs closer to parity with exogenous methods and generally increase the translational potential of EVs.

225. Utilizing the Endogenous NF1 Promoter for Widespread Cell Specific Expression

Rrita Daci[1], Simon Wentworth[2], Tyler Mola[3], Ana Rita Batista[2], Miguel Sena-Esteves[2]

[1]Neurosurgery, UMass Chan Medical School, Worcester, MA,[2]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[3]Neurology, UMass Chan Medical School, Worcester, MA

Introduction: Neurofibromatosis Type 1 (NF1) is among the most common human monogenetic diseases. These patients suffer from benign and malignant tumors, namely neurofibromas, gliomas, and malignant peripheral nerve sheath tumors (MPNST), among other cancers and diseases. While the monogenetic nature of NF1 makes it a good candidate for gene replacement therapy, there has been a paucity in its development for a variety of reasons. First, the NF1 coding sequence is ~8.5 kb, vastly exceeding the packaging capacity of adeno-associated virus (AAV) vectors. Secondly, neurofibromas are diffusely distributed throughout the body and arise from Schwann cells, which reside inside the avascular endoneurium making access of AAV to the cells challenging. Thirdly, neurofibromatosis affects patient's cerebrovasculature, as exemplified by the higher incidence of Moya-Moya in this group, and affects patients neurocognitive state, as exemplified by the higher presence of intellectual disabilities in these patients. Previous data in the literature has shown that the ubiquitously used Cag promoter is weak or non-functional in Schwann cells after systemic delivery of AAV9 vectors. In contrast, the rat P0 (from *Mpz* gene) promoter in AAV9 drives high efficiency gene expression in peripheral nerve by systemic administration. **Methods:** To overcome the obstacle associated with cell specific targeting and to better target both Schwann cells and neurons, the Sena-Esteves lab has analyzed publicly available single cell ATACseq data to identify regions of open chromatin with exceptionally high homology in NF1 genes across species. We have selected two sequences that incorporate human sequences with high homology to the mouse gene and identify them hereafter as NF1pL (long) and NF1pS (short). Ideally, we would use a promoter capable of driving gene expression in both Schwann cells and elsewhere in the body to mimic the ubiquitous expression profile of the NF1 gene. Five AAV9-GFP vectors were created utilizing the Cag, rat p0, Mecp2, and the novel NF1pL and NF1pS promoters. Adult mice were injected with $2x10^{12}$vg via tail vein injection with each vector-promoter combination (n=6 per group; 5 groups). There were equal numbers of male and female mice in each group. Endpoint was 6 weeks post injection, at which point the mice were necropsied. Analysis of liver, heart, brain, spinal cord, sciatic nerve and muscle was done with immunohistochemistry for GFP protein expression and RT-qPCR for GFP RNA expression. **Results:** As expected, all promoters had higher expression of liver than in any other organ. However, the

Cag promoter showed higher expression in the liver than all other promoters examined. Relative to the Cag promoter, other promoters appear to de-target the liver. The p0 promoter showed the least amount of expression in all tissue, except spinal cord and sciatic nerve, where the expression was similar across promoters. The novel NF1pL and NF1pS promoters showed similar expression profiles to the Mecp2 promoter across all organ groups. Immunohistochemistry of the brain of mice treated with AAV9-Cag-GFP showed a strong characteristic glial pattern of staining. However, immunohistochemistry in the brain of mice treated with other promoters showed predominantly neuronal staining. **Conclusion:** Due to widespread disease affecting multiple organ systems, treatment of NF1 requires targeting of several specific cell types including Schwann cells, endothelial cells, and neurons. Our preliminary results from treatment of 30 mice with 5 different promoters suggest that expression can be guided by unique and novel endogenous *NF1* promoters.

Cancer Immunotherapy and Cancer Vaccines

226. Interferon Alpha Gene Therapy Alters Tumor Metabolism in Bladder Cancer

Sharada Mokkapati[1], Alberto Martini[1], Come Tholomier[1], Seppo Yla-Herttuala[2], Nigel R. Parker[3], David McConkey[4], Colin P.N. Dinney[5]

[1]Department of Urology, The UT MD Anderson Cancer Center, Houston, TX,[2]Department of Urology, University of Eastern Finland - Kuopio Campus: Ita-Suomen yliopisto - Kuopion Kampus, Kuopio, Finland,[3]Hamesman Limited, Chinnor, United Kingdom,[4]Department of Urology, Greenberg Bladder Cancer Institute, Baltimore, MD,[5]M. D. Anderson Cancer Center, Houston, TX

Introduction IFNα gene therapy (Nadofaragene firadenovec) has been approved by the FDA in BCG Unresponsive Non-Muscle Invasive Bladder Cancer (NMIBC). Understanding and targeting mechanisms of resistance and identifying biomarkers predictive of response is a top priority. Using cell lines, preclinical models, and clinical samples we identified changes in the tumor metabolic pathway involved in fatty acid biosynthesis as one of the key pathways altered in end-stage tumors. FASN is a key enzyme in this biosynthetic pathway and was downregulated in both murine models and in human bladder tumors. To define the role of this gene in IFNα gene therapy, we generated conditional knockout cells for FASN in two bladder cancer cell lines: UC14 and RT112. We characterized these cells to better understand mechanisms that drive acquired resistance to IFNα gene therapy. We generated lipid profiles of urine from patients enrolled in phase 2 clinical trial to identify lipid biomarkers predictive of response. **Methods** Murine bladder cancer (BLCA) cell lines were treated with recombinant IFN (rIFNα) or Lentiviral IFNα (LV-IFNα) and sequencing was performed to identify differentially altered genes/pathways. Subcutaneous and intravesical tumor models were used to assess the effect of IFNα on tumor metabolism. Lipidomics, glucose uptake, and lactate levels were measured following treatment with IFNα in murine models. Lipidomics was carried out on phase 2 Nadofaragene firadenovec urine samples. We **Results** We identified several genes involved in fatty acid synthesis that were downregulated and several genes involved in glycolysis that were upregulated following treatment with IFNα in cell lines, in mouse models, and in human tumors treated with Nadofaragene. Q-PCR analysis was used to confirm changes in these genes. We performed lipidomics on mouse tumors treated with poly (I:C), an IFNα surrogate, and identified 79 lipids that were upregulated and 12 lipids that were downregulated. Three major classes of lipids were upregulated; phosphotidyl choline, spingomyelin and phosphotidyl ethanolamine; and one major class of lipid cardiolipin was downregulated. We also performed a preliminary analysis of lipidomics on urine samples collected from patients enrolled on the phase 2 Nadofaragene trial. We selected 7 responders and 6 non-responders and subjected urine samples collected at day1 (pre-treatment) and day12 (post-treatment) for lipidomics analysis. >592 lipids were detected in the urine samples tested. Our results show that the lipid profile is distinct between the responders and non-responders before treatment (day 1 samples) and the lipid profile is altered in non-responders after treatment. Finally, we found increased glucose uptake, and lactate levels suggesting increased glycolysis in BLCA cells treated with IFNα. **Conclusion** We uncovered a novel mechanism by which IFNα modulates tumor cell metabolism which may contribute to the development of resistance. Combining IFNα-based therapy with approaches targeting tumor cell metabolism might further facilitate in improving survival and overcome resistance.

227. Targeting AML with Naturally Occurring CD7 Negative T Cells Expressing CD7 CAR

Unmesha Thanekar[1], Chris Nevitt[1], Jaquelyn T. Zoine[1], Maksim Mamonkin[2], Masayuki Umeda[3], Jeffery Klco[3], M. Paulina Velasquez[1]

[1]Bone Marrow Transplant and Cell Therapy, St. Jude Children's Research Hospital, Memphis, TN,[2]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX,[3]Pathology, St. Jude Children's Research Hospital, Memphis, TN

Introduction: Finding the most effective surface antigen to develop chimeric antigen receptor (CAR) T cells for acute myeloid leukemia (AML) is challenging due to the high degree of overlap in antigen expression between AML blasts and normal hematopoietic progenitor cells (HPCs), leading to potential myelotoxicity. CD7 is a promising target as it is expressed in 30% of AML cases, correlates with worse prognosis yet is not expressed on HPCs. However, its expression on normal T and NK cells introduces the risk of fratricide. To bypass this fratricide, we have devised a strategy to select naturally occurring CD7-negative T cells and genetically modify them to express CD7CARs ($CD7CAR^{CD7-}$ T cells). Here we show that $CD7CAR^{CD7-}$ T cells effectively target AML. **Methods:** We mined a pediatric AML dataset to determine differential gene overexpression of CD7 in AML. We isolated CD7-negative T cells from peripheral mononuclear blood cells using a two-step magnetic bead separation process followed by selection of $CD3^+$ T cells from the CD7-negative fraction. These T cells were subsequently transduced with a CD7.CD28z retroviral vector and expanded with IL7-/IL15. Non transduced (NT) and CD19 CAR expressing CD7 negative T cells served as controls. We performed immunophenotypic analysis using flow cytometry and assessed effector function *in vitro* and *in vivo*. **Results:** We determined differential gene

overexpression of CD7 in a pediatric AML dataset comprising 887 TARGET and St. Jude cases and confirmed CD7 surface expressions in a panel of AML cell lines, PDX and pediatric AML blasts. We genetically modified CD7-negative T cells to express CD7 or CD19 CAR, with CAR expression ranging from 62.90% to 95.30% (N=4). CD7CAR^{CD7-} T cells predominantly showed CD4+ effector memory immunophenotype. They maintained viability over 85% and exhibited expansion kinetics similar to that of CD19-CAR and NT groups (N=4, p=ns), indicating the absence of fratricide. To assess effector function, we co-cultured CD7CAR^{CD7-} T cells with CD7+ AML cells (KG1a and THP-1). CD7CAR^{CD7-} T cells recognized and killed AML cells *in vitro*, as evidenced by consistent tumor lysis in 24-hour and repeat stimulation assays and by increased cytokine secretion in the presence of CD7+ targets ($p<0.0001$, N=4). To test anti-tumor activity of CD7CAR^{CD7-} T cells in an *in vivo* KG1a model, mice received a single iv dose of 3x10e6 T cells 7 days after tumor injection. CD7CAR^{CD7-} T cells substantially reduced tumor burden and showed significant survival advantage ($p<0.0001$) compared to tumor-only and control CAR T cells (CD19CAR^{CD7-}). **Conclusion:** Here we demonstrate that CD7 is overexpressed at a transcriptomic and protein level in a cohort of pediatric AML blasts. We show, for the first time, that naturally occurring CD7-negative T cells expressing CD7 CARs can effectively recognize CD7+ AML and have robust anti-tumor activity *in vitro* and *in vivo,* presenting a promising strategy for AML therapy.

228. A mRNA Lipid Nanoparticle Incorporated Nanofiber-Hydrogel Composite Generates a Local Immunostimulatory Niche for Cancer Immunotherapy

Yining Zhu, Zhi-Cheng Yao, Shuyi Li, Jingyao Ma, Christine Wei, Xiaoya Lu, Hai-Quan Mao

Johns Hopkins University, Baltimore, MD

Introduction: Immunotherapy has witnessed a paradigm shift recently with the advent of mRNA LNPs as an accelerated approach to combat cancer. The success of the two mRNA vaccines has demonstrated the effectiveness of stabilized mRNA constructs to deliver the encoded antigen(s) and the safety and potency of LNPs in delivering the gene therapeutics and vaccines to relevant cells in vivo. Nonetheless, the realization of the full potential of mRNA LNP cancer vaccines requires further optimization of LNP immune activation profiles and delivery strategies for more efficient programming of immune responses for potent efficacy. Herein, we propose an off-the-shelf mRNA LNP-loaded nanofiber-hydrogel matrix (LiNx), which promotes endogenous immune cell infiltration and creates a programmable immunostimulating microenvironment, therefore maximizing antigen presentation. Previously, we analyzed 1,080 LNPs to boost transfection efficiency in antigen-presenting cells (APC).[1] Our findings indicate that precise tuning of LNP composition enables the modification of transgene expression in both APCs and non-APCs, thereby influencing the elicited immune activation profile. In this study, we further loaded the selected D6 LNP into a previously developed nanofiber-hydrogel composite (NHC).[2] This configuration yields a more robust immune response with a single-dose vaccination in mouse colon carcinoma and melanoma models (**Fig. 1a**). **Methods:** To prepare LiNx, the synthesized NHC microgel (G′ of ~250 Pa) was combined with LNPs using a syringe extrusion method.[2] The kinetics of host cell recruitment was examined following injection of LiNx on C57BL/6 mice (30 μg mOVA per mouse, s.c.). To assess cell transfection, Ai9 mice were s.c. administered with LiNx (30 μg mCre per mouse). For tumor inhibition studies, mice aged 6-8 weeks were injected with MC38-OVA or B16-OVA cells (s.c.). Tumor growth was monitored every two days. **Results:** To investigate host cell recruitment in LiNx, we injected mOVA LiNx into mice and harvested composites at 3- and 7-days. Incorporating D6 LNP significantly increased host immune cell recruitment compared to LiNx itself (**Fig. 1b**). A substantial number of APCs, particularly macrophages, were detected on day 3 (**Fig. 1c**). We further assessed the delivery efficacy of LiNx using Ai9 mice and found that D6 LNPs loaded in the LiNx resulted in substantial tdTom$^+$ APCs in composites (**Fig. 1d**). The anti-tumor effect of D6 LiNx was investigated in MC38-OVA and B6-OVA therapeutic tumor models (**Fig. 1e**). D6 LiNx demonstrated potent tumor suppression, resulting in a median survival time of 75 days with 50% of mice remaining tumor-free beyond 100 days (**Fig. 1f**). Notably, D6 LiNx outperformed free LNPs (three doses, median survival time of 37.5 days), emphasizing the superior efficacy of this single-dose hydrogel vaccine. Additionally, D6 LiNx was assessed in a prophylactic melanoma model (**Fig. 1g**), showing a prolonged median survival time of 30 days (**Fig. 1h**).

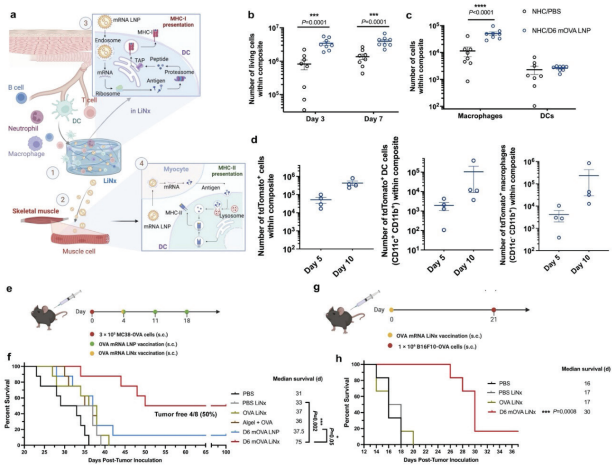


Fig. 1 | a, Schematic of the LiNx vaccination platform. **b-c,** The kinetics of host cell recruitment following injection of LiNx. **d,** Transfection of immune cells in the composite was analyzed using Ai9 mice. **e–h,** Schematic and results of therapeutic or prophylactic cancer vaccine models.

Conclusion: We engineered an mRNA LNP-incorporated microgel matrix as a programmable immunostimulating matrix that is capable of potentiating antigen expression, presentation, and immune cell cross-talks, and delivering potent antigen-specific immune responses and efficacy in murine cancer models. **Reference:** 1. Zhu, Y., et al. Nat. Biomed. Eng (2023). 2. Z.-C. et al. Small 2022, 18, 2202309.

229. Targeting EphA3 on Glioblastoma Stem Cells with CART Cell Therapy to Overcome GBM Recurrence

Ekene J. Ogbodo[1], Reona L. Sakemura[1], Elizabeth L. Siegler[1], Claudia Manriquez-Roman[1], Truc Huynh[1], James H. Girsch[1], Jennifer M. Feigin[1], Olivia L. Sirpilla[1], Kun Yun[1], Carli M. Stewart[1], Hong Xia[1], Omar Gutierrez Ruiz[1], Lionel Kankeu Fonkoua[2], Mehrdad Hefazi[1], Michael W. Ruff[3], Saad S. Kenderian[1]

[1]Hematology, Mayo Clinic, Rochester, MN,[2]Medical Oncology, Mayo Clinic, Rochester, MN,[3]Neurology, Mayo Clinic, Rochester, MN

Glioblastoma (GBM) is the most lethal and common form of brain cancer, with a median survival of < 2 years. Despite aggressive treatment strategies including surgical resection, radiation, and chemotherapy, GBM is often resistant to or recurs after these standard therapies. GBM stem cells (GSCs) have been demonstrated to be the major player in the recurrence and maintenance of GBM tumors. Ephrin receptor A3 (EphA3), a member of the Eph family, is overexpressed in multiple cancers, including GBM. Additionally, EphA3 has been shown to be expressed on GSCs. Given the strong expression of EphA3 on GBM and GSCs and minimal expression on healthy tissue, we sought to develop a targeted therapy by engineering chimeric antigen receptor T (CART) cells against EphA3. CART cell therapy has shown remarkable outcomes in certain hematological cancers, but clinical success in solid tumors remains a challenge. We hypothesized that targeting GSCs with EphA3-CART cells will result in durable antitumor efficacy in preclinical models of GBM. We first isolated GSCs from GBM patient-derived xenografts (PDX) and confirmed high expression of EphA3 by flow cytometry (>90% EphA3$^+$). We generated a CD28-costimulated EphA3-CART cells through lentiviral transduction of healthy donor T cells (>90% transduction efficiency). We then evaluated *in vitro* effector functions of EphA3-CART cells in cocultures with GSCs. EphA3-CART cells demonstrated potent anti-GSC activity compared to untransduced T cell negative control (UTD), as determined by CART cytotoxicity ($p<0.0001$, Fig. 1A), proliferation ($p<0.0001$, Fig. 1B), degranulation ($p<0.0001$, Fig. 1C), and cytokine production ($p<0.01$, Fig. 1D). We then assessed the anti-GSC of EphA3-CART cells *in vivo* by orthotopically implanting 1 x 10^4 luciferase$^+$ GSCs into the brains of NOD-SCID-$\gamma^{-/-}$ mice. Tumor engraftment was confirmed by bioluminescence imaging (BLI), and mice were randomized and treated intravenously with 5 x 10^6 EphA3-CART or UTD control. Tumor burden was monitored by serial BLI, and T cell expansion was determined through peripheral blood sampling. This *in vivo* orthotopic model showed that treatment with EphA3-CART cells resulted in a significant reduction in tumor burden ($p<0.0001$, Fig. 1E), increased CART cell expansion ($p<0.01$, Fig. 1F), and prolonged survival ($p<0.01$, Fig. 1G) compared to UTD treatment. In conclusion, we established efficient targeting of GSCs with EphA3-CART cell therapy. Given the importance of GSCs in contributing to GBM resistance to and recurrence after conventional therapies, EphA3-CART cell therapy demonstrates significant promise in overcoming these challenges in GBM treatment.

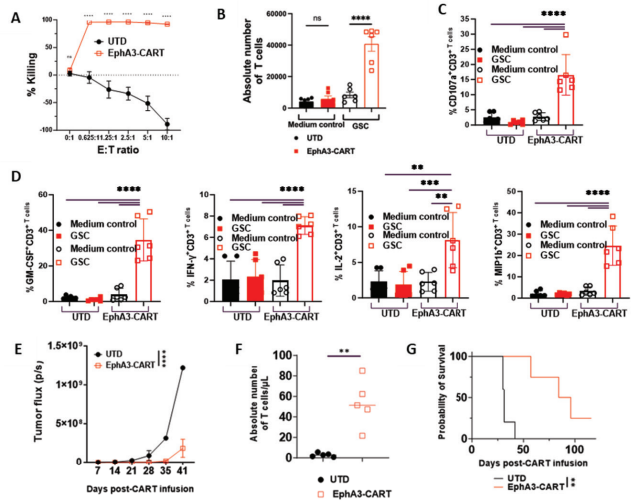


Fig 1. A. EphA3-CART or UTD were cocultured with luciferase$^+$ GSCs, and cytotoxicity was assessed by luminescence at 48 hours. **B.** Proliferation of EphA3-CART or UTD after a 5-day cocultured with GSCs was assessed by absolute counts of CD3$^+$ cells on flow cytometry. **C.** Degranulation of EphA3-CART or UTD after a 4-hour coculture with GSCs was assessed by CD3$^+$ CD107a$^+$ cells on flow cytometry. **D.** Cytokine production of EphA3-CART or UTD after a 4-hour coculture with GSCs was assessed by GM-CSF, IFN-γ, IL-2, or MIP1β on CD3$^+$ cells by flow cytometry. **E.** Antitumor activity of UTD or EphA3-CART was assessed by serial BLI as a measure of tumor growth. **F.** *In vivo* human T cell expansion in the peripheral blood was determined by absolute murine CD45$^-$ human CD45$^+$ human CD3$^+$ cell counts on flow cytometry. **G.** Kaplan-Meier survival curves of UTD- or EphA3-CART-treated mice are shown.

230. Combining the Anti-Viral and Anti-Tumor CD8+ T Cell Response Facilitates Immune Checkpoint Blockade Therapy and Oncolytic Virotherapy

Thanich Sangsuwannukul[1], Benjamin Kendall[1], Mason J. Webb[1], Jacob V. Vloten[1], Laura Evgin[1], Jason M. Tonne[1], Jill Thompson[1], Muriel Metko[1], Madelyn Moore[1], Maria P. Chiriboga Yerovi[1], Mark McNiven[1], Monga Satdarshan Pal Singh[1], Mitesh Borad[2], Roberts R. Lewis[1], Richard G. Vile[1]

[1]Mayo Clinic, Rochester, MN,[2]Mayo Clinic Cancer Center, Scottsdale, AZ

We tested the hypothesis that the heavily pro-inflammatory activity of vesicular stomatitis virus expressing interferon ß (VSV-IFNß) would convert the immune suppressive Tumor Micro-Environment (TME) of Hepatocellular carcinoma (HCC) to an immune stimulatory environment. In turn, this would recruit/prime/activate potentially anti-tumor T cells, the activity of which anti-PD-L1 Immune Checkpoint Inhibition (ICI) therapy would be able to potentiate further, thereby generating superior anti-tumor therapy to either therapy alone. This hypothesis is consistent with the model that inflammatory killing of tumor cells through viral oncolysis will recruit antigen presenting cells (APC) to the tumor site, release high loads of tumor associated antigens (TAA), and facilitate presentation of these TAA to CD8 and CD4 T cells in the draining lymph nodes, thereby generating systemically active anti-tumor T cell immunity. To test this hypothesis, we used a slow developing model of HCC, which resembles the etiology of human HCC, in which a Sleeping Beauty transposon integrates ß-catenin and hMet oncogenes into the livers of neonatal mice resulting in multi-focal tumor formation (SB-HCC) over 100-150 days. Tumor bearing mice were partially susceptible to anti-PD-L1 therapy (~50% long term cures), thereby mimicking at least one aspect of the human disease and current therapy. To our surprise, rather than observing even additive

benefit to therapy, and irrespective of the relative timings of both treatments, the addition of VSV-IFNß to anti-PD-L1 therapy largely abolished the therapeutic effects of anti-PD-L1 ICI alone. We show that VSV-IFNß treatment generated a highly significant expansion of one, or a few, dominant anti-viral effector $CD8^+$ T cell populations with the concomitant relative disappearance of those putative anti-tumor T cell populations which are the target of anti-PD-L1 treatment. We hypothesized that it would be possible to amalgamate the potent anti-viral response with an anti-tumor T cell response by expressing immunologically relevant tumor associated antigens from within the virus. Here we show that by expressing a multitude of putative, uncharacterized, tumor antigens within VSV using a cDNA library derived from three different SB-HCC explants (VSV-SB-HCC1,2,3) we observed highly significant improvements compared to anti-PD-L1 ICI alone - even in the absence of added anti-PD-L1 treatment. Our data provide a cautionary message for the use of highly immunogenic viruses as tumor-specific immune-therapeutics. We show that oncolytic virotherapy can induce anti-viral T cell responses which can actively inhibit, or obscure, weak anti-tumor T cell responses leading, potentially, to decreased anti-tumor therapy. However, by chimerizing anti-viral and anti-tumor T cell responses through encoding tumor antigens within the virus, at least a proportion of the anti-viral T cell response is co-opted into an anti-tumor response. In this way, oncolytic virotherapy can be purposed for very effective immune based tumor clearance and can generate anti-tumor T cell populations upon which immune checkpoint blockade can effectively work.

231. Adenovirus Capsid-Based Anti-Fentanyl Vaccine Protects Against Fentanyl-Induced Cardio-Respiratory Depression

Stephen M. Kaminksy[1], Isabelle Wilson[1], Cheyenne McKnight[1], Bishnu P. De[1], Tristan H. Lambert[2], Dolan Sondhi[1], Ronald G. Crystal[1]

[1]Genetic Medicine, Weill Cornell Medical College, New York, NY,[2]Chemistry and Chemical Biology, Cornell University, Ithaca, NY

Exposure to fentanyl, a highly potent synthetic opioid analgesic, resulted in the majority of the estimated 80,000 deaths caused by drug overdose in 2023. Fentanyl is not only associated with opioid use disorder due to its highly addictive qualities but also is added to other addictive drugs, resulting in an increase in deaths due to inadvertent use. Fentanyl causes bradycardia and respiratory depression in a dose-dependent fashion, often resulting in death. There is a need for a therapeutic that prevents the respiratory depressive effects of fentanyl and protects against overdose. We have developed a novel vaccine platform that sequesters fentanyl from mu-opioid receptors in the central nervous system to protect against lethal doses of the drug. The vaccine is based on direct covalent conjugation of a fentanyl analog (Hapten3) to the highly immunogenic serotype 5 $E1^-E3^-$ adenovirus after heat and detergent disruption (dAd5). dAd5-Hapten3 is produced by chemically crosslinking Hapten3 to the dAd5 hexon, fiber, and penton capsid proteins, thereby leveraging the adenovirus' naturally immunogenic properties to induce an immune response against fentanyl. Female C57BL/6J mice were administered dAd5-Hapten3 (8 μg, intramuscular) formulated with Adjuplex adjuvant at 0, 3, and 6 weeks. Serum collected prior to each vaccination was assayed for anti-fentanyl antibody titers. Vaccination generated titers $>10^6$ after a single dose and held steady after subsequent boosts. During weeks 8 to 13, mice were administered with fentanyl (2 mg/kg, intraperitoneal) and assayed for respiratory health parameters via pulse oximetry. Following fentanyl administration, unvaccinated controls had a decrease in heart rate (decrease 28.0 ± 14.1%), respiratory rate (decrease 42.8 ± 9.7%) and O_2 saturation (decrease 19.3 ± 7.9%). In contrast, vaccinated mice had less suppression of heart rate (fentanyl-induced decrease 13.0 ± 8.9%, $p<0.05$ compared to unvaccinated), respiratory rate (14.7% ± 16.6%, $p<0.01$) and O_2 saturation (9.3 ± 9.3%, $p< 0.05$). The vaccinated mice also demonstrated a decrease in fentanyl-induced mortality (44% unvaccinated, 80% vaccinated) despite the fact that the LD50 in mice is >100-fold greater than humans. In summary, capitalizing on the highly potent immunogenicity of adenovirus capsid proteins, we have developed a vaccine that produces antibodies specific to fentanyl, that can be used as a prophylactic against fentanyl-induced respiratory depression and resulting death.

232. The Novel Autologous AML Vaccine, TriLeukeVax, Stimulates Robust Anti-Leukemic Patient T Cell Responses

Karin L. Gaensler, Jacob Du, Uthpala N. Wijayaratna, Belinda Huang

UCSF, San Francisco, CA

There is an urgent need for more effective therapies for acute myeloid leukemia (AML), as relapse is frequent after chemotherapy. Evidence for the efficacy of immunotherapy is provided by the improved outcomes after hematopoietic stem cell transplantation (HSCT). However, this involves significant risks and toxicity, rendering many patients ineligible. To provide another strategy for stimulating anti-leukemic immunity, we developed a novel autologous cell vaccine (TriLeukeVax/TLV) by genetically engineering patient AML cells to express the co-stimulatory protein CD80 and the cytokine IL-15, with its receptor IL-15-receptor-α. Transduced AML can become potent antigen-presenting cells, capable of stimulating leukemia-specific responses, without the risks and toxicity of HSCT. Studies in leukemic mice showed 80% curative outcomes with serial TLV vaccination alone. In mice with advanced high-burden leukemia, non-curative chemotherapy followed by serial TLV vaccination eradicated residual disease in 40-50% of subjects. Ex vivo stimulation assays were used to test the efficacy of TLV in stimulating anti-leukemic activity in autologous patient T cells. TLV was generated by lentiviral transduction of diagnostic bone marrow or peripheral blood samples from five patients (47-69 y.o.). These cells were irradiated at 30 Gy and co-cultured with autologous post-remission T cells at an effector-to-target (E:T) ratio of 1:2, for 5 days. Controls to assess background levels of activation included co-culture of T cells with unmodified autologous AML. Proliferative responses were measured by flow cytometric analysis of effectors (CellTrace Violet) and targets (CFSE) stained with supravital dyes (Fig. 1). Primary co-culture with TLV stimulated greater CD4+ and CD8+ T cell proliferative responses than did co-culture with unmodified AML cells (Fig. 1). In our planned Phase 1 clinical trial, peripheral T cells in transplant ineligible AML patients in remission will be primed by serial vaccination with TLV. To recapitulate the induction of leukemia-specific cytolytic responses by primed T cells in patients, secondary ex vivo T cell stimulation assays were developed. Proliferating T cells previously co-cultured with TLV (primed T cells) in primary

stimulation assays were purified by fluorescence-activated cell sorting (FACS) and expanded in culture for 7-10 days. To determine whether TLV stimulation increased leukemia specific cytolytic activity, primed T cells were harvested and cocultured with unmodified autologous AML (E:T 2:1 for 24 hours). Background levels of cytolytic activity in non-primed T cells were compared with responses by primed T cells after co-culture with unmodified autologous AML (E:T 2:1 for 24 hours). Primed T cells showed significantly higher cytolytic activity against unmodified, autologous AML than did non-primed T cells (Fig. 2). These studies demonstrate that TLV mediates induction of robust proliferative and anti-leukemic cytolytic responses in autologous post-remission T cells. Investigational New Drug (IND)-enabling studies to support a future Phase 1 trial of TLV are currently underway.

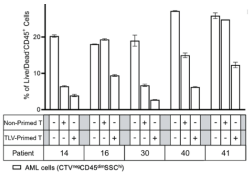


Figure 2. TLV enhances anti-leukemic responses in autologous T cells ex vivo. In secondary stimulation assays, the frequency of AML cells ($CTV^{neg}CD45^{dim}SSC^{hi}$) was quantified as % live $CD45^+$ cells in autologous AML cultured alone, or in AML co-cultured with TLV-primed T cells or non-primed T cells. In the 3 cultures performed per sample in triplicate, the left most bar indicates the frequency of AML cells as a % of total bone marrow or peripheral blood cells normalized to a 33% target cell plating frequency. The 2nd and 3rd bar in each set of samples depicts % live AML cells after co-culture with non-primed T cells (2nd) or TLV-primed T cells (3rd). Responder cells are shown in the table below the graph.

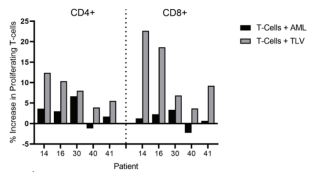


Figure 1. Increased T cell proliferation after primary co-culture with TLV versus unmodified autologous AML. Rested patient T cells were stained with CellTrace Violet (CTV) and co-cultured for 5 days with irradiated, carboxyfluorescein succinimidyl ester (CFSE)-stained AML or TLV at an effector-to-target ratio of 1:2. The frequency of proliferating cells (CTV^{dim}) was quantified in CD4+ or CD8+ T cells (Live $CFSE^-CTV^+$) cultured alone or co-cultured with autologous AML or TLV. The increase in proliferation is the % dividing T cells co-cultured with AML or TLV minus the %dividing T cells in the absence of target.

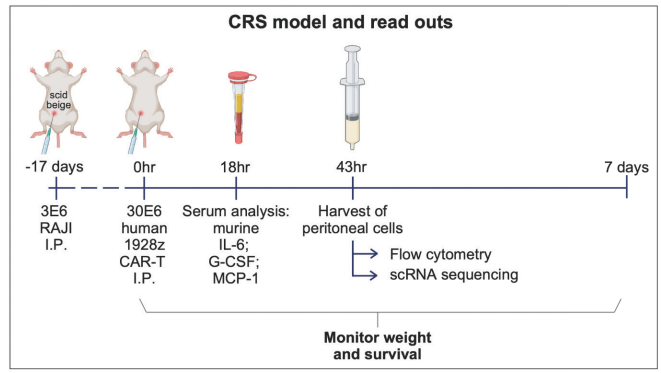
Pharmacology Toxicology Studies and Analytics Assay Development

233. CAR T-Cell-Derived TNF Drives Cytokine Release Syndrome and Enhances M1 Macrophage Differentiation

Pieter L. Lindenbergh[1], Theodoros Giavridis[1,2], Ophélie Vivier[1], Michael Lopez[1], Anton Dobrin[1], Michel Sadelain[1]

[1]Memorial Sloan Kettering Cancer Center, New York, NY,[2]Mnemo Therapeutics, New York, NY

Background: The pathophysiology of CAR T-cell-induced Cytokine Release Syndrome (CRS) is driven by cytokines and biochemical mediators produced by activated T-cells and macrophages. TNF serum levels are elevated in patients suffering from CRS and pre-clinical work from our group shows that CRS can be abated by TNF-blockade. To elucidate the role of TNF in CRS, we set out to identify its cellular source and its effect on macrophage activation. We tested the effect of TNF-blockade on the efficacy of several CAR T-cell designs to assess if TNF is a potential target for CRS prevention in the clinic. **In vivo models:** To study the role of TNF in CRS, we blocked TNF in a previously established model for CRS in which human 1928z CAR T-cells induce inflammatory cytokine release, weight-loss and occasional lethality (see figure). To assess the effect on therapeutic efficacy, we blocked TNF in a NALM6-leukemia model treated with 1928z, 19BBz or 1928z/4-1BBL CAR-T cells.



Results: To identify the cellular source of CRS-driving TNF, we treated the CRS model with clinically utilized agents that block human and murine TNF (etanercept) or only human TNF (infliximab). We observed that blockade of human TNF alone was sufficient to prevent cytokine release, weight-loss and lethality. Consistently, flow cytometric analysis of myeloid cells at the tumor site showed that human TNF-blockade abated CRS-induced macrophage accumulation. Because CD4 CAR T-cells produce more TNF than CD8 CAR T-cells, we hypothesized that CD4 CAR T-cells would be stronger inducers of CRS. We administrated sorted CD4 or CD8 CAR T-cells but observed no significant difference in cytokine release, weight-loss and lethality. Furthermore, CRS induced by both subsets was abated by etanercept. We used scRNA sequencing and the MacSpectrum annotation system to assess the effect of CRS and TNF on the M0/M1/M2 differentiation of macrophages at the tumor site. We found that CRS-induction universally polarized the macrophages towards a M1-like state, which was epitomized by the upregulation of *Nos2* (encoding the iNOSynthase of vasodilating NO). TNF-blockade attenuated the M1-like differentiation of these macrophages and significantly reduced *Nos2* expression. We assessed the effect of prophylactic TNF-blockade on CAR T-cell efficacy in a leukemia model treated with CAR T-cells. Mice received an induction dose of etanercept prior to CAR T-cell treatment and bi-weekly follow-up doses over the course of three weeks, covering the time window in which CRS occurs in the clinic. Whilst TNF-blockade reduced the efficacy of 1928z and 19BBz CAR T-cells, we observed that 1928z CAR T-cells modified to co-express 4-1BBL were not affected by TNF-blockade. **Conclusions:** Our results support a model in which CD4 and CD8 CAR T-cell-derived TNF is a crucial, positive regulator of CRS and enhances the differentiation of macrophages towards a proinflammatory state. The results of the preclinical efficacy experiments indicate that CAR T-cells can be modified to have uncompromised efficacy in the presence of long-term TNF-blockade. Thus, TNF is not only a nexus in the pathophysiology of CRS, but also a potential target for its prevention.

234. Identification of Cellular Factors that Contribute to Peripheral Blood-Derived CAR-NK Cell Mediated Inflammatory Toxicities

Supreet Khanal, Nirjal Bhattarai

FDA, Silver Spring, MD

Chimeric antigen receptor-expressing NK (CAR-NK) cells are increasingly being developed to treat a wide variety of human diseases. While CAR-T cells are known to activate bystander myeloid cells,

contributing to inflammatory toxicities such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), the potential of CAR-NK cells to induce similar effects remains uncertain due to limited clinical data. Here we studied human peripheral blood-derived CAR-NK cells and assessed their ability to cause inflammatory toxicities. Primary NK cells were isolated from healthy donors and activated using PMA/Ionomycin as well as using target cells such as Daudi and K562. Cell-free activated supernatants were collected and added to THP-1 cells and primary monocytes to assess myeloid activation, that was measured by the secretion of IL-6 and IL-1β using ELISA. Cytokine arrays were used for the identification of cellular factors. We found that following activation, NK cells secrete fewer inflammatory cytokines at lower levels compared to T cells obtained from the same donor, resulting in lower levels of myeloid cell activation and secretion of inflammatory cytokines IL-6 and IL-1β by myeloid cells. Nevertheless, activated NK cells demonstrated notable myeloid cell activation across all donors studied compared to resting NK cells. To assess the mechanism by which CAR-NK cells activate bystander myeloid cells, we analyzed the inflammatory cytokine profile of activated NK cells. Comparative analysis of secreted cytokines from activated and resting primary NK cells and NK cell lines identified key inflammatory factors with potential to cause myeloid cell activation. Antibody-mediated neutralization studies identified four NK cell factors, which, upon neutralization, significantly reduced NK-cell-mediated myeloid cell activation. This observation was confirmed through siRNA-mediated knockdown studies as knockdown of these four factors in peripheral blood-derived CAR-NK cells significantly reduced myeloid cell activation. Importantly, neutralization or knockdown of these four inflammatory factors in NK cells did not affect potency of CAR-NK cells. Together, these data suggest that peripheral blood-derived CAR-NK cells can activate bystander myeloid cells and contribute to inflammatory toxicities during CAR-NK cell therapy. Furthermore, this study identified four major inflammatory factors secreted by activated NK cells, which, upon inactivation, can significantly reduce CAR-NK cell-mediated inflammatory toxicities without affecting their potency.

235. Pharmacokinetics and Vector Shedding in NHPs Following a Single Intravenous Infusion of a CD20-Targeted Engineered Lentiviral Vector

Elaine M. Youngman, Leticia Kuri-Cervantes, Shanice Edwards, Meiling Miranda, Candice Schreiber, James Richardson, Tim Culp, Philip R. Johnson

Interius BioTherapeutics, Philadelphia, PA

Lentiviral vector (LVV) based *ex vivo* cell therapy is well established, and in this setting the carryover of infectious LVV particles into patients is low for clinical-grade material (Cesani et al. 2015). The *in vivo* administration of LVV to generate CAR cells inside the patient has the potential to circumvent manufacturing and administration challenges presented by *ex vivo* cell therapies but presents a potential risk of environmental contamination through shedding of infectious vectors in patient excreta. We developed an engineered replication-incompetent LVV clinical candidate (INT2104) that directs *in vivo* production of CD20-directed CAR-T and CAR-NK cells following a single-dose intravenous (IV) infusion. Here we report pharmacokinetics of vector capsid protein and RNA genomes in blood, and shedding of vector RNA genomes in urine, feces, and saliva of cynomolgus macaques after IV administration of 2 x 10^9 transducing units of INT2104. This dose represents the highest dose proposed for a FIH study planned to start in 4Q2024. We used ELISA and RT-qPCR methods to detect vector capsid protein and RNA genomes respectively in several LVV studies in cynomolgus macaques, including the GLP-Toxicology study. In all studies, LVV drug product was administered in a single-dose IV infusion. LVV capsid protein peaks in the blood 1 day after vector administration and is then rapidly cleared. Vector RNA genomes are cleared more rapidly than protein and are detected at much lower levels than expected if the all the capsid protein measured in circulation were associated with intact LVV particles. These data indicate that clearance of intact and therefore potentially infectious vector particles from circulation is rapid after IV administration of our replication-incompetent LVV. Shedding of vector RNA genomes into feces, saliva and urine was detectable in some animals at very low levels in the first 3 days following vector administration, suggesting very limited and transient LVV shedding. Taken together these data demonstrate rapid clearing of an in vivo-administered replication-incompetent LVV, with limited shedding in animal excreta indicating low risk of environmental contamination.

236. Characterization of Preclinical Models for Systemically Delivered Vectors Based on Human Adenoviruses HAdV-B3, HAdV-B11, HAdV-C5, HAdV-C6, and HAdV-D26

Margarita Romanenko, Jill Schappa Faustich, Kazuho Inoko, Sacha Robert, Melanie Graham, John P. Carney, Julia Davydova

University of Minnesota, Minneapolis, MN

Oncolytic adenoviruses (OAds) are promising cancer therapy agents that selectively lyse cancer cells while sparing healthy tissues. They also promote antitumor immunity by inducing immunogenic cell death and disrupting immunosuppressive microenvironment. Notably, their intravenous delivery targets metastases, enabling systemic treatment. However, OAds' clinical progress is hampered by the absence of reliable animal models that can predict OAds' therapeutic and immunomodulatory responses, particularly upon systemic administration. Ideally, preclinical models should support OAd replication, mimic OAd-induced immune responses, and represent OAd interactions with blood components. However, standard murine models don't support replication of human Ads, resulting in nonfunctional progenies. Furthermore, both mice and hamsters do not allow for the binding of species B Ads (e.g., HAdV-B3 and HAdV-B11) due to the absence of CD46 and human desmoglein-2, hDSG2, receptors. This limitation extends to chimeric OAd vectors targeting these receptors (e.g., HAd5/3). This study seeks to identify and characterize preclinical models for OAds evaluation. Our recent work shows porcine cells and tissues effectively support the binding, transduction, and replication of HAdV-C5- and chimeric HAd5/3-based vectors, at a level similar to human tissues. Here, we compared these findings across mammals, assessing binding, transduction, and replication of species B, C, and D Ads in mice, hamsters, pigs, and non-human primates. Focusing on systemic delivery, we explored Ads' interaction with blood factor X (FX), a pivotal element influencing

adenoviral transduction. Prior research has established that human FX binding notably augments cell transduction for species C Ads. In this study, we explored whether FXs derived from a diverse array of mammalian sources—including human, porcine, murine, canine, and cynomolgus monkey—could enhance the transduction efficacy of both wild-type and chimeric Ads, specifically HAdV-B3, HAdV-B11, HAdV-C5, HAdV-C6, and HAdV-D26, as well as the chimeric variants HAd5/3 and HAd5-RGD. We showed that porcine and canine FXs significantly improve the transduction efficacy of HAdV-C5, HAdV-C6, and chimeric HAd5/3 in CAR-, hDSG2-, and CD46-deficient CHO cells, matching human FX levels. Interestingly, while HAdV-B3 transduction remains unaffected by the presence of any FX variant tested, in the case of HAdV-B11, significant enhancement of transduction efficiency was observed when human and porcine FX was introduced. Unexpectedly, FX derived from cynomolgus did not exhibit any enhancement in the transduction capabilities across the Ad types we tested. We are currently examining Ad types' hemagglutination with red blood cells, RBCs, sourced from humans, porcine, hamsters, mice, rhesus macaque, and cynomolgus monkeys. Preliminary results indicate that porcine, murine, and hamster RBCs don't hemagglutinate with any of the explored Ad types (HAdV-B3, HAdV-C5, HAdV-C6, HAdV-B11, HAdV-D26, HAd5/3, HAd5-RGD), unlike human RBCs, which do with HAdV-C5, HAdV-C6, HAdV-D26, and HAd5-RGD. Notably, non-human primate (cynomolgus and rhesus macaque) RBCs do hemagglutinate with only HAdV-B11. Based on our current and previous research on the porcine model, we conclude that pigs represent a promising candidate for the preclinical evaluation of OAds, particularly for chimeric HAd5/3-based vectors. In light of these findings, the FDA has permitted the exclusive use of pigs for IND-enabling toxicology studies of the oncolytic HAd5/3 vector.

237. Potency Assay Enabling Both *Ex Vivo* and *In Vivo* Genome Editing Therapeutics for Sickle Cell Disease

Utku Goreke[1], Dipti H. Kamath[1], Yaw O. N. Ansong-Ansongton[1], Nathan M. Perez[1], Umut Gurkan[2], Petros Giannikopoulos[1], David N. Nguyen[1]

[1]Innovative Genomics Institute, University of California Berkeley, Berkeley, CA,[2]Mechanical and Aerospace Engineering, Case Western Reserve University, Cleveland, OH

Sickle cell disease (SCD) stems from a point mutation of the two β subunits in adult hemoglobin (HbA) that leads to nucleated polymerization of sickle hemoglobin (HbS) under hypoxia. A critical unmet need for using gene editing-based genomics therapies to cure sickle cell disease is the ability to characterize red blood cell (RBC) quality to assess the potency, regardless of the editing modality. The standard DNA sequencing methods can assess the safety of the product, and conventional hematologic assays such as high-performance liquid chromatography (HPLC) can measure the abundance of various hemoglobin variants in a sample. There is sparse knowledge of the *in vitro* functional characterization of RBCs. Still, these could assess the biophysical and rheological properties of RBCs derived from genome-edited SCD HSPCs at the single-cell level. We delivered CRISPR Cas9 RNPs targeting the BCL11a enhancer locus (or controls) to commercially obtained healthy donor peripheral blood mobilized (CD34+) HSPCs to enhance fetal hemoglobin expression. Edited HSPCs were differentiated into erythroid cells using a 21-day 3-phase cytokine cocktail system (including IL-3, SCF, and erythropoietin). Differentiation, proliferation, and enucleation were compared between control and gene-edited samples with flow cytometry. Adhesion and deformability of enucleated RBCs were studied using the SCD-Biochip Assay, a suite of microfluidic devices capable of functionally characterizing several biochemical and biomechanical phenotypes of RBCs namely: adhesion, deformability, and sickling under hypoxic and normoxic conditions [1, 2, 3]. Editing efficiency was estimated as high as ~60% via electroporation. The mean cell amplification of CD34+ cells reached a 695-fold expansion by Day 16. Differentiation of the immature erythroblasts into mature RBCs continued from day 16 to 21, marked by the progressive loss of CD71 expression; ~50% of CD235a+ cells were enucleated. We observed more microfluidic occlusion index (a surrogate for deformability) with glutaraldehyde crosslinked RBCs compared to untreated controls. We found that RBC occlusion and adhesion (to fibronectin) are significantly greater under hypoxia than under normoxia for HbSS RBCs. Here, we present a matrix-type potency assay to address the critical need for a universal potency assay for editing-based SCD treatments. The assay will be anchored by a suite of novel microfluidic biochip devices that can functionally assess the health of individual RBCs in a clinically relevant human capillary model in unprecedented sensitivity for precision diagnostics. We demonstrate the feasibility of adopting the SCD-Biochip platform to evaluate pertinent biomechanical functions of RBCs derived from CRISPR-edited HSPCs. The application of these functional assays could result in a robust and accurate precision diagnostic that addresses the critical need for a universal potency assay for editing-based SCD treatments. References: [1] Goreke, U., Iram, S., Singh, G., Domínguez-Medina, S., Man, Y., Bode, A., ... & Gurkan, U. A. (2023). Catch bonds in sickle cell disease: shear-enhanced adhesion of red blood cells to laminin. Biophysical Journal. [2] Man, Y., Wu, D. H., An, R., Wei, P., Monchamp, K., Goreke, U., ... & Gurkan, U. A. (2023). Microfluidic concurrent assessment of red blood cell adhesion and microcapillary occlusion: potential hemorheological biomarkers in sickle cell disease. Sensors & Diagnostics, 2(2), 457-467. [3] Sekyonda, Z., Goreke, U., An, R., Oshabaheebwa, S., Man, Y., & Gurkan, U. A. (2022). Sickling Dynamics Differ Among the Different Sickle Cell Disease Genotypes. Blood, 140(Supplement 1), 5401-5402.

238. Association Between Potency Critical Quality Attributes and Clinical Efficacy Across Lentiviral Vector Cell and Gene Therapy Products

Ilya A. Shestopalov, Hancheng Jiang, Marisa Gayron, Marc d'Anjou, Kelly Kral, Francis J. Pierciey, Richard Colvin

bluebird bio, Somerville, MA

Introduction: Identifying drug product Critical Quality Attributes (CQAs) that associate with clinical efficacy has been a challenge with cell and gene therapy (CGT) products. Betibeglogene autotemcel (beti-cel), lovotibeglogene autotemcel (lovo-cel), and elivaldogene autotemcel (eli-cel) are distinct CGT products with differentiated safety and efficacy profiles that leverage lentiviral gene addition in CD34+ hematopoietic stem and progenitor cells for treatment of severe genetic diseases. Beti-cel and lovo-cel utilize an LVV construct

with a tissue-specific promoter to address β-thalassemia and sickle cell disease, whereas eli-cel has a constitutive promoter to treat cerebral adrenoleukodystrophy. The choice of promoter contributes to distinct safety profiles. A matrix of potency assays has been developed for each of these drug products to capture their multi-faceted mechanisms of action. **Objective:** To identify common potency-related CQAs that are associated with clinical safety and efficacy of beti-cel, lovo-cel, and eli-cel. **Methods:** This post-hoc, exploratory analysis included data from a total of 155 patients across 6 clinical studies: beti-cel, 41 patients from HGB-207 (NCT02906202) and HGB-212 (NCT03207009); lovo-cel, 47 patients from HGB-206 (NCT02140554) and HGB-210 (NCT04293185); eli-cel, 67 patients from ALD-102 (NCT01896102) and ALD-104 (NCT03852498). Potency-associated attributes studied were vector copy number (VCN), percentage of transduced cells (lentiviral-vector positive cells, %LVV+ cells), vector copy number per transduced cell (VC/TC), therapeutic protein expression, percent colony forming cells (%CFC), and disease-specific potency assays that measure changes in biological function related to the mechanism of action of the product (red blood cell (RBC) differentiation for beti-cel, RBC morphology for lovo-cel, and metabolism of very long chain fatty acids for eli-cel). Univariate logistic regression analyses were preformed between potency-associated attributes and clinical outcomes: occurrence of vaso-occlusive events (VOEs) for lovo-cel; development of major functional disabilities (MFD) or myelodysplastic syndrome (MDS) for eli-cel . For beti-cel, a multi-variate approach was used to predict achievement of a peripheral blood VCN ≥0.75 copies per diploid genome, a stringent surrogate biomarker of transfusion independence (TI). Associations between CFC activity and engraftment were also studied. **Results:** Measures of transduction efficiency (VCN and %LVV+ cells) were significantly associated with clinical endpoints across the products, including preventing MFDs in eli-cel, resolution of VOEs in lovo-cel, and achievement of a stringent surrogate biomarker of TI in beti-cel. Transduction efficiency based on protein expression (% of cells positive for therapeutic adrenoleukodystrophy protein ALDP) was also associated with preventing MFDs in eli-cel. VCN and %LVV+ cells were not associated with cases of MDS that are unique to eli-cel. VC/TC was not associated with efficacy or safety-related outcomes and CFC had no association with time to engraftment in all three products. In contrast to transduction efficiency measurements, product-specific functional potency assays had variable associations with clinical outcomes. **Conclusion:** Our findings indicate that all three products (beti-cel, lovo-cel, and eli-cel) share measures of transduction efficiency as the common potency-associated CQAs that highly correlate with product efficacy. These results highlight the value of transduction efficiency assays as surrogate potency measurements and can help guide the establishment of rigorous and meaningful product quality acceptance criteria for complex CGT products.

239. Transcriptional Changes in Non-Human Primate Tissues After Intrathecal Delivery of Serotype 9 Adeno-Associated Viral Vector: Insights into Organ Toxicities

Fumiaki Aihara[1], Matthew Mardo[2], Vera Ruda[1], Megumi Onishi-Seebacher[1], Philippe Couttet[1], Keith Mansfield[1], Kelley Penraat[1], Eloise Hudry[1]

[1]Novartis, Cambridge, MA,[2]University of Colorado, Aurora, CO

Adeno-associated virus (AAV) based gene therapy has brought transformative therapeutic benefits to patients with otherwise untreatable genetic diseases. Despite these life changing effects, AAV-driven organ toxicities remain a safety concern in the clinic and the mechanism of action remains unknown. To gain further insights into the potential molecular mechanisms underpinning organ toxicity, we conducted RNAseq analysis in non-human primates administered scAAV9-CB-GFP intrathecally as well as empty viral particles and 'promotorless' AAV particles. Tissues primarily transduced by the viral vector (liver, dorsal root ganglia, heart, skeletal muscle, and spleen) were collected and processed. Initial pathway analysis confirmed that many of the upregulated pathways were related to a viral immunological response towards the full AAV, which persists at least a month after dosing across liver, dorsal root ganglia, and heart tissues. Evaluating the top upregulated genes after AAV dosing revealed several immunological genes associated with interferon signaling, phagocytic activity, antigen presentation, and T cell/B cell activity. Several of these significantly upregulated genes were expressed across the three organs of interest, suggesting that there is a common response towards the AAV's transgene expression. We have also observed a striking difference in transcriptional signals between fully functional AAV and the empty viral particles and 'promotorless', with far more significant changes seen in the group given the fully functional AAV. This distinction was most prominently seen in the dorsal root ganglia, liver, and heart, where there was the highest expression of the transgene within the organ. This suggests that lasting transcriptional changes are mostly observed when the transgene is expressed (early capsid-dependent transcriptional alterations were not captured in our analysis). Taken together, this study provides clearer insight into the transcriptional responses, with distinct signatures for interferon signaling and antigen presentation, within key tissues following administration of high-dose AAV.

Cell Therapy and Cell-Based Gene Therapy Trials

240. FVIII Expression through Autologous CD34+ Hematopoietic Stem Cells Transduced with a Lentiviral Vector with a Novel F8 Transgene for Gene Therapy of Hemophilia A - A First-in-Human Clinical Trial

Alok Srivastava[1,2], Aby Abraham[1], Fouzia Aboobacker[1], Gurbind Singh[2], Tulasi Geevar[1], Uday Kulkarni[1], Sushil Selvarajan[1], Anu Korula[1], Rutvi Dave[1], Mohanashankar Mahalingam[2], Ramachandran Shaji[1,3], Sukesh Chandran Nair[1], Gabriela Denning[4], Pete Lollar[4,5], Christopher Doering[4,5], H. Trent Spencer[4,5]

[1]Christian Medical College Vellore, Vellore, India,[2]Centre for Stem Cell Research Unit of inStem Bengaluru, Vellore, India,[3]Centre for Stem Cell Research unit of inStem Bengaluru, Vellore, India,[4]Expression Therapeutics, Tucker, GA,[5]Emory University, Atlanta, GA

Liver directed adeno-associated virus (AAV) vector-based gene therapy for hemophilia A has several limitations: age and anti-AAV antibody

related eligibility restrictions, inter-individual variation in FVIII:C expression and its durability as well as unexplained transaminitis. We demonstrated sustained FVIII expression from hematopoietic stem cells (Doering *et al Hum Gene Therapy 2018; 29:1183*) using a self-inactivating lentiviral vector (LV) encoding a novel *F8* transgene designed for enhanced FVIII secretion (ET3) and driven by a CD68 promoter. The vector, designated as CD68-ET3-LV, is being evaluated in a first-in-human clinical trial of this novel gene therapy approach for severe hemophilia A (NCT 05265767; CTRI/2022/03/041304). This report describes the outcome of the first 4 treated participants. $CD34^+$ cells selected from mobilized peripheral blood stem cell harvests were transduced *ex-vivo* with the CD68-ET3-LV vector and cryo-preserved targeting a final cell dose of $>5x10^6$ $CD34^+$ cells/kg. Transductions for the first two participants (G1) were without enhancer while for the next two participants (G2) transductions were with enhancer and a 50% reduction in vector dose. Conditioning for the hematopoietic stem cell transplant (HSCT) consisted of treosulfan and fludarabine. The cytopenic period was supported with rFVIII along with other standard of care measures for HSCT. As engraftment ensued and blood counts normalized, rFVIII replacement was gradually stopped. Post engraftment, FVIII:C levels were measured by one-stage and chromogenic substrate assays. The genomic integration profile of the CD68-ET3-LV vector was also assessed. The four treated participants ranged between 22-34 years and had >100 prior exposures to FVIII with a median annual bleeding rate of 33 (range:20-120). $CD34^+$ cell doses administered for HSCT ranged from 5.3-6.2 x 10^6 cells/kg. The vector copy numbers (VCN) in the therapeutic products were 0.57 & 1.02 in G1 and 0.62 & 1.49 in G2. The HSCT procedure was well tolerated. Neutrophil and platelet engraftment occurred at 10-12 and 12-15 days after HSCT and the duration of severe neutropenia and thrombocytopenia was 7-11 and 1-4 days, respectively. There were no bleeding episodes. No Grade >2 non-hematological toxicities occurred during the engraftment period. The last day of rFVIII replacement ranged from 11-20 days after HSCT. FVIII:C levels peaked between days +30 to 60 after HSCT and then remained stable within a small range. The median FVIII:C after day +30 at last follow-up for G1 participants are 5.2% (18 mo.) and 1.7% (12 mo.), while for those in G2 was 32.6% (6 mo.) and 11% (4 mo.) with comparable one stage and chromogenic substrate assays. The peripheral blood VCN at day +30 was 0.12 and 0.25 in G1 and 1.45 and 2.5 in G2 correlating with the measured FVIII:C levels. The vector integration profile showed no safety concerns. None of the participants reported any bleeds post-HSCT through the last follow-up. FVIII mRNA levels in blood cells were highest in $CD14^+$ cells, lower in $CD15^+$ cells and none in $CD3^+$ cells. This ongoing first-in-human clinical trial of a lentiviral vector transduced HSC-based gene therapy for hemophilia A establishes feasibility of this approach with no early safety signals, and very promising therapeutic efficacy with clinically significant stable levels of circulating FVIII:C within a month after treatment.

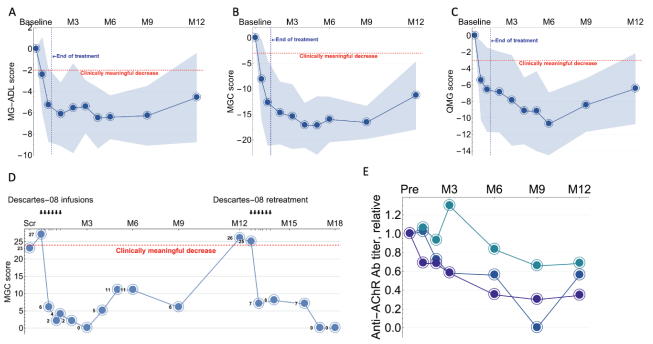
241. Investigation of mRNA Cell Therapy as a Treatment for Autoimmune Disease in Patients with Myastenia Gravis

James F. Howard[1], Tahseen Mozaffar[2], Tuan Vu[3], Nizar Chahin[4], Gregory Sahagian[5], Marc H. Feinberg[6], C. Andrew Stewart[7], Metin Kurtoglu[7], Milos D. Miljkovic[7], Christopher M. Jewell[7]

[1]University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]University of California Irvine, Irvine, CA,[3]University of South Florida, Tampa, FL,[4]Oregon Health and Sciences University, Portland, OR,[5]Neurology Center of Southern California, San Diego, CA,[6]SFM Research, Boca Raton, FL,[7]Cartesian Therapeutics, Gaithersburg, MD

Autoimmune diseases occur when the immune system mistakenly attacks host tissue. Myasthenia gravis (MG) is an autoimmune disease in which auto-antibodies attack the neuromuscular junction. We recently reported on the safety and preliminary clinical activity of an mRNA-engineered chimeric antigen receptor (CAR) T-cell therapy in patients with MG. This strategy is differentiated from DNA-based approaches in the use of mRNA to encode the CAR, which results in transient expression and avoids permanent genomic integration. As a result, this therapy is administered in the outpatient setting and does not require lymphodepleting chemotherapy. In particular, these engineered cells target the B-cell Maturation Antigen (BCMA) expressed on PC that produce the pathogenic autoantibodies during MG and many other autoimmune diseases. In the Phase 2a part of our study, 7 participants (4 acetylcholine receptor [AChR]+, 2 muscle-specific kinase [MuSK]+, 1 seronegative, by history) who received six weekly infusions of Descartes-08 exhibited clinically meaningful improvement in common MG severity scales at Month 3. Further, three of the 7 participants achieved minimal symptom expression (MSE). Here, we report the results of the final per-protocol one year follow-up. Descartes-08 maintained a favorable safety profile, with no new product-related adverse events reported during the 12-month follow-up period. At Month 9 follow-up - approximately 7 months after the final infusion - all participants continued to experience substantial clinical responses as measured by the MG Activities of Daily Living Score (Figure 1A), MG Composite Score (Figure 1B), Quantitative MG scores (Figure 1C), and others. Notably, at Month 12, 5 of the 7 participants maintained clinically meaningful improvement, including one with MSE. Two participants exhibited worsening disease at Month 12 and became eligible for retreatment. One opted for retreatment, which led to a rapid decrease in MG-specific clinical scores and MSE. The MSE is ongoing at Month 6 of retreatment follow-up (Figure 1D). All three participants with detectable anti-AChR levels at baseline also showed lasting reductions in these auto- antibody levels (Figure 1E). In summary, we observed continued clinical improvement and autoantibody reductions after BCMA-directed rCAR-T treatment that persisted through the one-year follow-up period. A patient with clinical relapse at Month 12 again achieved MSE after redosing. The favorable safety profile of this mRNA CAR-T treatment contrasts with DNA-based CAR-T treatments, which require lymphodepletion chemotherapy with potential hematologic toxicities and oncogenic risk from genomic integration of CAR DNA. Our observations suggest that using rCAR-T therapy to target BCMA-positive cells—including PCs—can result in durable depletion of autoantibodies and clinically meaningful improvement in MG severity scores without severe toxicity,

agammaglobulinemia, or increased risk of infection. These data support continued development of Descartes-08 in myasthenia gravis and other autoantibody-associated autoimmune disorders.



242. Safety, Tolerability, and Clinical Assessment of Bemdaneprocel for Parkinson's Disease: Results up to 18 Months from a Phase 1 Study

Claire Henchcliffe[1], Harini Sarva[2], Andres Lozano[3,4], Alfonso Fasano[4,5,6], Suneil Kalia[3,4], Kenny Kwok Hei Yu[7], Cameron Brennan[7], Whitney Stemple[8], Nauman Abid[8], Marcus Yountz[8], Ahmed Enayetallah[8], Antoine Lampron[8], Viviane Tabar[7]

[1]Department of Neurology, University of California, Irvine, Irvine, CA,[2]Department of Neurology, Weill Cornell Medicine, New York, NY,[3]Division of Neurosurgery, University of Toronto, Toronto, ON, Canada,[4]Krembil Brain Institute, Toronto, ON, Canada,[5]Division of Neurology, University of Toronto, Toronto, ON, Canada,[6]Edmond J Safra Program in Parkinson's Disease, Morton and Gloria Shulman Movement Disorders Clinic, Toronto Western Hospital, Toronto, ON, Canada,[7]Department of Neurosurgery and Center for Stem Cell Biology, Memorial Sloan Kettering Cancer Center, New York, NY,[8]BlueRock Therapeutics LP, Cambridge, MA

Background: Bemdaneprocel is an investigational cellular therapy comprising pluripotent stem cell-derived dopaminergic neuron precursor cells under development for the treatment of Parkinson's disease. This first-in-human phase 1 study assesses the safety, tolerability, and clinical outcomes of bemdaneprocel in participants with Parkinson's disease. **Methods**: In this open-label, 24-month, non-controlled study, 12 participants received 1 of 2 doses of bemdaneprocel bilaterally delivered to the post-commissural putamen and a 1-year immunosuppression regimen. The primary objective was safety and tolerability at 1 year. Exploratory clinical outcomes were also evaluated. **Results**: In the primary analysis at 1 year (previously presented), 2 serious adverse events (SAEs) unrelated to bemdaneprocel were reported, 1 in each cohort: a hospitalization due to COVID-19 and a transient seizure 1 day after surgery. Treatment-emergent adverse events (TEAEs) reported by 11/12 participants were mostly mild or moderate in severity (65/66), and none were considered related to bemdaneprocel. Early trends toward clinical improvement (median [Q1, Q3] change from baseline) at 12 months included: Movement Disorders Society-Unified Parkinson's Disease Rating Scale (MDS-UPDRS) Part III OFF scores and patient-reported Good ON times of 1.0 (−26.0, 13.0) and +0.7 (−0.4, 1.0) hours, respectively, in the low-dose cohort, and −13.0 (−20.0, −4.0) and +2.2 (−0.4, 2.2) hours, respectively, in the high-dose cohort. Median (Q1, Q3) change from baseline in patient-reported OFF time was −1.6 (−1.7, −0.4) and −1.9 (−2.2, 0.4) hours in the low-dose and high-dose cohorts, respectively. **Conclusions**: Bemdaneprocel was well tolerated, with no major safety issues in all 12 participants through 1 year. Exploratory clinical measures suggested improvements in MDS-UPDRS Part III OFF scores and patient-reported Good ON time and OFF time. Safety and clinical outcomes with 18 months of follow-up, 6 months post discontinuation of immunosuppression, will be presented.

243. Eighteen-Year Follow-Up of GD2 Chimeric Antigen Receptor-Cytotoxic T Lymphocyte Therapy in Patients with Neuroblastoma

Che-Hsing Li[1,2,3], Sandhya Sharma[1], Andras Heczey[1,3], Chrystal Louis[1], David Steffin[1,3], Bambi Grilley[1,4], Sachin Thakkar[1], Mengfen Wu[4], Tao Wang[4], Cliona Rooney[1,4], Malcolm Brenner[1,4], Helen Heslop[1,4]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine–Texas Children's Hospital–Houston Methodist Hospital, Houston, TX,[2]Program in Immunology & Microbiology, Baylor College of Medicine, Houston, TX,[3]Center for Advanced Innate Cell Therapy, Department of Pediatrics, Baylor College of Medicine, Houston, TX,[4]Dan L. Duncan Comprehensive Cancer Center, Baylor College of Medicine, Houston, TX

In a clinical trial open to accrual from 2004 to 2009 (NCT00085930) we treated children with neuroblastoma using immune effector cells genetically modified with chimeric antigen receptors (CARs) directed to the GD2 antigen expressed by neuroblastoma cells. We administered two different types of immune effector cells: EBV-specific cytotoxic T lymphocytes and CD3 activated T cells - each genetically modified with one of two distinguishable barcoded transgenes encoding the same CAR. We previously reported the initial and 5-year follow-up of this study in 19 patients with high-risk neuroblastoma: 8 (42.1%) in remission at infusion and 11 (57.9%) with active disease; we now report a median follow-up of 13.9 years (range 8.7 - 18.2 years) post-infusion for surviving patients. Of the 11 patients with active disease at the time of infusion, 3 patients achieved a complete response. One subsequently relapsed but two had sustained responses with one for 8 years until lost to follow-up and one for 18+ years. Of 8 patients with no evidence of active disease (NED), 5 had a history of relapsed disease and 3 were infused after completing therapy for high-risk disease. Five of these patients are disease free at their last follow-up with a median follow-up of 13.9 years (range 10.1 - 14.0 years) post-infusion. The median overall survival was 2.5 years (95% CI: 0.5 - NA years for all patients) (0.8 year, 95% CI: 0.2 - 6.8 years for patients with active disease, but not reached for patients with NED, $p = 0.019$, **Figure 1**). The median event-free survival was 0.9 year (95% CI: 0.2 - NA years for all patients) (0.3 year, 95% CI: 0.1 - 4.9 years for patients with active disease, but not reached for patients with NED, $p = 0.044$). The toxicities observed during this long-term follow-up were attributable to previous chemotherapy, with sensorineural hearing loss the commonest. One patient developed invasive ductal carcinoma of the breast 12 years after infusion. We detected low levels of GD2CAR transgene in the tumor

sample likely representing long-persisting CAR-T cells, an observation consistent with reports that breast cancer stem cells may express their targeted antigen, GD2. In conclusion, patients with refractory/relapsed neuroblastoma achieved long-term disease control after receiving GD2 CAR-T cell therapy including a patient in remission of relapsed disease for >18 years without subsequent therapy.

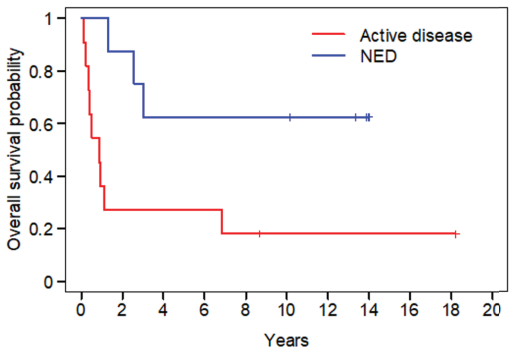


Figure 1. The median overall survival is 2.5 years (95% confidence interval 0.5 - NA years for all patients; 0.2 – 6.8 years in patients with active disease and not reached in patients with no evidence of active disease; $p = 0.019$, Log-rank test).

244. Efficacy and Safety of BRL-101, CRISPR-Cas9-Mediated Gene Editing of The BCL11A Enhancer in Transfusion-Dependent β-Thalassemia

Biao Zheng[1], Rongrong Liu[2], Xinhua Zhang[3], Bin Fu[4], Yajing Xu[4], Jun Shi[5], Xiaoqin Feng[6], Dali Li[1], Yuxuan Wu[1], Mingyao Liu[1], Yongrong Lai[2]

[1]BRL Medicine Inc., Shanghai, China,[2]The First Affiliated Hospital of Guangxi Medical University, Nanning, China,[3]923rd Hospital of the People's Liberation Army, Guangxi, China,[4]Xiangya Hospital, Central South University, Hunan, China,[5]Institute of Hemalology & Blood Diseases Hospital, Chinese Academy of Medical Sciences, Tianjin, China,[6]Nanfang Hospital Southern Medical University, Guangzhou, China

BACKGROUND: β-Thalassemia is an inherited hemolytic disease that is prevalent worldwide. Over 200 mutations in the HBB gene, which encodes adult hemoglobin (HbA), result in β-thalassemia. Hereditary persistence of fetal hemoglobin (HbF) can alleviate the symptoms of anemia. CRISPR-Cas9-mediated disruption of the BCL11A erythroid enhancer results in the reduction of BCL11A expression and the induction of fetal γ-globin, which is a practicable therapeutic strategy for treating transfusion-dependent β-thalassemia (TDT). ***METHODS:*** We obtained mobilized autologous CD34$^+$ cells from 9 TDT patients sponsor initiated in phase I/II clinical trial (NCT05577312) and 6 TDT patients in investigator initiated clinical study(NCT04211480, NCT04205435)respectively. These cells were edited with CRISPR-Cas9 RNP at the +58 erythroid specific enhancer region of the BCL11A gene and then reinfused after the patients had undergone myeloablative busulfan conditioning. We subsequently monitored adverse events, neutrophil and platelet engraftment. ***RESULTS:*** Between October 15, 2019, and August 24, 2023, 15 patients with TDT were enrolled and received BRL-101 with a median age of 11.5 years (6-26). Among all the treated patients, 8 patients were β^0/β^0 phenotype, 3 patients were β^0/β^+ phenotype,3 patients were β^+/β^+ phenotype, and 1 patient was β^0/β^E phenotype. As of November 27, 2023, the median follow-up was 9.8 months (3.2-43.6 m) (Tab.1). The longest duration of TI was 41.6 months. The levels of mean HbF increased significantly from 2.1 to 150.2 g/L and the mean total hemoglobin inceased from 114.1 to 152.0 g/L after BRL-101 infusion (Fig.1). At 5 months after BRL-101 infusion, the proportion of HbF-expressing red blood cells in peripheral blood had reached 96.5%, and then continued to rise and remained around 98-99% (Fig.2). Treatment-related adverse events were typical of those associated with myeloablation and autologous stem-cell transplantation, mainly manifested as hematological toxicities (Tab.2, Tab.3). 3 patients experienced adverse events (AEs) grade ≥3 which related to BRL-101. 4 patients experienced serious AEs (SAEs), including decreased platelet count, shock, febrile infection, soft tissue infection, and veno-occlusive liver disease. Only decreased platelet count may related to BRL-101, the others were due to busulfan treatment. All the SAEs were resolved. No study drug-related withdrawals or deaths occurred during treatment. ***CONCLUSIONS:*** Whether genotype was β^0/β^0 or non-β^0/β^0, BRL-101 demonstrated clinically meaningful increases in total Hb and HbF which occurred early and have been maintained over time, the safety profile of BRL-101 is generally consistent with that of myeloablative conditioning and autologous hematopoietic stem cell transplant. The updated data with 15 patients reported here are consistent with previous reports and support continued investigation of BRL-101 as a potential functional cure for patients with TDT.

Figure 1. Substantial increases in mean total Hb and HbF level



Figure 2. Mean (range) % peripheral F-cells, % circulating RBCs expressing HbF

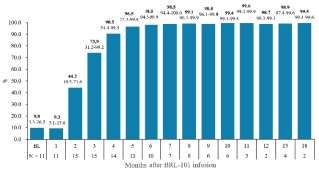


Table 1. Patient Baseline and Treatment Characteristics

Patient characteristics			Treatment characteristics		
Genotype, n	β0 / β0	8	Drug product cell dose,	Median (range)	
	β0 / β+	3	CD34+ cells × 10⁶/kg	8.63 (3.9-14.8)	
	β+ / β+	3			
	β0 / βE	1	Neutrophil engraftment Study Day	19.0 (14-29)	
Gender, Female/Male, n		7/8			
Age at consent, years		11.5 (6.0-25.0)	Platelet engraftment Study Day	41.0 (21-165)	
Pre-study pRBC transfusions Units/year, median (range)		36.0 (23.5-95.0)	Duration of follow-up, Months	9.8 (3.2-43.6)	

Table 2. Safety profile of BRL-101

Post-BRL-101 AEs Overview	n=15
Patients with any AEs, n(%)	15 (100%)
Patients with AEs related to BRL-101, n(%)	6 (40.0%)
Patients with SAEs, n(%)	4 (26.7%)
Patients with SAEs related to BRL-101, n(%)	1 (6.7%)
Patients with AEs Grade ≥3, n(%)	15 (100%)
Patients with AEs leading to death, n(%)	0

Table 3. Patients with AEs Grade 3/4 and SAE Sorted by SOC and PT

AEs Grade 3/4 and SAE	n=15	AEs Grade 3/4 and SAE	n=15
Investigations		**Vascular disorders**	
White blood cell count decreased	15 (100%)	Shock*	1 (6.7%)
Platelet count decreased*	15 (100%)	**Metabolism and nutrition disorders**	
Neutrophil count decreased	15 (100%)	Hypokalaemia	4 (26.7%)
Neutrophil percentage decreased	3 (20.0%)	**Respiratory, thoracic and mediastinal disorders**	
Lymphocyte count decreased	2 (13.0%)	Unilateral pleural effusion	1 (6.7%)
Alanine aminotransferase increased	1 (6.7%)	**Vascular disorders**	
Erythrocyte sedimentation increased	1 (6.7%)	Epistaxis	1 (6.7%)
Blood and lymphatic system disorders		**Infections and infestations**	
Febrile neutropenia	3 (20.0%)	Febrile infection*	1 (6.7%)
Anemia	2 (13.3%)	Pneumonia	2 (13.3%)
Gastrointestinal disorders		Infections	1 (6.7%)
Stomatitis	4 (26.7%)	Sepsis (6.7%)	1
Oral bacterial infection	2 (13.3%)	Upper respiratory tract infection*	1 (6.7%)
Tongue thrust	1 (6.7%)	**Skin and subcutaneous tissue disorders**	
Upper gastrointestinal haemorrhage	1 (6.7%)	Soft tissue infection*	1 (6.7%)
Lower gastrointestinal haemorrhage	1 (6.7%)	**Hepatobiliary disorders**	
Psychiatric disorders		Veno-occlusive liver disease*	1 (6.7%)
Dysphoria	1 (6.7%)		
Reproductive system and breast disorders			
Heavy menstrual bleeding	1 (6.7%)		

*Including SAEs (all resolved).

245. Lentiviral-Mediated Gene Therapy (RP-L102) for Fanconi Anemia [Group A], is Not Associated with Insertional Mutagenesis

Agnieszka Czechowicz[1,2,3], Julián Sevilla[4,5], Claire Booth[6], Rajni Agarwal[1,2,3], Josune Zubicaray[4], Paula Rio[5,7,8], Susana Navarro[2,7,8], Kritika Chetty[6], Gráinne O'Toole[6], Jinhua Xu-Bayford[6], Philip Ancliff[6], Elena Sebastián[4], Michael Rothe[9], Antonella L. Bastone[10], Philipp John-Neek[10], Axel Schambach[9], Wei Wang[11], Grace Choi[12], Miriam Zeini[12], Eileen Nicoletti[12], Cindy R. Eide[13], John E. Wagner[13], Gayatri R. Rao[12], Adrian J. Thrasher[6], Jonathan D. Schwartz[12], Maria-Grazia Roncarolo[1,2,3], Juan A. Bueren[5,7,8]

[1]Ctr for Definitive and Curative Med, Stanford Univ, Stanford, CA,[2]Dept of Pediatrics, Div of Hematology, Oncology, Stem Cell Transplantation, and Regenerative Med, Stanford Univ Sch Med, Stanford, CA,[3]Lucile Packard Children's Hosp, Palo Alto, CA,[4]Hematología y Hemoterapia, Fundación para la Investigación Biomédica, Hosp Infantil Univ Niño Jesús (HIUNJ), Madrid, Spain,[5]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain,[6]UCL Great Ormond Street Inst of Child Health, London, United Kingdom,[7]Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain,[8]Instituto de Investigación Sanitaria Fundación Jiménez Díaz (IIS-FJD), Madrid, Spain,[9]Hannover Medical School, Hannover, Germany,[10]Inst of Exp Hematology, Hannover Med Sch, Hannover, Germany,[11]Genewerk GmbH, Heidelberg, Germany,[12]Rocket Pharmaceuticals, Cranbury, NJ,[13]Univ Minnesota, Minneapolis, MN

Fanconi anemia (FA) is an inherited deoxyribonucleic acid repair disorder that results in progressive bone marrow failure (BMF) within the first decade of life in 80% of patients. Allogeneic hematopoietic stem cell transplant is curative for FA-related BMF with survival that exceeds 80% in experienced transplant centers, however adverse effects including 100-day mortality, severe infections, graft-vs-host disease and increased cancer risk present persistent limitations. We previously reported that autologous hematopoietic stem and progenitor cells (HSPCs) transduced with a lentiviral vector carrying the *FANCA* gene (RP-L102), administered without antecedent conditioning, conferred genetic and phenotypic correction as well as hematologic stability in at least 6 patients with FA-A treated in Rocket-sponsored studies with ≥1 year of follow up. Given that insertional mutagenesis is a potential risk associated with lentiviral (LV)-mediated gene therapy, we now provide data from longitudinal integration site analyses (ISA) conducted to assess clonal diversity and insertion site genomic location within patients' hematopoietic cells following RP-L102 administration. Patients with *FANCA* mutations, age ≥ 1 year, no HLA-matched sibling donor and ≥30 CD34+ cells/μL in bone marrow (BM) were eligible. Peripheral blood (PB) cells were collected via leukapheresis. Following CD34+ enrichment, HSPCs were transduced with a LV vector carrying the *FANCA* gene, and infused without cryopreservation or conditioning. Patients are followed for 3 years in the parent study and then in a long-term follow-up study. Peripheral blood mononuclear cell (PBMC) ISA was conducted regularly in a central laboratory to assess for overall clonality and any potentially predominant clonality, defined as an integration site with a VCN-normalized relative contribution ≥10%. Data are presented for all 23 patients treated with RP-L102 across Rocket-sponsored studies and FANCOLEN-I, a prior Investigator Initiated Study. As of Sept, 2023, mean [SD] follow-up was 3.57 [1.96] years (range: 0.4-7.7). Overall clonality and diversity indices (Gini, Shannon and UC50) indicated predominantly polyclonal integration patterns (range: oligoclonal to highly polyclonal). There was no evidence of RP-L102 related oncogenesis in any patient. Sequential ISA in patients treated with RP-L102 for up to 7.7 years demonstrated no insertional oncogenesis. These data contribute to the continuing favorable overall benefit:risk profile of RP-L102 as an *ex vivo* LV gene therapy for treatment of FA-A.

246. Autologous *Ex-Vivo* Lentiviral Gene Therapy for Pediatric Patients with Severe Leukocyte Adhesion Deficiency-I Provides Sustained Efficacy with a Favorable Safety Profile

Donald B. Kohn[1], Julian Sevilla[2,3], Gayatri R. Rao[4], Maria Chitty Lopez[4], Elena Almarza[2,3,4,5], Dayna R. Terrazas[1], Josune Zubicaray[2,3], Marta González-Vicent[2,3], Kritika Chetty[6], Gráinne O'Toole[6], Jinhua Xu-Bayford[6], Eileen Nicoletti[4], Caroline Y. Kuo[1], Satiro De Oliveira[1], Theodore B. Moore[1], Grace Choi[4], Miriam Zeini[4], Cristina Mesa-Núñez[3,5,7], Adrian J. Thrasher[6], Juan A. Bueren[3,5,7], Jonathan D. Schwartz[4], Claire Booth[6]

[1]Dept Pediatrics, Univ of California, Los Angeles, CA,[2]Fundación para la Investigación Biomédica, Hospital Infantil Univ Niño Jesús (HIUNJ), Madrid, Spain,[3]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER-ISCIII), Madrid, Spain,[4]Rocket Pharmaceuticals, Cranbury, NJ,[5]Unidad Mixta de Terapias Avanzadas, Inst de Investigación Sanitaria Fundación Jiménez Díaz (IIS-FJD), Madrid, Spain,[6]UCL Great Ormond Street Inst of Child Health, London, United Kingdom,[7]Centro de Investigaciones Energéticas Medioambientales y Tecnológicas (CIEMAT), Madrid, Spain

Background: Severe leukocyte adhesion deficiency-I (LAD-I) is a primary immunodeficiency caused by mutations in the *ITGB2* gene that encodes the common subunit of β2-integrins (CD18), which is essential for neutrophil adhesion to inflamed endothelium and migration into tissues. Severe LAD-I, predominantly defined as <2% of normal CD18 polymorphonucleocyte (PMN) expression, is characterized by frequent and often refractory bacterial and fungal infections, and significant pediatric mortality in the absence of allogeneic hematopoietic stem cell transplant (alloHSCT) which is curative but limited by donor availability, graft-versus-host disease, and graft failure. The gene therapyRP-L201 employs autologous CD34+ cells transduced with a lentiviral vector carrying *ITGB2* to restore CD18 expression. **Methods:** Patients ≥3 months old with severe LAD-I were enrolled in this Phase I/II study (NCT03812263). Hematopoietic stem cells, collected via apheresis after mobilization with G-CSF and plerixafor, were transduced *ex-vivo* with Chim-CD18-WPRE-LV (RP-L201, marnetegragene autotemcel). Myeloablative therapeutic drug-monitored busulfan conditioning preceded RP-L201 infusion. Patients were followed for safety and efficacy, including survival in the absence of alloHSCT to ≥1 year post-infusion and to 2 years for patients <1 year old at enrollment; peripheral blood (PB) PMN CD18 expression; PB vector copy number (VCN), leukocytosis improvement

and decreased infection-related hospitalizations. **Results:** Nine patients (age 5 months to 9 years) received RP-L201with median (range) follow-up of 29.3 (21.7-44.8) months; eight patients have completed ≥2 years follow-up. RP-L201 cell doses ranged from $2.8x10^6$to $10x10^6$ CD34+ cells/kg with a drug product VCN of 1.8 to 3.8. AlloHSCT free survival is 100%. No patients have had graft failure or graft-versus-host disease. Pre-treatment leukocytosis improved uniformly. Infection-related hospitalizations and prolonged hospitalizations (≥7 days)were markedly reduced ($p < 0.0001$) following therapy. All nine patients had sustained restoration of PMN CD18 expression (median over 18 to 42 months post-infusion: 17.4%to 87.4%) with concomitantly sustained genetic markings (median VCN/PBMC over 18to 42 months: 0.7-4.0). No RP-L201-related serious adverse events were reported. No evidence of replication-competent lentivirus has been observed. Insertion site analyses indicate highly polyclonal integration patterns across the entire cohort. **Conclusion:** RP-L201 has a favorable safety profile and confers durable correction of the severe LAD-I phenotype in all nine pediatric patients treated, eight of whom have ≥2 years of follow-up, as demonstrated across all relevant clinical and laboratory parameters.

AAV Vectors - Preclinical and Proof-of-Concept: Technology Focus

247. S/MAR-Containing AAV Vectors Result in an Increase in AAV Episomes & a Reduction in AAV Integration Sites in a Mouse Model with a High Rate of Hepatocyte Proliferation

Andrea Llanos-Ardaiz[1], Aquilino Lantero[1], Marina Ruiz de Galarreta[1], Leire Neri[1], Itsaso Mauleón[2], Martin Bilbao[2,3], Blanche Tamarit[4], Veronica Ferrer[4], Anne M. Douar[4], Jean-Philippe Alex Combal[4], Rafael Aldabe[2], Gloria Gonzalez-Aseguinolaza[1,2]

[1]Vivet Therapeutics, Pamplona, Spain,[2]DNA & RNA Medicine Division, Centre for Applied Medical Research (CIMA), University of Navarra, Pamplona, Spain,[3]IdISNA, Navarra Institute for Health Research, Pamplona, Spain,[4]Vivet Therapeutics, Paris, France

Scaffold/matrix attachment regions (S/MARs) are highly conserved ubiquitous DNA sequences that are involved in chromatin organization and gene regulation. Studies have shown that these sequences are able to increase expression and facilitate nuclear retention of exogenous DNA that contain them, allowing the exogenous DNA to replicate in synchrony with the host genome. Adeno Associated Viruses (AAVs) are single stranded DNA vectors broadly used for gene therapy (GT) due to their good safety profile and therapeutic efficacy. AAVs have a very low integration rate, although some integration events have been observed with both wtAAVs and rAAVs. Due to the non-integrative nature of AAV, their genomes, that remain as episomes, are lost upon division of the transduced cells, and thus therapeutic efficacy in growing organs, such as the infant liver, is transient. Hereditary tyrosinemia type I (HT1) is an autosomal recessive disease arising from mutations in the fumarylacetoacetate hydrolase (FAH) coding gene resulting in disruption of the tyrosine catabolism pathway. This leads to the accumulation of toxic metabolites that cause liver failure as well as kidney damage. The standard of care consists of daily dosing of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) that prevents the production of toxic metabolites and ultimately of liver transplant. Using an HT1 mouse model (Fah1R Tyrc/RJ), in which corrected hepatocytes have a selective advantage, we questioned whether the presence of a S/MAR sequence in an AAV expressing hFAH would favour maintenance of the AAV genome as an episome while inducing its replication in synchrony with hepatocyte regeneration, leading to a sustained therapeutic effect in damaged livers. Mice were injected with a subtherapeutic dose (1E11 vg/kg) of either a control AAV (AAV-FAH) or an AAV with the S/MAR sequence inside (AAV-FAH-S/MAR-pA) or outside (AAV-FAH-pA-S/MAR) the transcriptional unit. NTBC was withdrawn 2 weeks post AAV injection (p.i.) and body weight (BW) and serum biomarkers (SB), were followed periodically until 20% BW loss or to 70 days p.i., at which point the mice were sacrificed. Viral genomes and hFAH expression levels in liver were quantified by qPCR. FAH^+ liver area was detected by IHC, and viral integration was analysed by S-EPTS/LM-PCR. Results showed that AAV-FAH-S/MAR-pA achieved higher therapeutic effect (66.7% survival), than either the control (15% survival) or the AAV-FAH-pA-S/MAR vector (40% survival). Three different phenotypes were observed upon NTBC withdrawal based on BW and SB: failed, recovered, and stable mice. Survivors showed BW and SB normalization after approximately 50 days p.i. and the number of viral genomes and hFAH expression levels were similar between experimental groups. IHC showed FAH expression in clusters of positive hepatocytes in recovered mice, suggesting potential AAV integration and clonal expansion. Importantly, S-EPTS/LM-PCR studies revealed higher presence of episomal AAV genomes, read as vector-vector junctions, in the group treated with AAV-FAH-S/MAR-pA (2-fold difference vs. the control group), furthermore the number of integration sites was reduced in comparison to the other two groups. These results indicate that not only the presence of the S/MAR sequence, but also its position within the therapeutic vector, influence the fate of the viral genome in the transduced cell.

248. A Durable Gene Therapy with a Robust AAV-LNP Delivery System Allowing for a Reduced AAV Dose

Matthew Nitzahn, Bernard Kok, Oscar Alvarez-Garcia, David Ebeid, Lucas Homa, Khalid Hajj, Zhibo Ma, Alicia Davis, Cindy Negron, Joshua Rychak

Poseida Therapeutics, San Diego, CA

Adeno-associated virus (AAV) vectors have strong potential for treating genetic diseases but can face dose-related toxicity hurdles, especially when a high proportion of cellular correction is needed. Achieving lifelong expression of the therapeutic transgene is also challenging with AAV, particularly when treating children. Technologies that enable genomic integration of a therapeutic transgene have the potential to provide durability and possibly boost transgene expression. Our piggyBac™ DNA insertion system is unique in its ability to integrate large cargo into regions of the genome that support robust expression. Super piggyBac (SPB) is an enhanced transposase that enables highly efficient genome integration. In the current studies, we explored the feasibility of combining existing AAVs with SPB and demonstrate substantial reduction in AAV dose in two animal models of disease.

We first developed a lipid nanoparticle (LNP) for SPB mRNA delivery and an AAV transposon vector for liver-targeted gene expression. *In vivo* tolerability and pharmacology studies were conducted in rodents and non-human primates to characterize novel LNPs in combination with liver-tropic AAV capsids (AAV8, AAV9, or KP1). Integrated vector copies were dose-responsive (1-3 per cell) with a genomic insertion profile similar between human and mouse cells, and no bias toward exon sequence. We next tested this hybrid system in neonatal and adult mouse models of Ornithine Transcarbamylase Deficiency (OTCD) and Phenylketonuria (PKU), respectively. Using a mouse model of PKU (B6.BTBR-*Pah*^{enu2}/MalnJ), adult animals were dosed an AAV-PAH transposon vector, or the same vector co-administered with SPB-LNP. We observed a striking increase (>10-fold) in the frequency of hepatocytes expressing the PAH transgene following AAV+LNP treatment relative to AAV-alone at matched dose levels. Subsequent multi-dose studies demonstrated correction with the AAV+LNP system at AAV doses 3 to 10 times lower than non-integrating AAV alone, demonstrating that piggyBac-mediated integration can enable excellent efficacy at reduced AAV dose levels. We separately evaluated the hybrid platform in a mouse model of severe OTCD to explore the feasibility of utilizing this system to treat early-onset disease. Mice were dosed on day 1 of life with an AAV-OTC transposon vector or the same vector co-administered with SPB-LNP. High (>2E13 vg/kg) doses of AAV alone did not enable survival into adulthood; in contrast, co-administration of 0.25 mg/kg of the SPB-LNP with ten times lower AAV dose (3E12 vg/kg) enabled 100% survival. Liver immunostaining showed broad (>55% cells) hepatocyte expression of OTC protein persisting for at least 6 months in hybrid-treated mice, while AAV-only treatment yielded <1% of hepatocytes expressing OTC protein ~6 weeks after treatment. These data demonstrate the utility of the hybrid platform for enabling treatment at lower AAV dose levels. Additionally, we demonstrate how the platform can extend the utility of AAV for treatment of disease early in life. The piggyBac DNA insertion system can be deployed effectively with a variety of AAV capsids and used to expand the utility of AAV-based gene therapy.

249. Novel Micro-Dystrophin Constructs for DMD Gene Therapy

Ruchita Selot, Subhradeep Sarkar, Chitra Gopinath, Riya Patra, Arkasubhra Ghosh

Center for Genetics and Gene Therapy, Grow Lab Narayana Netralaya Foundation, Bangalore, India

Introduction Duchenne Muscular Dystrophy is caused by loss of the Dystrophin protein essential for muscle function. Due to its large size, the native Dystrophin gene cannot be packaged into AAV vectors for gene therapy. Hence, smaller, truncated versions called Micro-Dystrophins have been designed and tested in animal models and clinical trials. Although such a Micro-Dystrophin therapy has received conditional approval, the results of the trial indicate that despite high doses, the functional improvement in patients is low, necessitating better designs. We have therefore tested a set of new Micro-Dystrophin vectors that differ by domain designs. **Methods** Four micro-Dystrophin constructs were designed with ubiquitous (CAG) or muscle-specific (Spc5-12) promoters and codon-optimized. The constructs GL1 (ΔR4-R15/R19-R22/H3/CTD), GL2 (ΔH2/R4-R15/R19-R23/H3/CTD), GL3 (ΔH2/R3-R15/R19-R23/H3/CTD) and GL4 (ΔH2/R3-R15/R19-R22/H3/CTD) had common N-terminal, Hinge 1 and 4, nNOS binding domains, and the cysteine rich domain. They differed in their sequences for binding to distinct domains for sarcolemmal lipoprotein interactions and microtubule-binding. Each micro-gene was packaged in AAV9 capsids and delivered to *mdx* mouse TA muscles at a dosage of 1x10^9vg/muscle. GL3 was further injected systemically at a dosage of 3.5x10^{13}vg/kg with AAV9.eGFP as control in *mdx* mice via tail vein. Post-sacrifice, muscle tissues were analyzed for transgene expression, DAG complex, immune infiltration, and centro-nucleation. **Results** Expression of Dystrophin was observed in all four constructs, with GL1 (65.5±3.89%) and GL3 (70.71±3.08%) exhibiting significantly higher levels compared to GL2 (42.79±5.08%) and GL4 (38.47±3.536%) [***=p<0.0001, one-way ANOVA]. Furthermore, GL1 (42.266±0.039%) and GL3 fibers (36.0±7.69%) displayed minimal aggregation of nuclei in muscle, in contrast to the control (69.65±0.08%). Additionally, GL1 (10.1±1.62%) and GL3 (8.3±1.60%) constructs demonstrated lower levels of macrophage infiltration compared to the control (24.80±2.059%) and significantly higher levels of nNOS and Dystroglycan restoration (p<0.05). This observation was supported by a significant increase (***=p<0.0002) in both distance and time on the treadmill for *mdx* mice with GL3 systemic injection compared to sham-injected mice, indicating enhanced muscle function. **Discussion and Conclusion** Our findings indicate that the strategic choice of Dystrophin domains [GL1(ΔR4-R15/R19-R22/H3/CTD) and GL3(ΔH2/R3-R15/R19-R23/H3/CTD)] facilitated efficient Dystrophin expression, and resulted in sarcolemmal localization resulting in restoration of DAG complex. We anticipate that GL1 and GL3 constructs will contribute favorably towards ameliorating the DMD phenotype.

250. Addressing the Translational Potential of Neurotropic AAVs Using Functionally Mature Human Stem Cell-Derived Blood-Brain Barrier Organoids

Angelo Iannielli[1], Martina Greco[1], Serena G. Giannelli[1], Mirko Luoni[1,2], Benjamin E. Deverman[3], Vania Broccoli[1,2]

[1]Ospedale San Raffaele, Milan, Italy,[2]Institute of Neuroscience, CNR, Milan, Italy,[3]Broad Institute, Cambridge, MA

Novel AAV9 engineered capsids have been selected for their superior ability to cross the blood-brain barrier (BBB) and diffuse into the brain parenchyma after intravascular administration. However, their behaviors have been assessed in mice up to non-human primates, but whether the same capacity remains unchanged in a human setting remains unknown. To address this, we established improved procedures to differentiate human pluripotent-stem cells (hPSCs) into endothelial, mural and astroglial cells by defining an original combination of small molecule cocktails with the expression of cell lineage-specific master regulators. To assemble the three cell types together we decided to generate organoids that recapitulate the spatial organization of the BBB on a transwell filter (fBBBOs). We demonstrated that fBBBOs display a tight and homogeneous endothelial cell layer with well-organized cell junctions clustering key endothelial cell junction components including Glut1 and Occludin. BBBOs developed high functional properties with high values of trans-epithelial electric resistance (TEER) and

impermeability to high molecular mass dyes. Furthermore, we tested the fBBBO endothelial integrity by assessing any eventual crossing of AAV2 and lentiviruses (LVs) that notoriously are incapable to trespass this biological barrier in vivo. Indeed, the fBBBOs effectively blocked the diffusion of both LVs and AAV2. In contrast, the AAV-PHP.B was able to efficiently transverse the fBBBOs only when expressing its transcytosis receptor Ly6A. These results demonstrate that BBBOs have acquired a highly selective permeability. Altogether, the deployment of cell lineage master improves the consistency of cell differentiation and subsequent maturation state. Spatially-defined cultures of endothelial, mural and astroglial cells produced a robust in vitro neurovascular model with long-lasting stability and functions. Human fBBBOs offer a neat system for testing the translational potential of new brain shuttling agents, including the new AAV engineered variants exploited in animals or in vitro.

251. *In Situ* Biodistribution and Localization of Bidridistrogene Xeboparvovec (SRP-9003) in LGMD2E/R4 Mice

Young Seo, Stephen Fasul, Chrissy Hopkins, Akansha Pradhan, Jasmine Wu, Alex Haile, Rachael Potter, Sarah Lewis, Stephen Baine, Louise Rodino-Klapac

Sarepta Therapeutics, Inc., Cambridge, MA

Background Limb-girdle muscular dystrophy type 2E (LGMD2E/R4) is caused by mutations in the β-sarcoglycan (*SGCB*) gene resulting in loss of functional SGCB protein, progressive muscle degeneration, and shortened lifespan. Bidridistrogene xeboparvovec (SRP-9003, rAAVrh74.MHCK7.hSGCB) is an investigational adeno-associated virus (AAV)-based gene therapy designed to restore functional SGCB expression in muscle and improve clinical outcomes in patients with LGMD2E/R4. RNAscope™ has recently emerged as a technology capable of determining tropism, transduction efficiency, and in-situ localization of targeted gene therapies. Here we report the use of RNAscope™ to elucidate the spatial biodistribution, cellular tropism, transgene expression, and durability of SRP-9003 following intramuscular (IM) and intravenous (IV) administration in LGMD2E/R4 mice. **Methods** The RNAscope™ in-situ hybridization (ISH) probes were designed to be non-overlapping for two-plex ISH. One probe detected AAVrh74 vector DNA only (anti-sense strand), while a second probe captured *hSGCB* transgene mRNA (sense strand) allowing evaluation of subcellular localization of vector genome DNA and *hSGCB* RNA for spatial analysis. Mouse tissues were assessed at 24 hours, 7 days, 14 days, 21 days, and 28 days following a single IM administration (3×10^{10} total vector genomes) and up to 240 days after single IV administration of bidridistrogene xeboparvovec (7.41×10^{13} vector genomes per kilogram [vg/kg]). Importantly, timepoints evaluated in the systemic study included day 60, which corresponds to the timepoint for biopsy procedure utilized in clinical studies. For RNAscope™, vector genome DNA and *hSGCB* RNA were quantitively analysed using fluorescence in-situ hybridization (FISH) and FISH- immunofluorescence (IF) algorithm with the HALO® Image Analysis Platform. Vector DNA was also quantified using digital droplet polymerase chain reaction (ddPCR) (copies per nucleus), *hSGCB* mRNA was measured using reverse transcription (RT)-ddPCR, and hSGCB protein expression was assessed using IF (as percent beta-sarcoglycan positive fibers [%PβPF] quantified with HALO® Muscle Fiber FL algorithm). **Results** Proof of principle for in-situ detection of bidridistrogene xeboparvovec was established following IM evaluation. Vector genome copies were detected in all bidridistrogene xeboparvovec-dosed animal tissues by both RNAscope™ and ddPCR 24 hours after injection with DNA levels decreasing at day 7. In contrast *hSCGB* mRNA transcript levels plateaued at day 14 using both RNAscope™ and RT-ddPCR. IF results showed consistent SCGB protein expression at day 14 through day 28 (average PβPF 54.9% and 47.6%, respectively). Systemic administration (7.41×10^{13} vg/kg) of bidridistrogene xeboparvovec revealed significant vector DNA and mRNA levels at day 60 in muscle tissue, which corresponded to robust hSGCB transgene expression and reinforced the day-60 biopsy to assess maximal SGCB expression following treatment. In addition, IV administration of bidridistrogene xeboparvovec led to significant vector DNA and mRNA expression up to 120 days post dosing in skeletal and cardiac muscle. Limited off-target effects of bidridistrogene xeboparvovec were demonstrated by lack of DNA or mRNA probe signal in selected organs (kidney, brain; D14-D120) highlighting the preferential targeting of muscle and durable efficacy of bidridistrogene xeboparvovec in LGMD2E/R4 mice. **Conclusion** This study demonstrates the applicability of RNAscope™ to determine the spatial, cellular organization, and molecular trafficking of bidridistrogene xeboparvovec. These data may be useful to inform future application of RNAscope™ for biodistribution evaluation of AAVrh74 gene therapy.

252. Transgene Protein Evolution as a Novel Strategy for Next-Gen Gene Therapy in Canavan Disease

Sarah Foley[1], Morgan Mooney[2], NhatAnh B. Nguyen[2], Anoushka Lotun[2], Guangping Gao[2,3,4], Dominic J. Gessler[2,4], Lee Coffey[1,5]

[1]Pharmaceutical and Molecular Biotechnology Research Centre, South East Technological University, Waterford, Ireland,[2]Horae Gene Therapy Centre, UMass Chan Medical School, Worcester, MA,[3]Li Weibo Institute for Rare Diseases Research, UMass Chan Medical School, Worcester, MA,[4]Co-corresponding author, Worcester, MA,[5]Co-corresponding author, Waterford, Ireland

Canavan Disease (CD) is characterised as a progressive neurodegenerative disease caused by the accumulation of N-acetylaspartate (NAA) as a result of the deficiency of aspartoacylase (ASPA) enzyme associated with mutations in the ASPA gene. ASPA is responsible for the supply of acetate for the oligodendrocytes and is essential for normal myelination. Recombinant adeno-associated (rAAV) gene therapy (rAAV9-ASPA) has been shown to rescue the CD knock-out mouse model from premature death. This has been supported by the normalisation of NAA levels and growth, improved motor function, and ASPA gene expression detected in the CNS of the treated CD mice. The advancements in the design of rAAV as a gene therapy delivery vehicle have provided significant potential for numerous rare diseases with unmet therapeutic options. One of the remaining challenges for rAAV gene therapies for CNS disorders is the high dose required to achieve sufficient transgene protein levels. The high cost associated with rAAV production and the risk of systemic toxicity is further exacerbated by the vector dose required. To offer solutions to the dosage-associated toxicity and costs, we have hypothesised that the amino acid sequence translated from the rAAV-delivered transgene can be evolved and optimised for

enhanced transgene protein activity. To explore if specific mutation(s) in the wild-type ASPA (WT-ASPA) amino acid sequence could achieve increased ASPA enzyme activity, we constructed a random mutant ASPA library (~10^3) using an optimised directed evolution approach to maximise mutation distribution and diversity. The use of a prokaryotic expression system allowed for ease of microscale protein expression and high throughput functional screening by a spectroscopic method benchmarked against the recombinant human WT-ASPA enzyme. This led to the identification of several evolved ASPA (ev.ASPA) enzyme candidates with increased enzymatic activity. Subsequently the ev.ASPA candidates underwent enzyme purification and secondary screening using a high-sensitivity chromatography method to confirm enhanced performance. To determine the *in vivo* performance of ev.ASPA, the leading candidate nucleotide sequences were cloned into rAAV9 (rAAV9-ev.ASPA) and delivered to CD mice on postnatal day 1 (P1) intravenously at a 3-fold lower dose than our clinical full dose vector expression WT-ASPA. Initial findings show disease rescue in the CD mouse model, supported by improvements in growth and motor function. In addition, none of the treated animals have succumbed to the disease. This contrasts with untreated animal's overall survival of around 24 days of age. Studies to directly compare ev.ASPA to our clinical vector is underway to determine the side-by-side *in vivo* performance of our transgene evolution approach. This approach could be a critical contribution to rAAV genome design for therapeutic applications. In terms of rAAV delivery to the CNS, this would allow for potential viral vector dose reduction, all while maintaining the therapeutic efficacy - providing a novel solution to CD and other gene therapy applications and benefiting the wider gene therapy research community.

253. Antibody Gene Therapy for Rabies Encephalitis

Jyoti Yadav[1,2], Amanda L. Gross[2], Giovanna Panepinto[2], Ywh-Min Tzou[2], S. Satheshkumar Panayampalli[3], L. Claire Godino[3], Kent R. Van Kampen[4], Nicholas J. Jones[5], April D. Davis[5], Miguel Sena-Esteves[6,7], Henry J. Baker[2], Douglas R. Martin[1,2]

[1]Department of Anatomy, Physiology, & Pharmacology, Auburn University, Auburn, AL,[2]Scott-Ritchey Research Center, Auburn University, Auburn, AL,[3]Centers for Disease Control and Prevention, Atlanta, GA,[4]The Van Kampen Group, Payson, UT,[5]Department of Health, Wadsworth Center Laboratory, Albany, NY,[6]Department of Neurology, University of Massachusetts Medical School, Worcester, MA,[7]Horae Gene Therapy Center, Worcester, MA

Introduction. Rabies virus is a neurotrophic virus with almost 100% fatality. Vaccination strategies currently used to prevent rabies infection majorly rely on the generation of the host serum neutralizing antibodies. The major caveat of these antibody-based therapy is that while the rabies virus penetrates the CNS, antibodies cannot cross the blood brain barrier, making them largely ineffective in the treatment of symptomatic encephalitis. We designed a recombinant AAV9 (rAAV9) vector to traverse the blood brain barrier and express a neutralizing human antibody against rabies glycoprotein in the brain. The goal is to produce antibody-mediated protection in the CNS, independent of the host immune system. Methods. A rAAV9 vector expressing a broadly neutralizing human antibody against rabies (CR57) was used as pre- or post-exposure treatment to determine the effect of single-dose intravenous (IV) delivery on prevention or rescue of the infection in rabies-challenged mice. C57BL/6J mice were treated with low and high IV doses (1x10^{12} and 1x10^{13} vg/kg, respectively) of rAAV9, 14 days prior to rabies virus inoculation. For the rescue study, mice were immunized with 1x10^{13} vg/kg of rAAV9, 3 days after rabies inoculation, and monitored for 70 days. We analyzed rabies neutralizing antibodies in serum using Fluorescent Antibody Virus Neutralization (FAVN) and in brain by IHC. Additionally, 4-month-old cats were treated with low (2x10^{12} vg/kg) or high (1x10^{13} vg/kg) IV doses of rAAV9 and tissues were analyzed. Results. The IV delivery of rAAV9 14 days prior to rabies inoculation prevented disease in 100% (16 of 16) and 88% (14 of 16) of mice in the high- and low-dose cohorts, respectively. Rabies-inoculated, saline treated control mice (6 of 7) reached a predetermined humane endpoint within 11 days and tested positive for the rabies virus in postmortem samples. The rabies neutralizing antibody level in mouse serum from the end of study was >1000 times higher than the protective titer recommended by the WHO (0.5 IU/ml) after the high dose of rAAV9 vector. The expression of neutralizing antibodies was further tested in brain cells by IHC. In the post-exposure study, 92% (11 of 12) of mice survived when treated with the high dose of rAAV9 3 days after rabies inoculation (*Fig 1*). In rAAV9-treated cats, the high-dose treatment group showed significant levels of neutralizing antibodies in serum for at least 15 months and in CSF for 3 months post-injection. The decline in rabies neutralizing antibodies in feline serum after 15-months is due to the development of cat immune response against human CR57. Nonetheless, rabies neutralizing antibodies were detected in the feline brain of the high-dose treatment group at endpoint by IHC (*Fig. 2*). Conclusions. An AAV9 vector injected intravenously in rabies-challenged mice expresses broadly neutralizing antibodies in brain cells and protects against rabies infection in mice. Cats treated by IV injection of the same rAAV9 vector express rabies neutralizing antibodies in serum and brain for at least one year.

Fig. 1

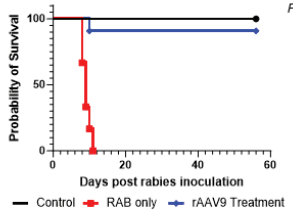


Fig. 2

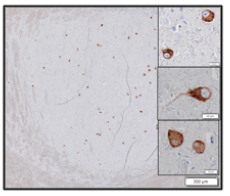


Fig. 1: Kaplan-Meier survival curve for rAAV9 treatment, 3 days post-lethal dose of rabies inoculation in mice.
Fig. 2: Analysis of broadly neutralizing rabies antibodies (represented by brown stained neurons) in the brain section of rAAV9 treated cats 24 months post injection.

On- and Off-Target Method Development

254. Precise Measurement of CRISPR Genome-Editing Outcomes through Single-Cell DNA Sequencing

Ayal Hendel[1], Nechama Kalter[1], Michael Rosenberg[1], Saurabh Gulati[2], Shu Wang[2], Benjamin Schroeder[2], Alex Li[2]

[1]The Institute for Advanced Materials and Nanotechnology, The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel,[2]Mission Bio, South San Francisco, CA

CRISPR-Cas genome-editing technology is rapidly integrated in diverse clinical applications for the treatment of various diseases. However, the clinical utilization of this technology encounters a bottleneck in the form of off-target activity, the inadvertent cleavage of the Cas nuclease at genomic loci beyond the intended targets. Off-target activity results in the formation of insertions and deletions (inDels) and structural variations (SVs), potentially driving oncogenic mutations. Many studies have characterized off-target activity at a cell-population level using bulk-sequencing-based methods. Nevertheless, these approaches lack sufficient sensitivity to study the off-target implications at the resolution of individual cells. To address this gap, we have harnessed the Tapestri technology, a single-cell DNA sequencing (scDNA-seq) platform which enables the sequencing of CRISPR-edited cell products at a single-cell level. This technology offers high-resolution insights into the co-occurrence of on-target and off-target editing, zygosity of editing profile, precise editing activity measurement per cell and per allele, and cell clonality. Furthermore, unlike conventional amplification-based bulk sequencing methods, which only report SVs presence, single-cell data enables the capture of the SVs frequency while integrating the inDels data. The enhanced resolution is primarily pivotal in CRISPR experimental designs aimed to simultaneously edit multiple biological targets, to ensure a genome-editing product that is edited at all specified targets. Hence, we have engineered human primary T-cells donors using a multiplex three guide RNAs (gRNAs) complex, designed to knock-out the *PDCD1*, *TRAC*, and *TRBC* anti-cancer targets. To address the off-target activity of these guides, we created a panel comprising of 111 putative off-target sites based on prior GUIDE-seq experiments, and sequenced these sites using the Tapestri platform, generating output data of ~10,000 edited cells per donor. Next, we evaluated the inDel profile at each allele, and quantified the CRISPR editing activity. Interestingly, different gRNAs showed different ratios of mono-allelic versus bi-allelic editing. Furthermore, only a minority of the cell population harbored bi-allelic frameshift mutations across all on-target sites. Single-cell analysis also revealed a heterogenous spectrum of CRISPR outcomes, with a unique on-target and off-target profile per cell, highlighting the importance of thoroughly characterizing the genome-engineered cell product. We also identified translocations and long-deletions between on-target and off-target loci. CRISPR editing-activity and translocations results were validated using bulk DNA sequencing using rhAmp-Seq technology, attesting to a high degree of reproducibility. In summary, the utilization of single-cell DNA sequencing for the assessment of off-target activity outperforms prevailing standards by (1) facilitating precise measurement of editing activity at both on- and off-target sites; (2) comprehensive profiling of the editing outcomes, encompassing the co-occurrence of editing outcomes; and (3) sensitive SVs identification. This comprehensive high-resolution methodology represents a leap forward in our understanding of CRISPR outcomes, while also serving as a platform for a highly sensitive quality control assessment of genome-engineering products, holding the potential to enhance and optimize therapeutic applications of the CRISPR-Cas platform.

255. Heterogeneous On-Target Genomic Rearrangements Occur upon Long Range Gene Editing in T-Cells

Daniele Canarutto[1], Claudia Asperti[1], Stefano Beretta[1], Chiara Gaddoni[1], Gabriele Pelosi[1], Elisabetta Rovelli[1], Simona Porcellini[1], Samuele Ferrari[1], Chiara Brandas[2], Maria Carmina Castiello[1,3], Anna Villa[1,3], Marina Radrizzani[1], Luigi M. Naldini[1,4]

[1]SR-TIGET Telethon Institute for Gene Therapy, Milan, Italy,[2]Translational and Molecular Medicine (DIMET), University of Milano-Bicocca, Monza, Italy,[3]UOS Milan Unit, Istituto di Ricerca Genetica e Biomedica (IRGB), CNR, Milan, Italy,[4]Vita-Salute S. Raffaele University, Milan, Italy

Introduction Long-range gene editing by homology driven recombination (HDR) of primary haematopoietic cells is a promising and currently almost unique strategy for the targeted integration of long DNA sequences under clinically feasible conditions. HDR-based gene editing is expected to result in precise integration of a single copy of the donor template in the target locus, and bona-fide HDR is commonly assessed by droplet-digital PCR (ddPCR). However, this approach is blind to imprecise and heterogeneous structural outcomes. Here we unbiasedly assessed genomic outcomes with optical genome mapping (OGM) and Oxford Nanopore sequencing (ON) upon long-range HDR editing of T-cells with Cas9 and an integrase defective lentiviral vector (IDLV), overcoming the limitations of PCR-based technologies. **Methods** Human male CD4 T-cells were edited at the X-linked *CD40LG* locus with Cas9 ribonucleoprotein and IDLV donor template carrying a functional truncated CD40LG cDNA for corrective knock-in into the locus coupled with LNGFR reporter, in GMP-like conditions. Correctly edited cells were then enriched by immunomagnetic selection and expanded as previously reported. OGM effective coverage was >1400X; data was analyzed with the rare variant pipeline. For ON, up to 6 gRNAs were designed to frame a region of interest of 3.8-7kb in the *CD40LG* locus. High molecular weight DNA was extracted and processed with modified Oxford Nanopore Cas9 sequencing kit. Reads were aligned using the wf-aligment Epi2me pipeline and then annotated manually. **Results** Having previously validated OGM for assessment of genomic rearrangements, we first uncovered the presence of template concatemers at the target site upon editing. We then sought to sequence on-target outcomes by ON. After workflow optimization, we acquired ≈340k reads/sample with an on-target enrichment of ≈0.1% per run. Median on-target read length was 7kb (range 0.2-76). As expected, a small percentage of the reads mapped to the native locus with or without small indels. About half of the on-target reads spanned the entire locus and supported bilateral HDR events at both the 5' and 3' homology arms, and HDR frequency was found to be similar to that measured by ddPCR. Interestingly, IDLV concatemers containing up to 6 long-terminal repeats were found in about 1/8

of the reads. Moreover, about 10% of the reads were unique and contained heterogeneous rearrangements, involving the native locus, the donor template, and/or other chromosomal regions. Notably, as the cell product underwent purifying selection for reporter expression from the hemizygous targeted locus, the vast majority of outcomes were compatible with transgene expression and may thus represent a fraction of those generated in the editing process. **Conclusions** IDLVs may constitute concatemers that can be integrated on-target. Complex on-target rearrangements can occur upon HDR editing of T-cells, and occasionally include genomic sequences from distant loci. Such trapping of unexpected sequences may also occur also with Cas9 alone, alternative donor templates, and different primary cell types. While some of these outcomes may not raise safety concerns and even be compatible with the intended goal of editing, the extent and breadth of heterogeneity occurring at the targeted locus defies the expectation of precision engineering and supports coupling the process to a natural or engineered purifying selection for safe clinical translation.

256. Selecting Highly Conserved and Specific Guide RNAs for CRISPR/CasX-Mediated Gene Editing of the HSV-1 Genome

Meltem Isik, Kevin Luk, Nadia Amrani, TJ Cradick, Jennifer Gordon, Guoxiang Ruan

Excision BioTherapeutics, Watertown, MA

Herpes simplex keratitis (HSK), caused by herpes simplex virus type 1 (HSV-1) infection of the cornea, is a major cause of blindness worldwide. After the primary infection of the ocular surface, HSV-1 is transported into trigeminal ganglia (TG) and other ganglia to establish lifelong latency, from which reactivation of the virus causes recurrent disease. Current anti-HSV-1 therapies, such as acyclovir, valacyclovir, and famiciclovir, interfere with the replication of viral DNA. However, these antiviral agents do not eliminate the latent HSV-1 reservoirs, and thus are only suppressive rather than curative. CRISPR-Cas-mediated gene editing offers the potential to address the underlying causes of the disease by eliminating the latent HSV-1 reservoirs directly. CasX (type V Cas12e) from Planctomycetes (PlmCasX) has recently garnered significant attention because of its compact size (less than 1000 aa) and potential for high specificity. The large size (~152kb) of the HSV-1 genome and the genetic diversity of infections collected globally (1-4% variation in nucleotide identity between viral genomes) have made it challenging to fully understand the HSV-1 virus from a clinical genomics perspective. Many studies have examined the global genetic diversity of HSV-1, with ongoing research uncovering new genotypes as more viruses are sampled worldwide. Therefore, it is important to consider HSV-1 genetic diversity when selecting conserved lead guides for therapeutic genome editing of the HSV-1 genome. In this study, we used the CRISPR/CasX gene editing system to inactivate the latent HSV-1 virus by co-targeting two essential HSV-1 genes, ICP0 and ICP27. To perform conserved guide design, we built an Herpesviridae family CDS database using the VIPR database (version Feb, 2022; 46,027 strains and 62,554 Genbank IDs). This database was used to choose highly conserved guide RNAs. 22 PlmCasX guide RNAs targeting ICP0 and 20 PlmCasX guide RNAs targeting ICP27 were found with a perfect sequence match in over 70% of HSV-1 strains. They were also found with perfect match to the consensus sequences of ICP0 and ICP27. The 440 guide RNA pairs formed with these 42 PlmCasX guide RNAs were tested in tissue culture for DNA excision efficiency. The PlmCasX guide RNAs from the most efficacious guide RNA pairs were evaluated for possible off-target editing using homology-dependent methods (computational nomination, hybrid capture, and Amplicon-seq) and homology-independent methods (GUIDE-seq). The results were analyzed using in-house developed bioinformatics pipelines for GUIDE-seq and targeted amplicon sequencing. These pipelines are built using Nextflow, a workflow tool to run tasks across multiple compute infrastructures in a very portable manner, with direct integration of AWS S3 buckets for automated data ingestion and helps with maintaining a database for reproducible results. The off-target assessment identified several highly specific PlmCasX guide RNAs that showed no detectable off-target editing activity. In summary, our comprehensive bioinformatics platform has helped identify highly conserved and specific PlmCasX guide RNAs for inactivating the HSV-1 virus.

257. *In Silico* Prediction Tool for Meganuclease Off-Target Sites

Zhe Zhang, R. Jason Lamontagne, Jenny A. Greig, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

The disruption of genetic coding sequences via sequence-specific nucleases represents a promising genome editing approach to reduce specific protein levels for therapeutic benefit, or produce specific double-strand breaks for targeted gene insertion. We extensively evaluated the long-term safety profile of an engineered ARCUS meganuclease (M2PCSK9) expressed from an adeno-associated virus (AAV) vector in liver of rhesus macaques. The M2PCSK9 meganuclease recognizes and edits a 22-bp target site within exon 7 of the *PCSK9* gene. As part of our assessment of the safety and efficacy of the M2PCSK9 meganuclease, we have previously utilized a genome-wide assay called inverted terminal repeat sequencing (ITR-seq), to identify the M2PCSK9 meganuclease off-target sites within the macaque genome when it is delivered by an AAV vector. Following identification of off-target sites, we performed amplicon sequencing analysis to confirm and quantify editing at individual sites. In an effort to further characterize the activity of this meganuclease, we developed an *in silico* approach to predict potential off-target sites that were not detected in our ITR-seq datasets. Using this approach, an M2PCSK9 meganuclease recognition motif of 22 bases was established via a training data set of 2,530 unique off-target sites identified from 93 ITR-seq libraries. We searched for matches to this motif in both the rhesus macaque and human reference genomes and calculated a weighted matching score to each hit to measure its potential as M2PCSK9 meganuclease off-target site. Upon further investigation of the locations and matching scores of these predicted sites, we found that they were enriched around known genes. While most predicted off-target sites were not yet identified by ITR-seq training data, we did observe a strong correlation between M2PCSK9 meganuclease activity and the matching score to the 22-base motif at individual sites that have been identified. To further verify our findings, genome editing at a selection of top-scoring sites was directly assessed by amplicon sequencing in AAV-delivered, M2PCSK9 meganuclease-treated rhesus macaques. Together, this study demonstrates that the off-target activities of the M2PCSK9 meganuclease can be predicted

according to sequence similarity to a consensus motif and that *in silico* prediction of the off-target sites may be a novel and valuable tool for evaluating gene editing safety.

258. Validating EPI-321 Safety: A Comprehensive Off Target Characterization Platform for Epigenetic Gene Therapies

Linsin A. Smith[1], Tyler Borrman[1], Melanie R. Silvis[1], Emily Dabe[2], Kaitlin Pensabene[1], Siddaraju V. Boregowda[1], Abhinav Adhikari[1], Christopher Still[1], Tengyu Ko[1], Timothy Daley[1], Robin W. Yeo[1], Alexandra Collin de L'Hortet[1], Yanxia Liu[1]

[1]Epic Bio, South San Francisco, CA,[2]Excelra/BISC Global, Iselin, NJ

Genome and epigenome editing hold immense potential for precisely altering DNA and modulating gene expression, enabling therapeutic applications for previously untreatable diseases. However, there remain significant barriers to realizing this potential including developing highly precise genome and epigenome editing tools. One well-recognized obstacle is the risk of off-target effects associated with CRISPR-based editors, where unintended binding to non-target regions of the genome can lead to undesirable and potentially dangerous outcomes. Epic Bio is advancing the field of epigenetic editing to control gene expression and treat diseases that are beyond the reach of genome editing. Facioscapulohumeral muscular dystrophy (FSHD), which affects 800,000 globally with no available cure, is a prime example. FSHD is linked to hypomethylation of the D4Z4 microsatellite region on chromosome 4, which leads to the inappropriate expression of DUX4 and progressive muscle wasting in skeletal muscles. Leveraging our proprietary GEMS platform, Epic Bio has developed EPI-321, an epigenetic therapy treatment for FSHD that targets the D4Z4 region, permanently suppressing DUX4 expression by restoring DNA methylation to healthy levels. To validate the safety of EPI-321, we conducted genome-wide off-target characterization and found no direct off-target effects mediated by EPI-321. Unlike genome editing technologies employing double-strand breaks for off-target profiling, epigenome editing technologies demand more comprehensive characterization based on the intended epigenetic modification mechanism. Here, we present our all-encompassing platform for off-target evaluation and showcase its application in off target characterization of EPI-321. Our comprehensive platform integrates in silico off-target prediction, genome-wide expression profiling, genome-wide bisulfite sequencing, and targeted validation assays across various model systems in order to robustly identify putative off target effects. Application of the off-target characterization platform to EPI-321 revealed no direct off-target events caused by EPI-321 treatment, highlighting the safety and precision of our GEMS technology.

259. Off-Target Analysis Shows Favorable Safety Profile of Prime Editing

Maria Collier, Jacob Stewart-Ornstein , Marina Lilieholm, Matthew Irby, Hari Prasanna Subramanian, Jack M. Heath, Justin G. Tedeschi, Billy Law, Alan Wilhelm, Dewi Harjanto, Thomas Aunins, Deepak Reyon, Jennifer L. Gori, Jeremy S. Duffield

Prime Medicine, Inc., Cambridge, MA

Off-target assessment is critical for evaluating the precision and specificity of gene editing technology as it moves into the clinic. Compared to other gene editing approaches, Prime Editing may offer fundamental safety advantages at the target site and at potential off-target sites because double stranded breaks in DNA are not required and editing is gated on further homology recognition beyond the spacer sequence. Here we describe a comprehensive empirical evaluation of Prime Editing specificity that supports the theoretical arguments in favor of a superior safety profile of Prime Editing. We present a suite of assays developed specifically for Prime Editing to comprehensively assess off-target activity with high sensitivity and specificity. We will present data from two single-stranded nick based biochemical approaches developed at Prime Medicine to detect potential Prime Editor induced nicks across the genome: Library of In Silico Targets sequencing (LIST-seq) and 3-Prime End liGation sequencing (PEG-seq). In addition, we will show data from the Single End Extension sequencing (SEE-seq) assay developed at Prime Medicine for high precision detection of chromosomal alterations, such as large deletions and translocations. Such events are observed in CRISPR double-stranded break-based genome editors in typical culture conditions at 1-5%, and have not been observed with Prime Editing, therefore requiring the implementation of highly sensitive assays for assessment. In addition, we will show data from whole genome and transcriptome analyses of Prime Edited cells, which further highlight the minimal levels of genome-wide off-target activity. Finally, we will present results from the off-target analysis of the late-stage preclinical program for Chronic Granulomatous Disease (CGD) as well as preliminary analysis from other advanced preclinical programs at Prime. Collectively, our assessment supports the specificity and minimal, if any, off-target activity of Prime Editing, which has potentially important implications for patient safety.

260. Casmini-Tool: A Comprehensive Database for Efficient and Specific Guide RNA Design Using dCasMINI

Spencer Lopp[1], Tyler Borrman[1], Nishant Jha[2], Xiao Yang[1], Melanie Silvis[1], Gabriella Alvarez[1], Courtney Klappenbach[1], Ryan Swan[1], Yanxia Liu[1], Daniel O. Hart[1], Lei S. Qi[3], Timothy P. Daley[1], Robin W. Yeo[1]

[1]Epicrispr Biotechnologies, South San Francisco, CA,[2]Nitro Bio, Pasadena, CA,[3]Stanford University, Stanford, CA

The dCasMINI protein is a compact, nuclease-inactivated CRISPR-Cas system engineered for transcriptional modulation and epigenetic editing. The small size of dCasMINI (529 amino acids), less than half the size of comparable Cas9 molecules, makes it ideally suited for AAV-based therapies which are frequently limited by AAV's small cargo capacity. Unlike Cas9 or Cas12a, there are no available computational

tools for designing dCasMINI guides which presents a significant barrier to its ease-of-use by academic and industry researchers alike and limits its clinical utility as a potential tool for gene therapy. To facilitate and accelerate the development of dCasMINI-based applications, we synthesized knowledge regarding dCasMINI guide design and built a web application to assist researchers in designing optimal guides for dCasMINI-based experiments for transcriptional inhibition (CRISPRi) and activation (CRISPRa). Effective CRISPR-Cas targeting requires recognition of a protospacer adjacent motif (PAM) in the target DNA sequence; in the case of dCasMINI, previous studies have reported it recognizing a TTTR PAM. Considering that other PAMs may permit dCasMINI binding and subsequent target regulation, we developed a pooled PAM screening assay using a synthetic reporter, FACS, and NGS to evaluate all possible nucleotide arrangements of the PAM. In addition to the canonical TTTR PAM, dCasMINI was found to recognize several other relaxed PAMs, less potent than TTTR but sufficiently strong for target regulation. We incorporated canonical and relaxed PAMs into our guide design algorithm to expand the set of candidate on-target guides and increase the sensitivity of off-target prediction. Off-target guide binding can lead to undesirable and potentially dangerous therapeutic outcomes. It follows, accurate prediction of off-target regions is critical to successful guide selection. To address this, we experimentally evaluated nucleotide substitutions and deletions for each nucleotide position of the guide RNA in the context of CRISPRa. We identified a high fidelity 6 bp seed region in the guide which was intolerant to mismatches or deletions. Our off-target prediction algorithm integrates the seed region, relaxed PAMs, and gapped alignment to report a comprehensive list of high confidence off-targets. Overall, the casmini-tool supports guide design, selection, and off-target prediction with functions and parameters optimized for the dCasMINI CRISPR-Cas system. We anticipate that this web-based tool will be of value to the CRISPR-Cas research community and will allow researchers to more easily and quickly design CRISPRi/a experiments with dCasMINI. The tool is freely available at casmini-tool.com.

AAV Vector Biology and Development II

261. Multi-Step Engineered Adeno-Associated Virus Enables Whole-Brain mRNA Delivery

Weiya Bai[1], Yaqing Zhao[1], Zhen Liu[1], Dong Yang[1], Guoling Li[1], Haifeng Quan[1], Yingsi Zhou[1], Tong Li[1], Shaoran Wang[1], Xuan Yao[1], Alvin Luk[1], Linyu Shi[1], Hui Yang[1,2]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: Messenger RNAs (mRNAs) have emerged as a new category of therapeutic agents to combat a wide range of incurable diseases. However, systemic injection of mRNA-delivering lipid nanoparticles (LNPs) or virus-like particles (VLPs) has limited therapeutic applications due to liver accumulation, a significant barrier in the development of therapeutically efficacious RNA drugs and limitation to deliver drugs to challenging tissue targets such as the brain due to the presence of the blood-brain barrier (BBB). Adeno-associated virus (AAV) vectors are commonly used for DNA delivery for gene therapy applications, with several advantages over other viral vectors. The use of naturally occurring or newly engineered AAV capsids is a promising strategy for targeting non-liver tissues, such as the brain, skeletal muscle, and heart. Here, we developed a system that enables the AAV shell to package mRNAs by introducing RNA-packaging components into the AAV production system (triple-plasmid system). **Methods:** We replaced the DNA packaging signal of AAV (inverted terminal repeat, ITR) with RNA packaging signals (RPSs) and enabled the binding of Rep78/68 proteins to RPS-harboring mRNAs to convert the AAV into an RNA-packaging virus. To improve the production efficiency and packaging specificity of the system, we conducted multi-step engineering of AAV Rep proteins. A comprehensive assessment of the properties of the resultant mRNA-carrying AAVs (RAAV) was further conducted. Furthermore, RAAVs were applied in gene editing. **Results:** Through multi-step engineering, we achieved a ~6000-fold increase in mRNA loading and a ~14,100-fold reduction in DNA loading with RAAV, compared to the conventional AAV (Fig. 1a). The RAAVs retained most properties of conventional AAVs, including capsid composition, virus morphology, and tissue tropism (Fig. 1b, c and Fig. 2). These RAAVs mediated mRNA transfer into the target cells and tissues, leading to transient expression of the functional protein (Fig. 1d, e). In addition, RAAVs enabled efficient CRISPR gene editing in cells with reduced off-target effects (Fig. 1f). Importantly, intravenously injected RAAV with capsid PHP.eB efficiently crossed the BBB and infected the whole mouse brain (Fig. 2). **Conclusions:** We have developed an efficient, engineered RAAV vector from the DNA virus AAV that specifically and safely delivers mRNAs for therapeutically relevant applications. The RAAVs combined the transient nature of mRNA with a variety of tissue tropism of the AAV capsid, making them ideal for either broad-spectrum or tissue-specific mRNA delivery. Our results demonstrate the feasibility of using rational engineering to change the virus genome type. Our strategy in developing RAAVs could be extended to other DNA viruses to endow them with mRNA packaging capability.

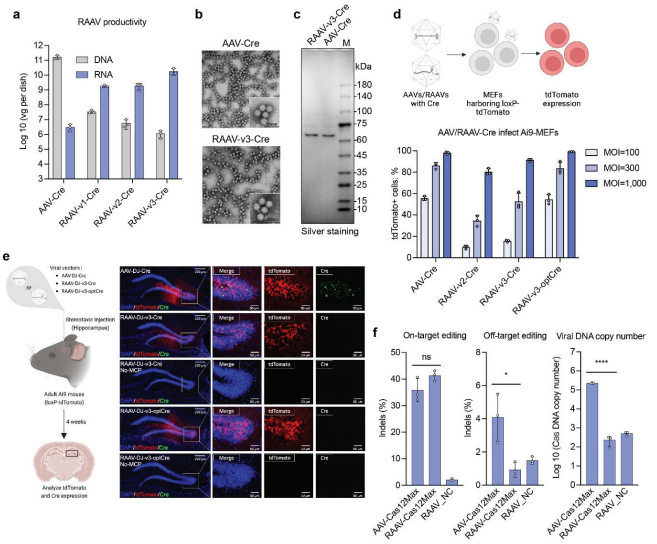


Figure 1. Developing RAAV as an efficient mRNA delivery tool. a. Quantification of the Cre RNA and Cre DNA encapsulated in RAAVs by RT-qPCR and qPCR. b. Morphology analysis of RAAV and AAV by transmission electron microscopy. Scale bars, 50 nm. c. Analyzing the protein composition of AAV and RAAV by silver staining. d. Investigating the infectivity of RAAVs by analyzing the percentage of tdTomato+ Ai9-MEFs 5 days after infection by cytometry. e. RAAVs enable efficient transfer of mRNA to the mouse hippocampus to transiently express functional proteins. f. RAAVs enable efficient CRISPR gene editing in cells with reduced off-target effects.

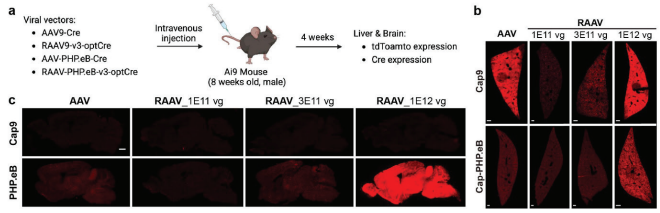


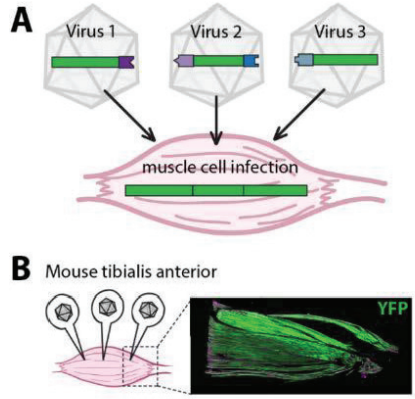
Figure 2. RAAVs enable tropism-dependent mRNA delivery to target tissues/organs to transiently express functional proteins. a. Schematic of in vivo systemic RAAV infection assay. b and c. Transduction of RAAVs/AAVs in the liver (b) and brain (c) of Ai9 mice 4 weeks after intravenous injection. N=2 mice. Scale bars, 1000 μm.

262. Optimization of RNA End Joining (REJ) to Safely and Efficiently Express Large Proteins

Lukas C. Bachmann[1], Kip J. Hermann[2], Ryan Hsu[3], Claire Williams[4], Samuel L. Pfaff[5]

[1]Gene Expression Laboratory (GEL-P), The Salk Institute, Vertuis Bio, and currently Insmed, La Jolla, CA,[2]The Salk Institute, La Jolla, CA,[3]Salk Institute & UC San Diego, La Jolla, CA,[4]Salk Institute for Biological Studies, La Jolla, CA,[5]Gene Expression Laboratory (GEL-P), The Salk Institute, La Jolla, CA

Background: Adeno-associated viruses (AAVs) have many favorable characteristics as a delivery vector for gene therapy, but have a limited cargo capacity of ~4.7 kb. Because many genetic diseases such as Stargardt, Usher 1F (Ush1F), and Duchenne Muscular Dystrophy (DMD) are caused by loss-of-function mutations in large genes, it has proven difficult to devise effective gene replacement strategies to treat these patients. Consequently a range of approaches are being undertaken including isolating functional mini-genes, re-engineering AAV capsids, and molecular strategies with DNA, RNA, and proteins that join smaller pieces together to produce larger proteins. Here we report an RNA based solution for safely and efficiently expressing large proteins by manipulating RNA trans-splicing. *Results:* Our studies to overcome the size constraints of AAV have focused on co-opting the cell's intrinsic RNA splicing machinery to join RNA fragments encoded by separate AAVs that coinfect target cells. This is achieved by breaking genes into fragment sizes compatible with efficient AAV packaging (<4.7kb) and appending the ends of each RNA segment with short ~250 nucleotide cis-element sequences termed RNA End Joining (REJ) modules that promote RNA trans-splicing (see Figure). REJ elements use the cell's intrinsic splice machinery to join separate RNAs, thereby generating long mRNAs capable of encoding proteins >300kd. The design features of this platform gene expression system include: (1) REJ modules are short; (2) REJ modules contain strong, but selective, RNA::RNA interaction elements to "trick" the spliceosome into splicing non-covalent RNAs; (3) the REJ system can be inserted at nearly any position, thereby allowing genes to be segmented without screening to account for protein domain structure; (4) REJ generates mRNAs that encode the exact endogenous protein without introducing a residual "scar"; (5) REJ functions without the need for foreign proteins, and (6) REJ modules are engineered to suppress translation of un-joined RNA fragments thereby preventing expression of truncated proteins that could oppose full length protein function by acting as dominant negatives. We find that the REJ system has a nearly undetectable level of off-target splicing using RNA sequencing, yields approximately 50% of the protein level compared to single linear RNAs, is a platform system that has reliably worked in >20 different genes tested in mouse and human cells, and has functioned well *in vivo* in every mouse tissue examined. *Conclusions:* The REJ system has shown excellent promise as a tool for treating genetic diseases with gene replacement (see Hsu et al. and Williams et al. abstracts) and *in vivo* gene editing with CRISPR. In addition the system is well suited for gene complementation, including applications involving intersectional genetics for precisely targeting specific cell populations. *Figure Legend:* REJ proof-of-concept. (A) The yellow fluorescent protein (YFP) gene was divided into 3 segments (green) and packaged into separate AAVs. Each RNA segment was appended with REJ domains (purple/blue). Viruses were mixed at equal concentration and injected into the tibialis anterior muscle of the mouse leg (B) Two weeks after virus injection YFP was detected.



263. Encapsidation of, and Transgene Expression from, Generation Z (Genz) Single-Stranded AAV Vectors is AAV Serotype Capsid-Specific

Keyun Qing[1], Jakob Shoti[1], Aneesha Nath[1], Geoffrey D. Keeler[1], Arun Srivastava[2]

[1]University of Florida, Gainesville, FL,[2]University of Florida College of Medicine, Gainesville, FL

We have previously reported the development of a generation Z (GenZ) ssAAV DNA genome in which the D-sequence in the AAV inverted terminal repeat (ITR) was replaced with a sequence obtained from a random nucleotide library (*Mol. Ther.*, 31: 234-235, 2023). The GenZ ssAAV genome overcomes the rate-limiting step of the viral second-strand DNA synthesis, and mediates significantly higher levels of transgene expression. Thus, it behaves more like a scAAV genome, but without the size-limitation. However, we observed that the extent of transgene expression from different GenZ AAV serotype vectors varied widely. For example, as shown in **Figures 1A** and **Figure 1B**, whereas the transduction efficiency of GenZ ssAAVrh74 and GenZ ssAAV3 vectors was ~20-fold and ~22-fold, respectively, compared with that of their wild-type (WT) counterparts, the transduction efficiency of GenZ ssAAV2 and GenZ ssAAV6 vectors was only ~3-fold and ~4-fold, respectively, compared with that of their WT counterparts (**Figures 1C** and **Figure 1D**). These observations prompted us to examine whether different AAV capsids modulate transgene expression. Previous studies by others have documented that AAV2 (*J Virol.*, 88:10711079, 2014; *J Virol* 2016;90:7196-7204) and AAV9 (*Mol. Ther.*, 28:1373-1380, 2020; *Hum. Gene Ther.*, 31:1155-1168, 2020) capsids indeed play a role in

transcription and second-strand DNA synthesis. To test our hypothesis that AAV2 and AAV6, but not AAVrh74 and AAV3 capsids, negatively impact the transgene expression from GenZ ssAAV vectors, the following strategy was used. GenZ ssAAVrh74 vectors were used to transduce HeLa cells in triplicates at $3x10^3$ vgs/cell, incubated with and without highly purified empty capsids of AAVrh74, AAV2, AAV3, and AAV6, either before or during transduction. Transgene expression was visualized under a fluorescence microscope 72 hrs post-transduction. These results are shown in **Figure 1E**. As is evident, whereas AAVrh74 and AAV3 empty capsids had no effect, both AAV2 and AAV6 empty capsids led to a significant decrease in transgene expression from GenZ ssAAVrh74 vectors. These data not only provide a mechanistic insight into the observed difference in the extent of transgene expression mediated my different GenZ ssAAV serotype vectors, but also further corroborate the studies cited above. However, given the absolute requirement of the D-sequence for the optimal rescue, replication, and packaging of the AAV genome (*Mol. Ther.*, 9: 2628-2630, 2021), we also observed that the efficiency of rescue and replication of the GenZ AAV genome from recombinant plasmids was significantly reduced, and depending on the AAV serotype capsid, the efficiency of GenZ AAV genome packaging ranged between ~10% to ~60% that of the AAV genome containing the D-sequence. Despite this limitation, which warrants further studies for its mitigation, the availability of the GenZ AAV genome has important implications for its potential use in achieving high-efficiency transgene expression from ssAAV vectors.

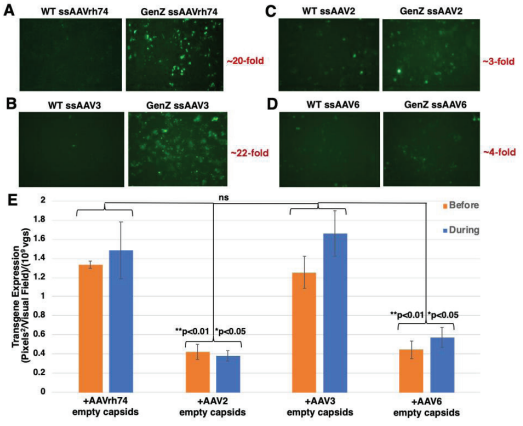


Figure 1: Transduction efficiency of WT and GenZ ssAAVrh74 **(A)**, AAV3 **(B)**, AAV2 **(C)**, and AAV6 **(D)** vectors in human HeLa cells *in vitro*. Cells were transduced with each vector at 1,000 vgs/cell and transgene expression was visualized under a fluorescence microscope 72 hrs post-transduction. **(E)** HeLa cells were transduced with WT and GenZ ssAAVrh74 vectors at 3,000 vgs/cell, with and without pre-incubation with AAVrh74, AAV2, AAV3, and AAV6 empty capsids, respectively. Transgene expression was visualized and quantitated by ImageJ analysis. ns=not significant.

264. Development of ITR-Modified AAVrh74 Vectors with Improved Transgene Expression in Primary Human Skeletal Muscle Cells *In Vitro* and in Murine Skeletal Muscles *In Vivo*

Jakob Shoti[1], Geoffrey D. Keeler[2], Keyun Qing[1], Dongsheng Duan[3], Arun Srivastava[4]

[1]University of Florida, Gainesville, FL,[2]University of Florida, Gainesville, FL,[3]University of Missouri School of Medicine, Columbia, MO,[4]University of Florida College of Medicine, Gainesville, FL

We have previously reported that the distal 10-nucleotides in the D-sequence in the the wild-type (WT) AAV2 inverted terminal repeat (ITR) share partial homology with the consensus ½ site of the glucocorticoid receptor-binding element (GRE), and that replacement of the distal 10 nts with a full GRE site leads to generation Y (GenY) ssAAVrh74 vectors that mediate up to 6-fold higher transgene expression in primary human skeletal muscle cells compared with those containing the WT D-sequence (*Mol. Ther.*, 30: 237-238, 2022). In the current study, we designed a modified AAV ITR, designated as DeltA ITR, in which the A-domain was made complementary to the GRE sequence, and the D-sequence was replaced with the GRE sequence (**Figure 1A**). This allowed the terminal resolution site (*trs*) to be conserved (**Figure 1B**), for AAV Rep-mediated resolution, rescue, replication of the AAV genomes. WT ITR and DeltA ITR genomes containing a muscle cell-specific promoter (CDK8)-driven hrGFP expression cassettes were packaged into AAVrh74 capsids. The extent of the transgene expression from the DeltA AAVrh74 vectors was ~34-fold higher than that from the WT AAVrh74 vectors in differentiated primary human skeletal muscle cells *in vitro* (**Figure 1C**). The transduction efficiencies of the WT and the DeltA AAVrh74 vectors were also evaluated in a mouse model *in vivo.* $1x10^9$ vgs of WT or DeltA-CK8-hrGFP AAVrh74 vectors were administered into gastrocnemius (GA) muscles in C57/BL6 mice (n=6 each). Cyro-sectioning and imaging of muscle tissues were performed 2 weeks post-injections. These results, shown in **Figure 1D**, document that DeltA-ITR AAVrh74 vectors averaged ~24-fold increased transgene expression than that from the WT-ITR AAVrh74 vectors. These data suggest that this novel ITR-modification subverts the need for the AAV D-sequence and is a useful strategy to achieve improved transgene expression from ssAAV vectors. However, since we have previously documented the essential role of the D-sequence in AAV genome encapsidation (*Mol. Ther.*, 9: 2628-2630, 2021), the deletion of this sequence significantly reduced the titers of the DeltA AAVrh74 vectors. Studies are warranted to further optimize the DeltA-ITR design to improve the vector titers. Additional studies are also underway to encapsidate the DeltA-ITR genomes into capsid-modified NextGen AAVrh74 vectors (*Mol. Ther. Meth. Clin. Dev.*, 31: 101147, 2023) leading to the generation of optimized DeltA-ITR vectors (OptΔ) AAVrh74 vectors for high-efficiency transduction as well as augmented transgene expression following systemic delivery, which has implications in the optimal use of these vectors in the potential gene therapy of muscular dystrophies in humans.

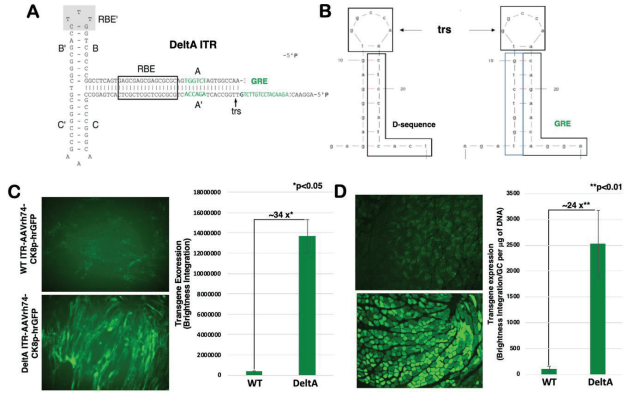


Figure 1: (A) Schematic structures of the DeltA AAV-ITR. **(B)** Secondary structures of the *trs* sites in the WT and the DeltA ITRs. **(C)** Transduction efficiency of WT and DeltA AAVrh74 vectors in primary human skeletal muscle cells *in vitro*. **(D)** Transduction efficiency of WT and DeltA AAVrh74 vectors in mouse gastrcnemius skeletal muscle cells *in vivo* following intramuscular injections. Data were quantitated using the Keyence software.

265. Transcriptional Crosstalk Between AAV Genomes Depends on Concatemer Formation and Enables Cell Type-Targeted Delivery of Oversized Cargo

Gerard M. Coughlin, Máté Borsos, Nathan Appling, Bre'Anna H. Barcelona, Acacia M. H. Mayfield, Elisha D. Mackey, Rana A. Eser, Xinhong Chen, Sripriya Ravindra Kumar, Viviana Gradinaru

California Institute of Technology, Pasadena, CA

Engineering of adeno-associated virus (AAV) capsids has enabled broad and efficient access to target organs following systemic administration. Inclusion of regulatory elements, such as enhancers mined from the host genome, can further refine the transduction profile of AAVs to target specific cell types. However, the limited packaging capacity of AAVs precludes use of enhancer-driven expression for delivery of certain large cargoes, including machinery for CRISPR-based gene editing and manipulation. Here we demonstrate that cell type-specific enhancer elements on one AAV genome can increase expression from minimal promoters present on another AAV genome. We term this behaviour "transcriptional crosstalk" and profile it across 11 different enhancers with cell type-specific activity in the mouse brain. To better understand the mechanisms of transcriptional crosstalk, we develop novel spatial genomics methods, AAV-Zombie and SpECTr, that enable high-resolution subcellular localization of AAV genomes and concatemers in cultured cells and in tissue. With these tools, we construct detailed views of the spatiotemporal dynamics of AAV genomes, concatemers, and cargo expression in primary neurons, and provide evidence that transcriptional crosstalk is dependent on AAV genome concatemerization. Lastly, we leverage transcriptional crosstalk for cell type-targeted delivery of a large Cas9 cargo following systemic administration of a BBB-penetrant engineered AAV. Using an established reporter assay, we demonstrate that this approach enables all-AAV cell type-specific gene editing. To demonstrate the efficiency of this approach, we disrupted *Cacna1a* in wildtype animals with Purkinje-cell specific Cas9 expression, recapitulating known phenotypes of genetic knockouts. Importantly, this paradigm does not require transgenic animals and thus can be used for cell type-specific gene disruption and manipulation in a range of models, including species that lack Cas9-expressing transgenic strains. This work synergizes with recent advances in AAV capsid engineering that have enabled genetic access to nervous systems of non-human primates, opening new directions for understanding cell type-specific gene function in a myriad of model organisms.

266. Newly Identified AAV Binders: Blood-Brain-Barrier Transcytosis Mediator LRP6 for Engineered AAVs and Immune Cytokine IL3 for AAV9-Host Interaction

Seongmin Jang[1], Timothy Shay[2], Tyler Brittain[1], Xinhong Chen[1], Beth Walker[3], Claire Tebbutt[3], Damien Wolfe[1], Cynthia Arokiaraj[1], Erin E. Sullivan[1], Xiaozhe Ding[1], Yaping Lei[1], Miguel Roberto Estella Chuapoco[1], Viviana Gradinaru[1]

[1]Division of Biology & Biological Engineering, Caltech, Pasadena, CA,[2]CLOVER Center, Caltech, Pasadena, CA,[3]Charles River Laboratories, Chinley, United Kingdom

Due to their native blood-brain barrier transcytosis ability, adeno-associated virus serotype 9 (AAV9) and its engineered derivatives have been used as potential gene therapy vectors for both basic science and clinical applications. However, AAV infection mechanisms remain poorly understood, thereby limiting further development and application. This is especially so for the many promising capsids engineered by mechanism-agnostic directed evolution. To address this problem, we screened wild type and engineered AAV capsids against the human extracellular proteome and secretome displayed as a cell microarray. Here, we report multiple newly-identified AAV interactions including two key findings: (1) low-density-lipoprotein-receptor-related-protein 6 (LRP6) as a lab-evolved receptor mediating blood-brain barrier transcytosis of AAV9-X1.1, AAV.CAP-Mac, and AAV-BI30, and (2) interleukin 3 (IL3) as a potential host immune reactor toward the natural serotype AAV9. Our screening effort also found other AAV binders outside of central nervous system that can help in understanding an AAV's off-target infection and side effects. This study provides a deeper understanding of AAV tropisms and the host's immune response to AAV, ultimately helping the engineering of highly brain-enhanced and safe AAVs for practical applications.

267. Creation of an AAV-Based Replication Origin for Novel AAV Vectors That Cannot Be Replicated in Nature

Leah R. Whitfield[1], Shani Inbari[1], R. Bryan Sutton[2], R. Jude Samulski[3], Matthew L. Hirsch[1]

[1]University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Department of Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX,[3]UNC Gene Therapy Vector Core, Chapel Hill, NC

Recombinant AAV (rAAV) mobilization is the phenomenon in which rAAV spreads from the recipient's cells to additional cells within the patient and potentially the environment. This is facilitated by the wild-type (wt) AAV genome, either naturally present or provided as a contaminant in the vector preparation, and a helper virus which

allows rAAV replication in *trans* to create *de novo* rAAV. As wtAAV serotype 2 ITRs are currently used in all clinical AAV gene therapies and are replication targets for AAV Rep proteins of many natural serotypes found across many species, the objective of this work is to eliminate the potential for rAAV production in patients and therefore, excessive vector shedding and environmental spread. A previous report of AAV serotypes 2 and 5 Rep and ITR sequences identified the region between the Rep binding element and the base of the nicking stem and the length of the nicking stem as determinants that restrict ITR2 replication by Rep5 and vice versa. Additionally, analyses of the protein sequences reveal that Rep5 is truncated in the region implicated in ITR binding and nicking, with aa 135-160 reported as essential for ITR2/5 replication specificity. Given these insights, it was hypothesized that the addition of a single helical turn within Rep's nicking region would eliminate replication of wtITRs and confer replication of a unique ITR sequence selected from a mutant ITR library. First, Rep Y152 and Y156 were individually mutated to alanine to investigate their roles in replication. In contrast to previous reports which implicate Y156 as the endonucleolytic tyrosine, mutation of either residue eliminated AAV vector production. Next, mutations of Rep2 implicated in increased production titers were investigated. A S535A mutation showed no significant titer change compared to Rep2 while substitution of the Rep start codon to ACG resulted in a two-fold increase in production titers. Given these insights, mutated Rep proteins were rationally designed in the ACG context by extension of the nicking region through the addition of natural AAV alpha-helical turns. Using AlphaFold, repetition of the second turn of the endonucleolytic helix of multiple Rep serotypes failed to maintain the helix while inserting a corresponding endonucleolytic turn of Muscovy Duck AAV (mdRep) did and was used for further analyses. As desired, yet somewhat surprising, mdRep failed to produce wtITR2 containing vectors, despite the maintenance of both Y152 and Y156. Production runs using mdRep and capsid 2 on a mutant ITR library demonstrated rAAV production at relatively low titers. Currently, these mutant ITR genomes are being amplified through production cycling followed by nanopore sequencing. In addition to offering mechanistic determinants of ITR and Rep protein restriction, this work has the potential to create a capsid packaged mutant ITR flanked genome that can only be replicated in a laboratory environment thereby decreasing concerns associated with rAAV mobilization.

Neurologic Diseases III

268. AAV-Based Prion Protein-Lowering Gene Therapy for CJD

Jun Zhang[1], Manuel Camacho[1], Fengfeng Bei[2], Qingzhong Kong[1]

[1]Pathology, Case Western Reserve University, Cleveland, OH,[2]Neurosurgery, BWH, Boston, MA

Creutzfeldt-Jakob disease (CJD) is the most common human prion disease that is transmissible and always fatal, progresses rapidly and has no treatments. An effective and lasting treatment for CJD is urgently needed. The cellular prion protein (PrP) is essential for both prion replication and prion pathogenesis yet expendable for life, making it an ideal target for prion therapeutics development. Knocking down the PrP gene expression through RNA interference, such as with antisense oligonucleotides (ASOs), has been shown to be safe and effective against rodent prions in mouse models. But there has been no published report on PrP-lowering RNA interference or gene therapy for human prions. We designed a few shRNAs for the human PrP gene that showed ~90% knockdown of human PrP protein level in the M17 human neuroblastoma cell line. We created rAAVs carrying one of the shRNAs with the AAV.CAP-B10 (or B10) capsid. The rAAV.B10-shRNA was given as a systemic dose (1.6×10^{12} vg/mouse) via the retro-orbital route to a CJD-inoculated humanized CJD mouse model (Tg40h) at near clinical onset that led to a ~26% reduction of brain PrP level and a 9.6% extension of survival ($p=0.0003$), which is very significant given that the Tg40h mice overexpress human PrP at 3-4 fold of wild type level. We found that the same rAAV-shRNA packaged with the PHP.eB (or eB) capsid doubled the brain PrP knockdown (to 52.7%) at a much lower systemic dose (2.4×10^{11} vg/mouse) in the Tg40h mice, suggesting that a much more extensive survival extension of CJD-inoculated Tg40h mice or Tg40 mice (the hemizygous counterpart of Tg40h) should be achievable with a moderate systemic dose of rAAV.eB-shRNA. The recently reported AAV.CPP16 capsid (CPP16) that can effectively cross the BBB and transduce the brain tissues of both mice and macaques offers a good chance of translation for treatment of human CJD patients. Experiments treating CJD-inoculated Tg40 or Tg40h mice with a systemic dose of rAAV-shRNA packaged with the eB or CPP16 capsid are ongoing. Our data suggest that a single systemic dose of an optimized rAAV-shRNA targeting human PrP is a highly promising and realistic approach for effective CJD treatment when given at or near clinical onset. Acknowledgements: This study is supported by CJD Foundation. We are very grateful to Drs. Timothy Shay and Viviana Gradinaru at Caltech for sharing the plasmids for two AAV capsids.

269. Effective *In Vivo* Gene Silencing of HTR2A Reduces Anxiety and Enhances Memory Performance

Troy Townsend Rohn, Dean Radin, Tracy Brandmeyer, Peter Seidler, Barry Linder, Tom Lytle, John Mee, David Pyrce, Fabio Macciardi

Cognigenics, Boise, ID

Short-hairpin RNA (shRNA) molecules, tailored for the targeted silencing of specific genes, offer substantial potential for the development of precise therapeutic interventions for a variety of neurodegenerative diseases. Yet, the capability of shRNA constructs to alter neuronal pathways that control certain behaviors remains to be investigated. We engineered shRNAs to silence the *HTR2A* gene *in vitro* within neurons derived from induced human pluripotent stem cells (iPSCs). Subsequent analysis with Multi-electrode arrays (MEA) indicated that suppression of the 5HT-2A receptor was associated with reduced spontaneous neural firing. In a mouse model, the intranasal administration of adeno-associated virus serotype 9 (AAV9) vectors encapsulating shRNA led to diminished anxiety-like behavior in mice, as well as a marked enhancement of memory performance, as evidenced by a 104% improvement in mice and a 92% improvement in rats over control groups. This research not only showcases a groundbreaking non-invasive shRNA delivery method that successfully circumvents the

blood-brain barrier but also has potential implications for the treatment of a multitude of neurological and psychiatric conditions. Particularly, the inhibition of the HTR2A gene emerges as a promising new strategy for managing chronic anxiety and combatting cognitive deterioration associated with mild-cognitive impairment patients.

270. Development of an Adeno-Associated Virus Gene Therapy for the Treatment of CDKL5 Deficiency Disorder

Janine M. Lamonica, Eileen Workman, Rachel S. White, Mariya Kostiv, Ralf S. Schmid, Juliette Hordeaux, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

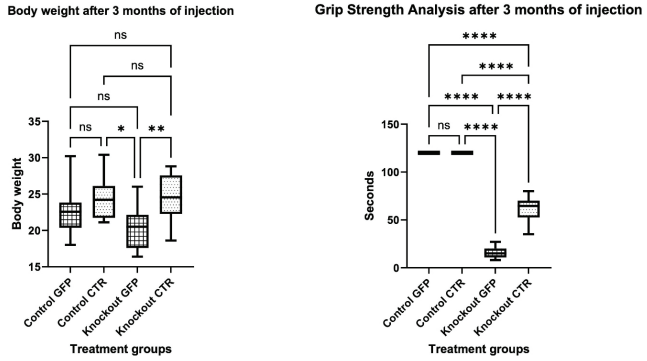
Loss-of-function mutations in the X-linked cyclin-dependent kinase-like 5 (CDKL5) gene cause a severe neurodevelopmental condition known as CDKL5 deficiency disorder (CDD). CDD is characterized by early-onset epilepsy, intellectual disability, autistic features, and impaired motor function. Presently, there is no cure for CDD, and treatment options are limited to reducing seizure burden. Research has shown that restoring CDKL5 protein expression in Cdkl5-deficient mice leads to a reversal of behavioral phenotypes, pointing to *CDKL5* gene replacement as a possible therapeutic strategy for CDD. To this end, we are developing an adeno-associated virus gene therapy vector, a clade F AAVhu68 vector encoding a human codon-optimized *CDKL5*. We performed pharmacology and minimum effective dose studies following delivery of our vector via intracerebroventricular injection into neonatal *Cdkl5* knockout (KO) mice at four escalating doses, ranging from 7.5×10^9 to 9.75×10^{10} genome copies (GC). At 11 and 15 weeks of age, the mice underwent a series of behavioral tests. Compared with vehicle-treated KO mice, vector-treated KO mice exhibited improvements in locomotor activity, hindlimb clasping severity scores, and nest-building ability in a dose-dependent manner. We are currently performing Western blotting and immunohistochemical analyses to detect hCDKL5 protein levels and activity in the brain. We are also conducting a toxicological evaluation of our vector in rhesus macaques following intra-cisterna magna (ICM) administration. We administered our vector at low (4.5×10^{12} GC), intermediate (1.5×10^{13} GC), and high (4.5×10^{13} GC) dose levels. Three nonhuman primates (NHPs) per dose, plus one vehicle-treated control, will be euthanized at Day 90 post-injection, and three additional high-dose NHPs plus one vehicle-treated control will be euthanized at Day 180. This study will provide critical data regarding the safety, tolerability, and expression of our vector following ICM administration in NHPs. Ultimately, the goal of these studies is to provide a safe and efficacious treatment option for patients with CDD.

271. Validation of an AAV Gene Therapy Designed to Complement the Loss of TDP-43 Splicing Repression for ALS-FTD

Aswathy Peethambaran Mallika[1], Philip C. Wong[2]

[1]Pathology, Johns Hopkins University School of Medicine, Baltimore, MD,[2]Pathology & Neuroscience, Johns Hopkins University School of Medicine, Baltimore, MD

Background: Emerging evidence support the notion that loss of splicing repression by TDP-43, an RNA binding protein that was first implicated in ALS-FTD, underlies their pathogenesis. Previously, we showed that delivery of an AAV9 vector at early postnatal day expressing a fusion protein (AAV9.RAV1-3'UTR termed CTR) comprised of the N-terminal region of TDP-43 and an unrelated splicing repressor termed RAVER1 complemented the loss of TDP-43 in mice lacking TDP-43 in spinal motor neurons (*ChAT-IRES-Cre;tardbp$^{F/F}$* mice). To translate this potential therapeutic strategy to the clinic, it will be important to demonstrate benefit of such AAV delivery of CTR to motor neurons in adult mice. Here we validate a therapeutic approach using a recently developed AAV.PHP.eB vector that enables efficient transduction in central neurons, including motor neurons by an intra-venous (IV) administrative route. **Method:** Intra-venous injection of AAV.PHP.eB.RAV1-3'UTR [(*ChAT-IRES-Cre;tardbp$^{F/F}$* (n=12) and *ChAT-IRES-Cre;Tardbp$^{F/+}$* (n=8)] and AAV.PHP.eB.GFP [(*ChAT-IRES-Cre;tardbp$^{F/F}$* (n=10) and *ChAT-IRES-Cre;Tardbp$^{F/+}$* (n=8)] was carried out at 6 weeks old littermate animals. Histology and immunohistochemistry to show the infectivity, RT-PCR and RNA in situ hybridization to show the rescue of splicing repression and finally the grip strength analysis were performed. **Result:** We show that IV delivery of AAV.PHP.eB.RAV1-3'UTR to adult *ChAT-IRES-Cre;tardbp$^{F/F}$* mice efficiently transduced spinal motor neurons, repressed cryptic exon inclusion and attenuated muscle weakness. **Conclusion:** These data support validation of our AAV gene therapeutic strategy to attenuate motor neuron disease, including ALS and potentially extended to other human disorders harboring TDP-43 pathology. Other relevant results, including rescue of motor neurons and extension of life span will be presented.



272. ATAD1 Gene Rescue of Zellweger Spectrum Disorders via Targeting of Shared Common Mechanism

Tamara Stevenson[1], Esther Nuebel[2], Nettie Pyne[3], Allison M. Bradbury[3], Joshua L. Bonkowsky[1]

[1]University of Utah, Salt Lake City, UT,[2]Noorda College of Osteopathic Medicine, Saratoga Springs, UT,[3]Nationwide Children's Hospital, Columbus, OH

Peroxisomal biogenesis disorders (PBDs), of which Zellweger Spectrum Disorders (ZSDs) are the most severe, are caused by mutations in the peroxin proteins, coded by PEX genes. The birth incidence of ZSDs is ~1/50,000. ZSDs present with severe clinical features, including seizures, leukodystrophy, hepatomegaly, mitochondrial defects,

retinopathy, and ultimately lead to death in infancy or early childhood. There are no curative treatments for ZSDs, and only a few drug trials are being pursued. Gene replacement therapy is an appealing option, as the ZSDs are largely autosomal recessive monogenic conditions. However, a major limitation in the development of gene therapies for ZSDs is the size of the PEX genes, which exceed the AAV payload. Further, with 14 known PEX genes, each form of ZSD would require a lengthy and expensive gene therapy development process. We have developed a novel approach for treatment of ZSDs, through targeting of a shared mechanism common to ZSDs. Using a PEX3$^{-/-}$ patient-derived fibroblast line, we showed that mitochondrial mislocalization of peroxins (PEX) contributes to the phenotype of ZSDs. Further, we showed that we could rescue PEX localization, restore mitochondrial function, and restore peroxisome function by expression of ATAD1, a mitochondrial chaperone AAA+ ATPase (Nuebel et al., 2022). Use of ATAD1 has two important, appealing features for gene therapy for ZSDs. First, its small size (~1kb) allows it to package efficiently in AAVs. Second, ATAD1 could potentially rescue multiple different ZSDs. To establish generalizability across the ZSDs, we tested ATAD1 exogenous expression in a PEX1-/- patient-derived fibroblast line. We found that ATAD1 expression corrected mitochondrial deficits of ZSDs and also found that peroxisomal dysfunction was corrected. We observed normalization of very-long chain fatty acid levels (a marker of peroxisome metabolic function); and improvement in plasmalogen synthesis (a marker of peroxisome anabolic function). We are testing ATAD1 AAV9-mediated gene therapy in a PEX1 ZSD mouse model (G844D mutation). We are using PEX1 as a control to compare outcomes with ATAD1 gene supplementation. Genes (GFP, ATAD1, or PEX1) were subcloned into a single-stranded AAV plasmid. We used 2 different promoters: an EFS (shortened EF1-α) promoter; or the ubiquitous promoter chicken β-actin with a CMV enhancer (CAG). Due to the large size of the PEX1 transgene we could only use the small EFS promoter for the PEX1 construct. In contrast, because of the smaller size of the ATAD1 transgene (1 kb), we have generated 2 comparison promoters, EFS and CAG. All constructs were packaged in AAV9 capsids. P1 wildtype mice were injected by intracerebroventricular (ICV) or intravenous (IV) delivery. Mice were sacrificed at 1-month post-injection to evaluate expression and biodistribution via immunofluorescence staining. The AAV9-CAG-GFP construct resulted in high expression levels throughout the neuroaxis, with on average approximately 75% of the brain positive for GFP. Additionally, the intensity of expression was robust. When LUTs were normalized, expression and intensity of GFP driven by EFS was significantly lower. Our results suggest that ATAD1 gene therapy has potential to rescue mitochondrial and peroxisomal dysfunction and their downstream phenotypic effects in PEX1, PEX3, and potentially other ZSDs.

273. AAV-Based Anti-RAN Antibody Therapy for C9orf72 ALS FTD

Lisa E. L. Romano[1], Camille Preston[1], Tala Ortiz[1], Ramadan Ajeridini[1], Hannah Golliher[1], Ellie Carrell[2], Megan Keiser[2], Elaine Ames[1], Beverly L. Davidson[2,3], Laura P. W. Ranum[1,4]

[1]Department of Molecular Genetics and Microbiology, University of Florida, Center for NeuroGenetics and the Genetics Institute, Gainesville, FL,[2]The Children's Hospital of Philadelphia, Center for Cellular and Molecular Therapeutics, Colket Translational Research Building, Philadelphia, PA,[3]Perelman School of Medicine, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA,[4]McKnight Brain Institute and the Fixel Institute for Neurological Diseases, College of Medicine, University of Florida, Gainesville, FL

Introduction: Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that causes paralysis and death ~3-5 years after diagnosis. The most frequent, known, genetic cause of familial and sALS is the *C9orf72* (C9) G_4C_2 repeat expansion. Strong evidence indicates toxic proteins expressed by repeat associated non-AUG (RAN) translation contribute to disease. Our previous studies show that targeting GA RAN proteins with a human antibody, α-GA1, improved disease in a BAC transgenic mouse model of *C9orf72* ALS/FTD (C9-BAC). Surprisingly, the α-GA1 antibody not only reduced GA aggregates, but also lowered GP and GR RAN proteins through improved proteasome and autophagy function. While passive immunotherapy is promising, high manufacturing costs, the need for frequent dosing, and limited blood-brain-barrier (BBB) penetration (~0.1%) highlight the need to develop improved immunotherapy strategies. **Methods:** Here, we report a second-generation immunotherapy strategy that targets GA-RAN proteins using an AAV-based approach. We engineered IgG and single-chain antibody variants with high affinity to GA-RAN proteins for AAV delivery. Cell overexpression, mouse, and organoid models were used to test target engagement and therapeutic efficacy of these AAV-IgG-GA variants. C9-BAC and non-transgenic (NT) littermates were injected intracerebroventricularly with AAV-IgG1-GA or AAV-IgG-GFP (isotype-control). Hanging-wire and Open-Field behavioral analyses were performed on 16 and 23-week-old animals. Immunohistochemistry (IHC) and IF were used to evaluate the effects of AAV-IgG1-GA in the brain and spinal cord. **Results:** Our data show these novel a-GA antibody variants (IgG1, IgG2, or single-chain scFv) reduce GA-RAN proteins ($n \geq 3$, $p<0.0001$) and their toxicity ($n \geq 3$, $p<0.0001$) in cells. A pilot study identified AAV-IgG1-GA as the most effective AAV-antibody for reducing GA-RAN proteins in C9-BAC mice ($n>5$/group, $p=0.0499$). In a larger study (n=30 mice/group), we found that animals injected with AAV-IgG1-GA improved performance in open-field (e.g., ambulatory distance, $n \geq 5$/group, $p<0.0001$), hanging-wire ($n \geq 5$/group, $p=0.0301$) and importantly increase survival ($n \geq 5$/group, $p=0.0044$). Histopathological studies show that the AAV-delivered IgG1-GA antibody is broadly distributed throughout the hippocampus and motor cortex and that GA aggregates in the brains of IgG1-GA-treated mice are reduced. **Conclusion and Discussion:** These data show that AAV-anti-RAN immunotherapy reduces RAN protein load, improves behavior, and increases survival of C9-BAC mice. We are continuing to analyze the histopathological and molecular phenotypes of the mice to fully understand the molecular effects of AAV-anti-RAN immunotherapy. AAV delivery of high-

affinity anti-GA antibodies provides a novel strategy to achieve broad and sustained CNS expression and biodistribution of therapeutic antibodies. These data open new possibilities for developing AAV-antibody therapies as a novel approach for C9orf72 ALS/FTD and for a broader group of disorders characterized by RAN protein pathology.

274. Systemic AAV Gene Therapy with CNS-Targeted Engineered Capsids Achieves Significant GCase Activity Increases in the Primate Brain to Support the Potential Treatment of GBA-PD

Kimberly McDowell, Reed Ressler, Brandon Wheeler, Shelby Thomas, Lubov S. Grigoryeva, Alexander Armendariz, Maeve Flynn, Hari Acharya, Paul Denis, Pasha Tchourilov, Swati Tole, Nick Goeden, Nicholas C. Flytzanis, Susan Catalano

Capsida Biotherapeutics, Inc., Thousand Oaks, CA

Parkinson's disease (PD) is the second most common neurodegenerative disorder with a prevalence of over 1.2 million people in the United States by 2030 and no approved disease-modifying treatments today. Current understanding of risk factors suggests approximately up to 15% of PD patients have mutations in the GBA1 gene, making this the most significant genetic risk factor for developing the disease. GBA1 encodes a lysosomal hydrolase enzyme, glucocerebrosidase (GCase), the reduced levels or aberrant function of which is associated with accumulation of alpha synuclein, loss of nigrostriatal dopaminergic neurons and development and spread of aggregated protein inclusions known as Lewy bodies, the pathological hallmarks of PD. GBA-PD is associated with the same motor and non-motor symptoms as idiopathic PD - bradykinesia, rigidity and resting tremor, cognitive decline, sleep disturbances, hyposmia, psychiatric symptoms, and autonomic dysfunction - but tends to present at an earlier age and with greater cognitive dysfunction. Growing clinical and preclinical data suggest that an effective treatment that restores GCase levels in the brain could slow or stop the progression of GBA-PD. A novel gene therapy candidate, CAP-003, may offer the potential to stably supplement wild-type GCase protein throughout the CNS with a single intravenous administration, enabling long-term disease modification and substantially slowing disease progression of GBA-PD with limited treatment burden. CAP-003, which consists of an engineered AAV capsid that is a product of our high-throughput, non-human primate (NHP)-based AAV engineering platform, packaging the human GBA1 gene, achieves breakthrough levels of neuronal transduction throughout the brain while de-targeting the liver 16-fold compared to AAV9. The CAP-003 candidate delivered at low to moderate doses successfully transduces up to 60% of dopaminergic neurons in the substantia nigra and up to 70% in other brain regions, raising bulk GCase enzyme activity levels up to 45% over wild-type in healthy NHPs. Estimated average levels of GCase activity increase within transduced neurons surpass 100% of wild-type levels and are expected to more than normalize the enzymatic activity deficit in human patients with PD. Importantly, these levels of GCase supplementation throughout the brain can be achieved at doses that are well tolerated, without any of the clinical pathology, immunogenicity or histopathological findings associated with higher dose systemic gene therapies. With the above profile, CAP-003 is being developed with the aim of achieving meaningful, disease-modifying clinical benefit for patients with GBA-PD through a convenient, single-dose, intravenous administration.

Advancements in Technologies for *In Vivo* Gene Therapies

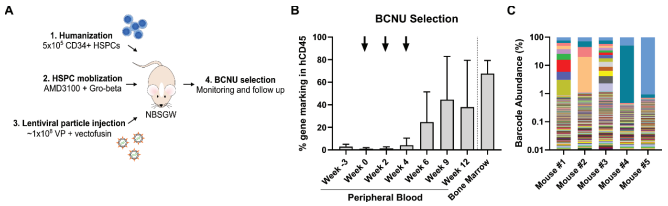
275. *In Vivo* Application of BaEVRLess-Pseudotyped Lentiviral Vectors for Hematopoietic Stem Cell Gene Therapy

Denise Klatt[1], Boya Liu[1], Adele Mucci[1], Marie Peschers[1], Giulia Schiroli[2], Axel Schambach[1,3], Chad Harris[1], Danilo Pellin[1], John P. Manis[4], Myriam Armant[5], David Scadden[6], Els Verhoeyen[7,8], David A. Williams[1], Christian Brendel[1]

[1]Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, Boston, MA,[2]Tessera Therapeutics, Cambridge, MA,[3]Hannover Medical School, Hannover, Germany,[4]Transfusion Medicine, Boston Children's Hospital, Boston, MA,[5]Trans Lab, Boston Children's Hospital, Harvard Medical School, Boston, MA,[6]Regenerative Medicine, Massachusetts General Hospital, Boston, MA,[7]INSERM/CIRI/EVIR, Lyon, France,[8]Université Côte d'Azur, Nice, France

Traditional *ex vivo* hematopoietic stem cell (HSC) gene therapy has effectively cured severe inherited blood disorders, such as sickle cell disease (SCD), but more widespread implementation is limited by high costs, scalability issues, and significant risks associated with myeloablative chemotherapy. *In vivo* HSC gene therapy, which aims to directly modify the cells *in situ*, has the potential to overcome these limitations. Here, we explore the use of retroviral vectors pseudotyped with the baboon endogenous retrovirus (BaEV) glycoprotein, and its derivative BaEVRLess, for stable gene transfer into HSCs *in vivo* (Figure A). Although BaEVRLess-pseudotyped retroviral vectors very efficiently transduce quiescent HSCs, their titers are often low due to toxicity associated with the high fusogenic activity of BaEVRLess during virus production. To overcome this problem, we generated a stable BaEVRLess-packaging cell line carrying knock-out of the viral entry receptor ASCT2. This resulted in a 2-fold increase in titers (10^8-10^9 infectious particles/mL) and a 3-fold reduction in cytotoxicity of virus preparations. Additional modifications to the packaging cells include the overexpression of CD47 and knock-out of B2M, which aims to reduce phagocytosis and immune responses *in vivo*, respectively. To increase *in vivo* HSC gene transfer, we mobilized humanized NBSGW mice with AMD3100 and Gro-beta, which achieved on average 20 hCD34+ cells/µL of blood. Upon mobilization, ~$1x10^8$ viral particles were injected intravenously into animals reaching up to 2.9±2.1% gene marking in peripheral blood hCD45+ cells within one week. To enrich gene-marked cells, we overexpressed MGMT-P140K in transduced cells, which mediates resistance to the alkylating agent BCNU. After three cycles of BCNU treatment, we observed a 38-fold enrichment of gene marked cells in the peripheral blood (Figure B). In bone marrow, gene marking reached 67.7% in hCD45+ cells and averaged 42.2% in hCD34+ cells. Barcode analysis showed the presence of 300-1000 unique barcodes and a polyclonal distribution in transplanted

mice (Figure C). Transduction and selection of *bona fide* HSCs was confirmed in secondary transplantations, where gene marking and clonality remained stable with 39.3-48.1% transduced hCD45+ cells. In proof-of-concept experiments, we targeted the transcription factors BCL11A and ZNF410 using miRNA-embedded shRNAs containing vectors. Downregulation of BCL11A and ZNF410 in erythroid cells led to sustained reactivation of gamma-globin and induction of fetal hemoglobin, which largely attenuates the hematologic effects of SCD. Following *in vivo* gene transfer and BCNU selection in humanized mice, we observed a significant upregulation of gamma-globin (35.6%) and fetal hemoglobin (25.1%) in transduced erythroid cells. In summary, we demonstrate that BaEVRLess-pseudotyped lentiviral particles can be applied for *in vivo* gene therapy to treat sickle cell disease.



276. LNP-Based Delivery of CRISPR/Cas12a for the Potential Treatment of Myocilin-Associated Glaucoma

Mariacarmela Allocca, Chi-Hsiu Liu, Chrysa Latrick, Xiaoqi (Kevin) Sun, Andrea Pinilla, Steve Bottega, Shrutokirti De, Khushboo Undavia, Alison Miller, Ruhong Dong, Eleanor Quenzer, He (Harry) Wang, Mark Jones, Steve Pietrasiewicz, Meng Wu, Stephen Sherman, Eugenio Marco, Kayla Delano, Jim Bochicchio, Georgia Giannoukos, John A. Zuris, Judith Newmark, Paul J. Wrighton, KaiHsin Chang, Heather MacLeod

Editas Medicine, Cambridge, MA

Glaucoma is one of the leading causes of blindness in the developing world. Mutations in the myocilin (*MYOC*) gene are the most common genetic cause of primary open angle glaucoma (POAG), accounting for 2-5% of the POAG cases. The mutations in the *MYOC* gene result in intracellular protein aggregates in the trabecular meshwork (TM) cells leading to endoplasmic reticulum (ER) stress, malfunction of the TM tissue, and elevated intraocular pressure (IOP). Medications to lower IOP are often not sufficient and surgery is required. Even then, the long-term prognosis is reduced vision and eventual vision loss. We hypothesize that editing of the *MYOC* gene to knock-out its expression would reduce ER stress and restore TM function, thus lowering IOP. We aim to develop an in vivo therapy for myocilin-associated POAG using lipid nanoparticles (LNPs) to deliver our engineered AsCas12a mRNA with a chemically modified guide RNA (gRNA) to edit the *MYOC* gene and reduce IOP. To this end, we developed in vitro and in vivo phenotypic models to evaluate the impact of editing on ER stress and IOP. The in vitro model was generated using HEK293T cells that stably express the Y437H mutant myocilin, which is one of the more aggressive mutations and is associated with early-onset of POAG. These cells exhibit intracellular buildup of the mutant myocilin protein and elevated levels of GRP78, a marker of ER stress. The in vivo mouse model was developed by replacing the mouse *Myoc* gene with the human Y437H mutant *MYOC* gene ($hMYOC^{Y437H}$) and presents with elevated IOP compared to wild-type control mice. We identified an ionizable lipid and LNP formulation that robustly targets the TM tissue in vivo by using GFP mRNA as cargo. The small AsCas12a gRNAs are subject to rapid degradation once released inside the cell resulting in lower editing efficiency. Therefore, we evaluated different chemical modifications to the gRNA in primary TM cells in vitro and selected those with improved editing efficiency for further testing in vivo in our humanized mouse model. LNPs encapsulating our engineered AsCas12a mRNA and gRNA with the top three modifications from in vitro testing were intracamerally injected into $hMYOC^{Y437H}$ mice and human *MYOC* mRNA levels were evaluated by RT-ddPCR. Finally, the modified gRNA leading to the greatest reduction in human *MYOC* mRNA in vivo was selected for the assessment of the reversal of the disease phenotype in vitro and in vivo. In our in vitro phenotypic assay, treatment of cells with increasing concentrations of LNPs encapsulating our engineered AsCas12a mRNA and the optimized gRNA resulted in concentration-dependent editing of the *MYOC* gene, reduction of *MYOC* mRNA and protein, as well as reduced GRP78 protein. The data demonstrate in vitro reversal of the ER stress phenotype that accompanies myocilin-associated glaucoma. We next tested our engineered AsCas12a mRNA with the optimized gRNA encapsulated in LNPs for in vivo efficacy in the $hMYOC^{Y437H}$ mice. Editing of the *MYOC* gene in $hMYOCY^{437H}$ mice resulted in a reduction of IOP at all doses tested. In summary, we demonstrate that editing of the *MYOC* gene, by LNP-mediated delivery of an optimized gRNA and engineered AsCas12a mRNA, reduces ER stress in TM cells in vitro and rescues IOP in $hMYOC^{Y437H}$ mice in vivo. These results suggest that *MYOC* gene knockout by LNP-based delivery of CRISPR/AsCas12a is a potential therapeutic approach for treating myocilin-associated glaucoma.

277. G-CSF-Free HSC Mobilization with WU-106/AMD3100 Allows for Safe and Efficient *In Vivo* HSC Prime Editing of the Sickle Cell Disease Mutation in a Mouse Model

Chang Li[1], Anna K. Anderson[1], Peter Ruminski[2], Michael Rettig[3], Darja Karpova[3], Hans-Peter Kiem[4], John F. DiPersio[3], Andre Lieber[1]

[1]University of Washington, Seattle, WA,[2]Washington University School of Medicine,, Saint Louis, MO,[3]Washington University School of Medicine, Saint Louis, MO,[4]Fred Hutch, Seattle, WA

We have reported direct repair of the sickle cell mutation *in vivo* in a disease model using vectorized prime editors after hematopoietic stem cell (HSC) mobilization with G-CSF/AMD3100. Despite its robustness in HSC mobilization, G-CSF is contra-indicated in patients with Sickle Cell disease (SCD), presenting a hurdle for the clinical translation of the approach. Additionally, G-CSF results in unselective bone marrow cell mobilization, which leads to leukocytosis and higher numbers of cytokine-producing cells in the periphery. This increases the number of cytokine-producing cells in the periphery that come in contact with intravenously injected viral particles, which in turn, contributes to higher cytokine levels in mobilized versus non-mobilized animals. Committed bone marrow cells mobilized to the periphery also sequester vectors thus reducing the effective dose

for primitive HSCs. Further, the five-day treatment regimen and high costs associated with G-CSF/AMD3100 justify the development of an alternative mobilization regimen. Here, we tested a G-CSF-free mobilization regimen using WU-106, a PEG-conjugated inhibitor of integrin VLA-4 (α4β1), plus AMD3100 for *in vivo* HSC prime editing in SCD mice (CD46/Townes). The all-in-one PE5max system expressed by a helper-dependent adenoviral vector (HDAd) was used to repair the sickle mutation (GTG>GAA conversion). Mobilization with WU-106+AMD3100 in CD46/Townes mice was rapid (with a peak at 2 hours) and efficient. In contrast to the G-CSF/AMD3100 approach, leukocytosis was minimal and serum cytokines (IL-6, TNF, IFN-γ) were not detectable after HDAd vector injection. Target site editing (T>A correction) of the SCD mutation was on average 23% in peripheral blood cells and bone marrow lineage-negative cells at week 16 after *in vivo* transduction and selection. The treated mice demonstrated phenotypic correction, reflected by normalized blood parameters and spleen size. Editing rates were significantly increased (29%) in secondary recipients indicating preferential mobilization/transduction of long-term repopulating HSCs. Using this approach, we found <1% of undesired indels and no detectable off-target editing at top-scored potential sites. Our study shows that *in vivo* transduction to treat SCD (including HSC mobilization and HDAd injection) can now be done within 2 hours involving only simple intravenous injections with an excellent safety profile. The same-day mobilization regimen makes *in vivo* HSC gene therapy more attractive for the resource-poor settings where SCD does the most damage.

278. Cas12a CRISPR Hybrid RNA-DNA (chRDNA)-Mediated *In Vivo* Genome-Editing Technology for Efficient and Functional Hepatic Gene Disruption

Marta Losa-Llabata, Prachi Rajmane, Liz Stanaway, Mary Coons, Antonio Munoz, Vaibhavi Garish, Paul Donohoue, Scott Gradia, Steven Kanner, Meghdad Rahdar

Caribou Biosciences, Berkeley, CA

Familial hypercholesterolemia (FH) is an autosomal dominant inherited disease characterized by high circulating low-density lipoprotein (LDL) cholesterol. Affected patients quickly develop atherosclerosis, leading to peripheral and coronary artery disease and often followed by heart attack, stroke, or other cardiovascular complications. Transthyretin amyloidosis (ATTR) is a progressive life-threatening disease characterized by accumulation of misfolded transthyretin (TTR) protein in the nerves and heart leading to neurodegeneration and cardiomyopathy. While significant progress has been made to demonstrate the utility of Class 2 Type II Cas9 for *in vivo* genetic intervention, the *in vivo* potential of Class 2 Type V Cas12a has not fully been realized. In these proof-of-concept studies, we targeted proprotein convertase subtilisin/kexin type *9* (*Pcsk9*), angiopoietin-like 3 (*Angptl3*), and transthyretin (*Ttr*) - three clinically validated gene targets for FH and ATTR, using a Cas12a CRISPR hybrid RNA-DNA (chRDNA) genome-editing technology. We have previously shown that CRISPR guides designed with a combination of RNA and DNA nucleotides, called chRDNAs, can be leveraged with Cas9 and Cas12a to enhance nuclease specificity and significantly reduce off-target genome editing. Upon identification of high-activity gene targeting Cas12a guides, optimization of the Cas12a mRNA sequence, and refinement of our lipid nanoparticle (LNP) formulation, we sought to demonstrate functional gene disruptions in wild-type Balb/c mice. A single intravenous dose of Cas12a chRDNA optimized to target *Pcsk9* resulted in nearly 60% hepatic editing, followed by a 50% reduction in circulating PCSK9 protein and a 30% reduction of plasma cholesterol in wild-type mice. Similarly, targeting *Angptl3*, we demonstrated a >70% hepatic editing efficiency followed by a >70% reduction of plasma ANGPTL3 protein, accompanied by a 30% reduction of plasma cholesterol. Surprisingly, a dual knockout of *Pcsk9* and *Angptl3* did not demonstrate increased reduction of circulating LDL cholesterol compared to single disruptions. Lastly, targeting *Ttr*, we observed 70% gene disruption with a single administration, leading to a 98% reduction in circulating TTR protein levels. With all the targets, we did not detect significant liver toxicity or chronic cytokine release. Furthermore, analysis of *in silico* predicted off-target sites by next-generation sequencing (NGS) did not identify off-target genome editing. In addition, no significant peripheral organ on-target genome editing was detected following tissue isolation and NGS analysis. Our studies demonstrate that LNP delivery of Class 2 Type V Cas12a mRNA in conjunction with an optimized chRDNA guide is a powerful tool for efficient and functional genome editing in hepatocytes and a tractable alternative platform to first-generation CRISPR-Cas9 genome-editing.

279. *In Vivo* panCAR™ Therapy Using Circular RNA for the Treatment of B cell Malignancies in Oncology and Autoimmune Disease

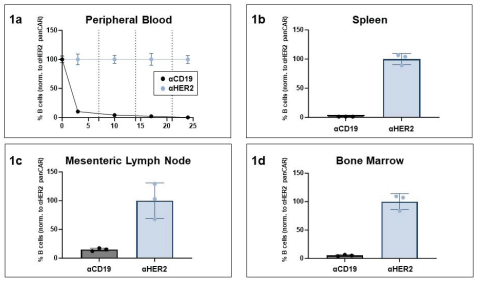
Amy Becker, Isin Dalkilic, Allen Horhota, Robert A. Wesselhoeft, Akinola Emmanuel, Thomas Lee, Blake Cohen, Ramya Elangovan, Akshi Thakkar, Magnolia Chinn, David Soto, Sharmistha Kundu, Kevin Kauffman, Meghan Richardson, Chris Wright, Kristen Ott, Jui Dutta-Simmons, Rena Schindler, Yessica Wiryawan, Trent Stevens, Ashley Wong, Ian Langer, Rahul Vungutur, Corey Ciullo, Zifiso Nyoni, Junghoon Yang, Emily Fitzgerald, Nelson Chau, Tom Barnes, Robert Mabry

Orna Therapeutics, Watertown, MA

Orna Therapeutics is developing off-the-shelf *in vivo* panCAR™ therapies for the treatment of cancers and other immunologic diseases. ORN-101, our first clinical candidate, is an αCD19 panCAR for the treatment of leukemia and lymphoma. While traditional *ex vivo* generated CAR-T cell therapies have had tremendous success in the clinic, the manufacturing, safety, and efficacy remains a challenge. Circular RNA (oRNA™)-enabled panCAR therapies promise a transient, re-dosable and scalable immune cell therapy, without requiring immunodepletion. panCAR combines a novel, synthetic, circular coding RNA platform (oRNA) and immunotropic lipid nanoparticles (LNP) to drive CAR expression on the surface of immune effector cells after i.v. administration, eliminating the need for external manufacturing. LNPs deliver oRNA payloads to multiple immune effector subsets, including T cells, NK cells, and macrophages in vivo, potentially increasing efficacy and broadening application beyond hematologic malignancies. Immunotropism is

preserved across multiple species, including mouse, rat, humanized mouse, and non-human primate models. oRNA technology uses a sequence based IRES element to initiate protein translation in target cells. To identify IRES sequences that drive high reporter payload expression in primary human T cells, we screened over 3000 oRNAs containing IRES sequences extracted from viral genomes using our proprietary FoRCE platform. Of hundreds of sequences identified, a subset was selected to generate CAR expressing oRNAs for functional testing in vitro and in vivo. In a PBMC-engrafted NALM6 CD19+ tumor-bearing model, we observed tumor regression in a dose- and antigen-dependent manner in mice treated with the αCD19 panCAR but not αHER2 panCAR. Importantly, tumor regression was observed at doses as low as 0.1 mg/kg using a bi-weekly dosing schedule. While T cells are the primary cell type in PBMC-engrafted immunodeficient mice, CAR-expressing NK cells can augment tumor clearance as well. To understand this, we developed a humanized NK panCAR mouse model, where Raji cells were engrafted with primary human NK cells. The NK model showed improved tumor control in i.v treated mice with αCD19 panCAR compared to control treated animals. Taken together, these data suggest that panCAR can target and engage multiple potent anti-tumor effector cells. Analogous to an immune competent host, we evaluated endogenous B cell depletion by treating human CD34+ hematopoietic stem cell engrafted mice with αCD19 panCAR vs negative control panCAR. With weekly dosing, the panCAR-treated mice exhibited significant depletion of CD19+ cells in the peripheral blood, spleen, mesenteric lymph node, and bone marrow (Figure 1). Extensive B cell depletion in these compartments reinforces the effectiveness of αCD19 panCAR for diseases like hematological cancers or B cell-mediated autoimmunity. Collectively, Orna Therapeutics' panCAR therapy shows promise as an efficacious, scalable therapy that eliminates manufacturing time, thus providing patients access to immediate immune modulating treatments.

Figure 1: Evaluation of endogenous B cell depletion by treating human CD34⁺ hematopoietic stem cell engrafted mice with αCD19 panCAR. Mice were dosed weekly at 2 mg/kg. B cell counts were analyzed in the peripheral blood (1a, days 1-24) and spleen (1b), mesenteric lymph node (1c), and bone marrow (1d) at study termination, and percentages were normalized to αHER2 panCAR (negative control).



280. *In Vivo* HSC Gene Editing for Correction of Sickle Cell Mutation Using RNA Gene Writers

Giulia Schiroli[1], Yaser Heshmati[1], Yu Cao[1], Lorenzo Tozzi[1], Micheal Monte[1], Apiwat Wangweerawong[1], James Rottman[1], Rahul Palchaudhuri[1], Jane Wang[2], Jean-Antoine Ribeil[3], John Manis[4], Bill Querbes[2], Anne Bothmer[1], Cecilia Cotta-Ramusino[1], Micheal C. Holmes[1]

[1]Tessera Therapeutics, Cambridge, MA,[2]Tessera Therapeutics, Somerville, MA,[3]Boston University Medical Center, Cambridge, MA,[4]Boston Children's Hospital, Boston, MA

Tessera Therapeutics is pioneering a new approach to genome engineering through the development of our Gene Writing and delivery platforms. RNA Gene Writers are inspired by the biology of naturally occurring non-LTR retrotransposons, a class of mobile genetic elements that can edit the genome through target-primed reverse transcription, or TPRT. We have engineered non-LTR retrotransposon proteins that are designed to introduce a broad range of edits to the genome, from writing in a transgene, inserting an exon, or introducing single nucleotide changes. We believe RNA Gene Writers could potentially be applied to sickle cell disease (SCD) to correct the SCD causing mutation, *HBB* E6V, in primary hematopoietic stem cells (HSCs). We first modeled the efficiency of *HBB* editing by introducing *HBB* Makassar variant (E6A) in wild-type (WT) CD34+ human HSCs from healthy donors. We observed an average of 74% correction *in vitro* after optimization of the RNA template, delivery and expression of multiple RNA Gene Writers. Phenotypic or functional impairment was not observed in edited HSCs as measured by colony forming assays, differentiation, or *in vivo* reconstitution studies in immunodeficient mice. We observed high engraftment levels and *HBB* correction was maintained in long-term repopulating HSCs and their multi-lineage progeny isolated from mice 16 weeks post bone marrow transplant. Comparable editing levels were also maintained after secondary transplant of the edited cells. As compared to treatment with nucleases, *HBB* editing with RNA Gene Writers did not induce a detectable p53-related response in HSCs as measured by RNAseq. We then demonstrated efficient editing of CD34+ cells from multiple SCD patients. We observed ~70% correction of sickle alleles to WT. Erythroid differentiation further demonstrated edited cells exhibited improved enucleation and, importantly, restoration of normal adult hemoglobin levels by liquid chromatography-mass spectrometry (LC-MS) and high performance liquid chromatography (HPLC). Phenotypic correction of SCD donor CD34+ cells were further confirmed by culturing differentiated erythrocytes under hypoxic conditions and demonstrating abrogation of cell sickling compared to untreated donor cells. RNA Gene Writers are designed to be delivered as an all-RNA composition, which enables *in vivo* non-viral delivery by lipid nanoparticles (LNP). Delivery of a reporter gene to a substantial fraction of HSCs *in vivo* was observed, including >90% of long-term HSCs in both humanized mice and non-human primates. *In vivo* studies performed using a surrogate reagent, *B2M,* demonstrated an average of ~15% rewriting of long-term HSCs in humanized mice with a single LNP dose. By combining our LNP delivery platform and RNA Gene Writers, we believe we may be able to correct the SCD mutation by targeting HSCs *in vivo*.

281. The Development of a *Staphylococcus epidermidis* Strain Expressing LEKTI-D6 (ATR12-351) for Netherton Syndrome

Sara Mootien, Roger Leger, Dorival Martins, Francisco Salva, Travis M. Whitfill, Mary Spellman

Azitra Inc., Branford, CT

Netherton syndrome (NS) is a rare autosomal recessive disorder caused by mutations in the serine protease inhibitor of Kazal type 5 (*SPINK5*) gene, which encodes the serine protease inhibitor lympho-epithelial Kazal-type-related inhibitor (LEKTI). Loss-of-function mutations in the *SPINK5* gene lead to unregulated kallikrein (KLK) proteolysis and activation of the KLK cascade, resulting in skin desquamation and increased transepidermal water loss. Harmful microbial and allergen penetration trigger the release of antimicrobial peptides and activation of inflammatory pathways, including by IL-36γ. There are no approved therapies for NS. ATR12-351 is a live biotherapeutic topical ointment comprised of an auxotrophic strain of *Staphylococcus epidermidis* (SE351) engineered to express domain 6 (D6) of the human LEKTI protein. Delivery of recombinant human LEKTI-D6 (rhLEKTI-D6) in the lower layers of the stratum corneum restores normal KLK activity levels, while the *S. epidermidis* strain reduces IL-36γ. ATR12-351 is expected to inhibit skin serine protease activity, improve skin barrier function, and reduce underlying cutaneous inflammation, reducing the signs and symptoms (skin appearance, pain, and pruritus) of NS. Early developmental studies were conducted to measure the potency of protease inhibition of SE351 in a skin matrix with added KLK5, which showed an IC_{50} of trypsin-like activity of 4-16 mol/mol of LEKTI/KLK5. In an *in vitro* assay using human keratinocytes monolayer, SE351 culture supernatant significantly reduced Poly I:C induced IL-36γ levels. SE351 culture supernatant reduced IL-36γ levels by 93% compared to Poly I:C alone (908 pg/mL vs. 70 pg/mL) and were similar to untreated cells (~50 pg/mL). Formulation studies were conducted to optimize rhLEKTI-D6 expression by SE351. In *in vitro* studies, growth of SE351 in the presence of various excipients as nutrient sources in the formulation was assessed, and the addition of colloidal oatmeal to the formulation of ATR12-351 increased rhLETKI-D6 expression by 5-fold compared to formulation without colloidal oatmeal ($p<0.001$). The efficacy of ATR12-351 was evaluated on an NS model consisting of human skin extracts supplemented with recombinant KLK5 (7-fold excess over normal human skin to model protease activity in NS) focusing on trypsin-like activity. The results demonstrate that trypsin-like activity was reduced to levels like those of normal skin ($p<0.01$). Toxicology and rhLEKTI-D6 expression were evaluated on minipigs with abraded skin treated with either topical ATR12-351 or vehicle for 14 days. There were no adverse safety findings when ATR12-351 was applied to skin abraded minipigs, and ATR12-351 was not detected in blood. rhLEKTI-D6 was also significantly increased at day 14 in ATR12-351 vs. vehicle on the surface of the pig skin (12 vs. 2.6 ng/cm^2, respectively, $p<0.01$). An ongoing proof-of-concept, randomized, double-blind, multicenter, vehicle-controlled clinical study (NCT06137157) assesses the safety, tolerability, and pharmacokinetics of topical ATR12-351 in approximately 12 adult patients with NS. ATR12-351 ointment is applied twice daily to a defined area of affected skin on one side of the body, and its vehicle is applied to similarly affected skin contralaterally during a 14-day treatment period. Throughout the study, including a 70-day follow-up period, clinical assessments include safety and local tolerability, the severity of the signs and symptoms of NS, local biomarkers (including KLK activity), and the presence of rhLEKTI-D6 in skin. The results of this clinical study will establish an initial safety and efficacy profile of ATR12-351 in NS patients to potentially support further clinical development.

Novel Production Platforms

282. Healthy Donor vs Patient Manufactured Autologous Deltex DRI Product; Immunophenotyping Gene Expression

Mariska A. Ter Haak[1], Branimir Popovic[1], Caitlyn C. Lucas[1], Becca B. Weekley[1], Wenjing Pan[2], Daniel Weber[2], Miranda Byrne-Steele[2], Lawrence S. Lamb[1], Kate Rochlin[1]

[1]IN8bio, New York, NY,[2]iRepertoire, Hunstville, AL

Gamma-delta T cell based therapies have been advancing clinically and have shown therapeutic promise but their molecular profiles have not been well characterized. Gene expression changes that occur throughout the manufacturing process which can be >10 days, could provide insight into the potency, effector and trafficking capabilities of these products along with the reproducibility and robustness of particular manufacturing processes. Deeper analysis of the gene expression changes through manufacturing has not previously been demonstrated but may identify predictors of γδ T cell expansion and/or correlate to potency or clinical outcomes. Further, such data may elucidate which profiles or cellular characteristics are imparted by donor selection versus those from the manufacturing process. We evaluated TCR repertoire and gene expression from both (a) the apheresis starting material and (b) final manufactured γδ T cell product from healthy donors and glioblastoma (GBM) patients (NCT 04165941). Gamma-delta T cell clinical products were manufactured from apheresis using proprietary expansion protocols. The cells were transduced with a Methylguanine-DNA methyltransferase (MGMT) construct, which conveys resistance to temozolomide chemotherapy. TCR libraries were prepared with VDJ-specific primers for α, β, δ and γ chains by dimer-avoided multiplex PCR (dam-PCR) using iRepertoire's RepSeq+. In parallel, bulk RNA phenotyping libraries were prepared by dam-PCR encoding +150 immune profiling genes and sequenced using Illumina's platform. Multigroup comparisons were performed by nonparametric statistical test while controlling for false discovery rate. At manufacturing initiation, the TCR repertoire consisted primarily of αβTCR clones. Gene expression levels from healthy donors seem to have increased BCL6, naïve cells, CD8, CD63, but overall gene expression was similar between healthy donors and GBM patients. The corresponding shift to γδ TCR expression was confirmed in the final products, where preferential expansion of Vγ9 and Vδ2 clones was observed from both healthy donors and GBM patients. Interestingly, gene expression analysis showed a significant increase in markers of cellular activation and cytotoxicity in both final products. These markers, indicating that the cells are active and primed to kill, include IFNγ, Perforin, Granzyme B, TNF, CD25, CD40L, CD71, 41BB, CD226, Eomes, CXCR3, ICOS, and IL-4, as well as LAG3, TIM3, and CTLA-

4, which are suggested to be γδ T cell activation markers p <0.01. Interestingly, the expression of immune trafficking and stimulation markers were significantly increased in the final product, including STAT-1, STAT-4, CCR6, Runx1, Tbet, GATA3, CD103, CD193, S1PR1, Il-5, Il-13, IL7-R, IL-12Rb2, and Il-23R, p <0.05, suggesting the cells are capable of tissue trafficking and immune cell recruitment. Products manufactured from healthy volunteers and GBM patients exhibited highly similar gene expression profiles, suggesting that the process is both robust and reproducible across different donor populations. Our studies show activated, cytotoxic γδ T cell products were successfully manufactured from different starting materials and exhibited similar gene expression profiles. In addition, immunophenotyping gene expression of DeltEx DRI product elucidated additional attributes such as cytotoxic and/or trafficking potential along with the ability to stimulate immune cell recruitment.

283. Enhancing Cell Transfection Efficiency via Modulation of Extracellular Fluid Viscosity

Jingyao Ma, Yining Zhu, Jiayuan Kong, Di Yu, Wu Han Toh, Milun Jain, Junkai Yang, Qin Ni, Zhuoxu Ge, Jinghan Lin, Joseph Choy, Konstantinos Konstantopoulos, Maximilian F. Konig, Sean X. Sun, Hai-Quan Mao

Johns Hopkins University, Baltimore, MD

Statement of Purpose: Gene therapy and cellular programming rely on effective cell transfection in vitro and in vivo. While numerous viral and non-viral vectors and transfection techniques have been developed to improve transfection efficiency, the extracellular fluid viscosity as a biophysical parameter has long been overlooked[1]. Here we report a generic effect of culture media viscosity on transfection efficiency of several gene delivery vehicles. In this study, we examined the effect of extracellular fluid viscosity ranging from 0.77 to 15 cP (at 37°C) on transfection efficiency across various cell types, using plasmid DNA (pDNA) or mRNA-based lipid nanoparticles, polyplex nanoparticles, and viral vectors. **Methods:** The LNP formulation developed by Moderna was utilized as a standard benchmark formulation. We synthesized LNPs and administered them to a diverse array of over 20 cell types, and the cells were cultured in media with varying viscosities, ranging from 0.77 to 15 cP (Fig. 1a). To verify whether the media viscosity-dependent enhancement effect applies to other transfection vectors or carriers, we substituted the LNP cargo with polyplex nanoparticles and viral vectors, and employed pDNA instead of mRNA as the genetic payload. In addition, we conducted pathway inhibition studies using chemical inhibitors for specific cell entry pathways. **Results and Discussion:** The transfection efficiency of mRNA LNPs was increased by 2 to 60-fold when subjected to a higher media viscosity, among cancer cell lines, immune cells, and other frequently used cell lines (Fig. 1a). The optimal media viscosity varied among different cell types, though within the range of 1 to 4 cP. Additionally, LVV-mediated transduction of peripheral blood mononuclear cells (PBMCs) showed a media viscosity-dependence effect (Fig. 1b). We then examined the transfection of human primary B cells using mCherry mRNA LNPs and showed that the percent of transfected cells increased by about 2-fold and MFI increased by about 80% at 2 cP compared with 0.77 cP condition (Fig. 1c-d). The pathway inhibition study revealed that at 2 cP, in B16F10 cells, the predominant pathways for LNP uptake shifted from an evenly distributed mold of uptake to clathrin-dependent endocytosis and micropinocytosis (Fig. 1e). **Conclusion:** We present a novel approach to enhance transfection or transduction efficiency in a wide range of cell types by adjusting media viscosity in in vitro or ex vivo processes and report a protocol to better mimic the in vivo cellular conditions in the current gene delivery research. **References:** 1. Bera, K. et al. Nature 611, 365-373 (2022).

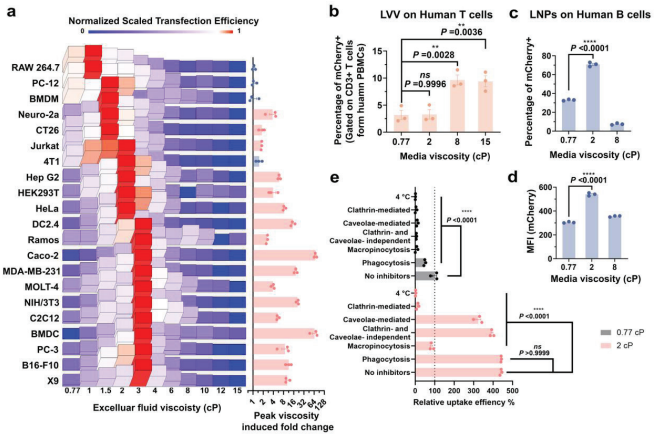


Figure 1. a, Transfection efficiency of LNPs in 21 cell types in media with different viscosity levels (0.77–15 cP). **b,** Transduction efficiency of LVV on CD3+ T cells of PBMCs transfected in media with different viscosity levels (MOI = 10). **c-d,** Percentage cells transfected (**c**) and MFI (**d**) of mCherry+ human primary B cells treated with mCherry LNPs in media with different viscosity levels (0.77, 2, 8, and 15 cP). **e,** Relative cellular uptake level of cells treated with specific pathway inhibitors and Cy5-labeled LNPs in media with 0.77 cP or 2 cP (n = 3).

284. A Short, Simple, and Cost-Effective CAR-T Cell Manufacturing Process

Ying Xiong, Yanping Xie, Yang Liu, Matthew Addington-Hall, Beatrix Ferencz, Tony Luo, Maggie Yates, Oxana Slessareva, Ibeawuchi Oparaocha, Zhongyu Zhu, Boro Dropulic, Rimas J. Orentas

Caring Cross, Inc., Gaithersburg, MD

Chimeric antigen receptor modified (CAR-T) cell therapy has shown tremendous clinical efficacy for hematological malignancies, and limited efficacy in solid tumor therapy. However, bottlenecks in the ability to manufacture and deliver these engineered cell products, combined with their high cost, have limited patient accessibility of CAR T therapy, especially to those in underserved areas or who are uninsured. Therefore, we are developing a flexible, cost-effective CAR T manufacturing process that generates sufficient CAR T cells numbers with excellent functionality.It is possible to achieve a high degree of T cell purity and recovery after a rosetting-based negative T cell selection protocol from either 450ml fresh whole blood or a 45ml (quarter size) leukapheresis product. We are in the process of developing an in-house reagent composed of bi-specific antibodies that rosette by means of binding to CD235 on red cells and to target antigens on B cells and cells of monocytic lineage, termed (T-Pure). Using the T-pure reagent and a Sepax™ C-Pro instrument (Cytiva) large-scale CAR-T manufacturing was attained. When T cells were enriched either by our negative selection method by T-pure or by positive selection using CD4/CD8 magnetic beads (Miltenyi Biotec), higher γδ T cell recovery was observed with T-pure reagent, which might indicate an advantage of the negative enrichment method.For large-scale T cell transduction with a CAR-encoding LVV, 1x 10^8 T cells were suspended in TexMACS GMP medium (Miltenyi Biotec) with 200 IU/ml IL-2 and transferred to

a G-Rex cell culture device (Wilson-Wolf), followed by the addition of T cell activation reagent (TransAct from Miltenyi Biotec, or Enceed from GenScript) and HIV gp120 DuoCAR lentiviral vector (MOI 40). On Day 3, a complete medium exchange or direct fresh medium addition (1:10 dilution) was carried out in the same G-Rex culture vessel. CAR-T were then cultured and expanded to Day 8, when the final cell product was harvested. On days 6 and 8, cultures were sampled to monitor T cell growth, phenotype, CAR expression, and function. Evaluating 3 healthy donors, similar cell growth was observed with either complete medium exchange (the "wash" condition) or medium addition alone (the "no wash" condition) on Day 3. In addition, comparable transduction efficiency, memory phenotypes (T_{scm}% and T_{cm}%) and target gp120 cell cytolysis were observed on both Day 6 and Day 8, indicating the feasibility of simpler manufacturing protocol integrated with medium dilution. Furthermore, equivalent transduction efficiency, similar memory phenotypes and comparable target gp120 positive cell cytolysis were demonstrated when alternative activation reagents were tested, confirming the robustness of the simplified CAR-T generation process. In conclusion, this simplified large-scale CAR T production process can provide the flexibility of an earlier culture harvest, as well as the use of alternate GMP reagents for manufacturing. Implementing this approach in a point-of-care manufacturing setting creates an economical process for CAR T production that can be administered to all who would benefit, especially in low- and middle-income countries. Future work will include evaluation of this process with patient-derived material.

285. An Efficient Way to Generate Virus- and Factor-Free Human iPSCs Using S/MAR DNA Vectors

Anna Hartley[1], Cornelia Wincek[1], Manuela Urban[1], Alicia Roig-Merino[1], Luisa Burger[1], Zhaoyu Du[2], Tracy Li[3], Lieke Dons[3], Martin J. Hoogduijn[2], Mehrnaz Ghazvini[3], Richard P. Harbottle[1]

[1]DNA Vector Lab, German Cancer Research Centre, Heidelberg, Germany,[2]The Rotterdam Transplantation Lab, University Medical Center Rotterdam, Rotterdam, Netherlands,[3]Erasmus MC iPS Core, University Medical Center Rotterdam, Rotterdam, Netherlands

Generating isogenic or autologous cells for modelling diseases or cell- and organoid-based therapies depends on efficiently reprogramming cells derived from patients. This was originally accomplished using integrating viral vectors, which pose risks of insertional mutagenesis and oncogenesis. Alternatively, non-integrative reprogramming commonly uses viral components (EBNA1/oriP, Sendai) or involves multiple transfection steps (RNA). However, these methods raise concerns; cells reprogrammed with viral components exhibit increased markers of cellular damage and immune activation, and repeated transfections escalate both costs and the risks of non-compliance with clinical quality standards. To mitigate these risks and enhance the clinical potential of cells, we have devised a user-friendly approach to reprogramming primary human fibroblasts using entirely non-viral DNA vectors, requiring only a single transfection. Our vectors are based on Scaffold Matrix Attachment Regions (S/MARs), sequences of human origin that enable autonomous episomal replication of the vector within the host cell nucleus. This facilitates sustained expression of the necessary reprogramming factors without integration, paving the way for virus-free, vector-free human induced pluripotent stem cells (hiPSCs). hiPSCs made using our S/MAR vectors are morphologically and phenotypically indistinct from EBNA/oriP-derived hiPSCs, and express markers of pluripotency as measured by immunofluorescence and qRT-PCR. On a transcriptional level, S/MAR iPSCs show close similarity with human embryonic stem cell lines. S/MAR iPSCs are also capable of directed tri-lineage differentiation *in vitro*. Copy number variations (CNVs) assessed by microarray in comparison with input somatic cells show minimal perturbation of iPSC genomes in most clones assessed. An additional advantage is that S/MAR vectors can be lost passively after reprogramming, generating vector-free hiPSCs without the need for selection. Our S/MAR DNA vectors can also be employed for the stable and heritable genetic modification of hiPSCs. Importantly, these vectors are maintained episomally at a low copy number, preserving the cells' pluripotent capacity without causing damage. The stability of the vector transgene expression in hiPSCs persists over an extended period (>150 days) even in the absence of selection and remains unaffected by cryogenic cell storage. Furthermore, transgene expression is stable across all stages of differentiation into target cell types. We have demonstrated the persistence of transgene expression *in vitro* in the trilineage differentiation of S/MAR-modified hiPSCs into germ layers, as well as in directed differentiation into lymphoid effector cells and kidney organoids. Modification of mouse embryonic stem cells using S/MAR vectors led to the generation of chimeric pups which retained transgene expression. S/MAR DNA vectors present a safe and stable alternative for efficient reprogramming of primary human fibroblasts into hiPSCs, as well as a heritable means of genetic modification of stem cells for clinical application. Future work will focus on guaranteeing the passive loss of reprogramming vectors after iPSC generation, further improving the safety profile of the vectors, and progressing towards virus-free, vector-free, factor-free iPSCs which are feasible to generate at clinical scale and grade.

286. Establishment of Automated Point-of-Care Manufacturing of ΔNPM1 TCR-Engineered T Cells for AML Therapy

Isabella Elias Yonezawa Ogusuku[1], Eva Agiro[2], Vera Herbel[1], Caroline Brandes[1], Sabrina Veld[2], Lois Hageman[2], Marieke Griffioen[2], Thomas Schaser[1], Rosa de Groot[2], Dominik Lock[1], Ian C. D. Johnston[1]

[1]Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany,[2]Department of Hematology, Leiden University Medical Center, Leiden, Netherlands

Acute myeloid leukemia (AML) is a heterogeneous malignancy that requires the expansion of available therapeutic options, especially for the elderly and patient subgroups with poor prognosis. A recently discovered T cell receptor (TCR) targeting a common mutant nucleophosmin 1 (ΔNPM1) neoepitope presents an attractive opportunity to design such a novel cancer antigen targeted cellular therapy. We have recently developed a rapid (8-day), closed and GMP-compliant, automated TCR-T cell manufacturing process using the CliniMACS Prodigy®. Starting from cryopreserved leukapheresis, 2E8 CD8-positive T cells from healthy donors were magnetically enriched, activated, transduced with lentiviral vector expressing an HLA-A*02:01 restricted recombinant ΔNPM1-specific TCR, expanded and finally formulated. In downscaled experiments, we also verified that the planned approach yielded functional and potent

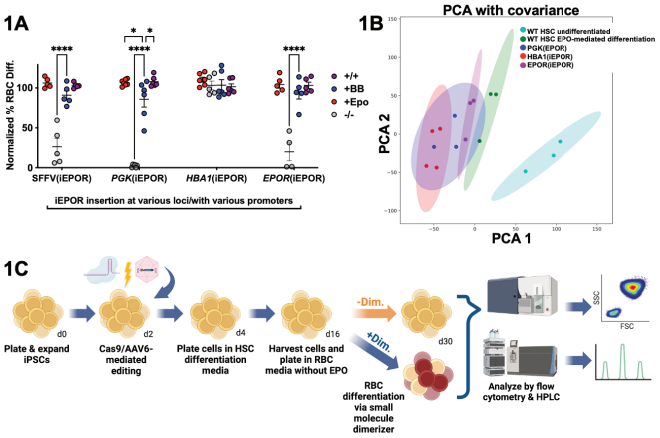
ΔNPM1-specific TCR-T cells when starting with frozen material from AML patients. After optimization of the manufacturing process, the protocols and associated quality control assays were transferred to the clinical manufacturing site at Leiden University Medical Center. As expected, the robust manufacturing process demonstrated comparable performance in all relevant IPC and QC assays yielding >5E9 CD8+ ΔNPM1+ T cells with >98% viability. We show that across independent donors, devices, manufacturing sites and operators, this process consistently yielded therapeutic doses of potent TCR-engineered CD8 T cells while maintaining the product's high viability, purity and favorable early memory phenotype, which is known to support in vivo persistence and efficacy. This novel state-of-the-art manufacturing process will now be used to manufacture autologous TCR-modified T cells for assessment in a phase I/II clinical trial to treat patients with relapsed or refractory NPM1-mutated AML.

287. Engineering Inducible Signaling Receptors for Erythropoietin-Free Erythropoiesis

Aadit P. Shah[1], Kiran R. Majeti[2], Matthew H. Porteus[2], Kyle Cromer[3]

[1]Stanford University School of Medicine, Stanford, CA,[2]Stanford University Department of Pediatrics, Stanford, CA,[3]University of California San Francisco Department of Bioengineering & Therapeutic Sciences, San Francisco, CA

Introduction: Blood cell transfusion is vital to medicine. In the US, more than 12 million red blood cell (RBC) units are used annually. Yet, availability relies on donor material, resulting in supply constraints and safety concerns. Shortages are expected to worsen with aging populations and diminishing donor numbers. To address this need, *ex vivo* manufacture of RBCs from induced pluripotent stem cell (iPSC) producer lines emerges as a potential solution that offers benefits compared to donor blood, including lower infectious disease transmission, streamlined production, product uniformity, and ability to source or genetically engineer antigen-negative cells. However, this approach remains extremely costly, largely due to purified, clinical-grade cytokines required to stimulate producer cells for expansion and differentiation into erythroid cells. Because erythropoietin (EPO) signaling through the EPO receptor (EPOR) is indispensable to RBC development, of all components in erythroid-promoting media, EPO is by far the costliest (currently $300,000/unit). **Methods:** We used synthetic biology tools and genome engineering to devise a series of FKBP-EPOR chimeras, termed inducible EPORs (iEPORs), that exchanged the requirement for native EPO ligand with an exogenous, orthogonal small molecule (SM). These were site-specifically inserted *in vitro* into primary human hematopoietic cells (HSCs) via CRISPR/AAV-mediated genome editing. Using flow cytometry, ddPCR, and RNA sequencing-based assays, we determined the fidelity (responsivity to SM) and potency (ability to drive differentiation towards the erythroid lineage) of iEPOR constructs with different signal peptides, extracellular and intracellular domain modifications, and insertion at different HSC and RBC-relevant loci. **Results:** We optimized iEPOR constructs with a highly efficient IL6 signal peptide and naturally occurring intracellular domain truncation enhancing erythropoiesis. When integrated at the *CCR5* locus and driven by the SFFV promoter, we drove 100% differentiation into RBCs from engineered HSCs using SM without Epo. However, we observed 26.2% differentiation without any erythroid-biasing ligand. To increase iEPOR fidelity, driving expression via the PGK promoter ablated ligand-independent differentiation (Fig 1A). Identical HSC response to iEPOR in the presence of SM versus native HSC in the presence of EPO was confirmed by flow cytometry and measurement of hemoglobin production by HPLC. By RNA sequencing, iEPOR-integrated HSCs differentiated into RBCs clustered with natively differentiated HSCs compared to undifferentiated HSCs by PCA covariance plots (Fig 1B). We observed effectively identical counts of hemoglobin RNA by EPO or SM-mediated differentiation supporting the HPLC findings. Finally, we integrated iEPOR into an iPSC line and devised a consolidated differentiation strategy from iPSCs to HSCs and, ultimately, RBCs (Fig 1C). This proves our approach serves as an *ex vivo*, EPO-independent RBC production strategy that closely mimics native EPOR signaling by surface biomarkers, relevant protein output, and transcriptional profile. Our iEPORs provide a starting point to address one of the greatest cost barriers to meeting the global demand for blood by transforming *ex vivo*-manufactured RBCs into a scalable and renewable source for transfusion medicine.



288. Non-Viral Engineering of Primary Human Macrophages Using a cGMP-Compliant Electroporation Platform

Ashley B. Strickland, Cornell Allen, Camille Ciesliga, Jason Papademetriou, Lauren E. Unsworth, James Brady

MaxCyte Inc., Rockville, MD

Macrophages are highly plastic innate immune cells that can exhibit pro- or anti-inflammatory effector functions depending on their activation state. For this reason, macrophages can be used for various therapeutic applications ranging from cancer treatment to regenerative medicine. To engineer macrophages for these purposes, biomolecules or other genome editing tools must be delivered into these cells. Electroporation, also known as electropermeabilization, is an effective non-viral transfection method that utilizes an electric field to facilitate the passage of molecules across biological membranes. Here, it is demonstrated that highly efficient delivery of mRNA, DNA and CRISPR ribonucleoproteins (RNPs) into primary human macrophages can be achieved using the MaxCyte® ExPERT GTx™ system - a cGMP-compliant electroporation platform. In fact, use of the ExPERT GTx™ to transfect macrophages with mRNA or SIRPα-targeting CRISPR RNPs

resulted in efficiencies greater than 90% with minimal impact on cell viability. This system also enabled delivery of DNA, ranging in size from 2.5-kb to 9.2-kb, into macrophages with transfection efficiencies between 60% and 90%. These efficiencies were inversely related to plasmid size and were accompanied by a dose-dependent decrease in cell viability. Despite this, electroporation was determined not to directly impair migration, activation/polarization, or phagocytosis in viable macrophages. For further proof-of-concept, the ExPERT GTx™ was also used to generate clinically relevant chimeric antigen receptor (CAR)-expressing macrophages targeting the tumor antigen CD19. These CAR macrophages were found to be functional and highly effective at eradicating $CD19^+$ target cells. Together, these demonstrate that the MaxCyte ExPERT GTx™ electroporation platform can be used to engineer macrophages for use in cell therapy applications.

AAV Manufacturing III

289. Optimizing for > 95 % rAAV Empty Capsid Removal at Process Scale with Anion Exchange Chromatography Using a Mechanistic Model

Vijesh Kumar, Ohnmar Khanal, Mi Jin
Spark Therapeutics, Philadelphia, PA

The current recombinant adeno-associated viral vector (AAV) system produces empty capsids in greater quantities than the genome-containing capsids, making the large-scale separation of empty and full capsids challenging. Anion exchange chromatography (AEX) is currently the industry standard for removing empty capsids. Despite the recent surge in published AEX methods for removing empty capsids, companies still struggle to find a robust and scalable AEX method that withstands load composition and quality variation, often seen over the product development lifecycle. Furthermore, an AEX process that requires peak fractionation is difficult to scale up and transfer, rendering the process less desirable. We present here a computational strategy, namely mechanistic chromatography modeling, which is based on the well-established and detailed fundamental understanding of chromatography, to achieve >95% removal of empty capsids. Our detailed model accurately describes the interaction between AAVs and the stationary phase. Given that AAVs are large and do not wholly penetrate the commercially available AEX resins designed for use with smaller proteins, we predicted the partial penetration of AAVs by modeling the resin particle as a core-shell bead using an advanced general rate model. This geometric representation was suitable for the widely used POROS XQ resin. Our mechanistic model describes the interaction between neighboring AAV capsids on the resin surface as well as the resin-AAV interaction using the comprehensive colloidal isotherm. These two interaction terms were the only two parameters calibrated using breakthrough curves at various experimental conditions, ensuring that our model was not over-fitted. The isotherms account for the capsid radius and were calibrated separately for empty and full capsids, allowing for the selective optimization of full capsid retention. The distinct isotherms for empty and full capsids showed that employing a higher ionic strength buffer during loading and a lower ionic strength buffer during washing leads to >95% removal of empty capsids with the optimal retention of full capsids. We will demonstrate the further utility of the model by providing model-derived solutions for empty capsid removal in flowthrough, step, and gradient-based operations.

290. A Proprietary HEK293 AAV Production System Can Achieve Greater Than 50% Full Capsids with Greater Than 1e15vg/L at Harvest Enabling Scalable Chromatography-Based Polishing with High Yield and Purity

Matt Edwards[1], Michael White[1], Paul Freeman[1], Esther Aribilola[1], Matt Bennett[1], Patrick Bresnahan[1], Rong Cong[1], Ramin Kamran-Sami[1], Ghazi Abuzarifah[2], Graham Lilley[2], Dan Linnehan[2], Shahrzad Parker[2], Jordan Shufro[2], Rob May[3]
[1]Process Science, Affinia Therapeutics, Waltham, MA,[2]Analytical Science, Affinia Therapeutics, Waltham, MA,[3]Technical Operations, Affinia Therapeutics, Waltham, MA

Manufacturing of AAV gene therapies has long been a challenge which has only increased with the discovery of novel AAV variants. Innovative solutions to vector production are needed to realize the potential of AAV gene therapy. The current barriers to AAV manufacturing are low vector yields, lack of scalability in downstream polishing and meeting the predefined CQA thresholds for percentage of full particles, detectable rcAAV, host cell DNA (hcDNA), host cell protein (HCP), and residual plasmid (rPlasmid) limits. These contribute to the high cost of goods and regulatory uncertainty. Varied efforts across the CGT field have attempted to address these challenges through novel cell culture additives, media and transfection optimization, plasmid engineering and cell line development. Here we discuss an Affinia Therapeutics developed HEK293 suspension manufacturing platform that meets these requirements more fully than any reported to date. Using this platform, we have achieved harvest titers equal to or greater than 1e15vg/L across numerous WT and engineered AAV capsids. In addition, we commonly see 40-60% full particles at harvest for WT and engineered capsids which enables chromatographic polishing that delivers high enrichment (>90% full) without the use of ultracentrifugation (UC). Analysis of vector quality shows an equal or increased reduction in hcDNA, HCP, rDNA, rcAAV and potency compared to other AAV production platforms. This platform is flexible and can be readily implemented into standard AAV manufacturing processes across serotypes, payloads, and scales.

291. Identification and Control of AAV Charge Heterogeneity through Optimized Bioreactor Operation

Thomas Thiers, Alex Meola, Eli Wiberg, Sarah Laughlin, Luke Mustich, Guang Yang, Qi Zhang, Iraj Ghazi, Lucas Govert, Alejo Zacarias, Danny Lassiter, Eugenia Fandunyan, Richa Jaiswal, Rob Horton
Oxford Biomedica (US) LLC, Bedford, MA

Small charge differences in recombinant adeno-associated virus (rAAV) capsids are often exploited with anion exchange (AEX) chromatography to separate empty and full capsids. During the development of this

step for rAAV9, we discovered that capsids are not uniform in charge and can be found in various AEX fractions eluted at variable salt concentrations. The observed broad distribution of capsids suggested a heterogenous charge profile. To therefore understand the root of this heterogeneity, we investigated the production time in the bioreactor and where in the system the heterogeneity is greatest (intracellular or extracellular). HEK293 cells were transfected using a transient transfection platform and incubated for a duration of either 3 or 6 days before harvest. In one arm, a detergent lysis was performed at day 3 post-transfection and the full lysate was collected. For the other arm, the cell and supernatant were separated by centrifugation at days 3 and 6 post-transfection. The cell pellets were resuspended and lysed to extract intracellular capsids. After harvest, all feed-streams were purified using affinity chromatography followed by an AEX purification step to enrich for full capsids and examine the observed charge distribution. We found that (i) empty capsids had more heterogeneous charge distributions than DNA-containing capsids and (ii) that surprisingly the intracellular capsids were more negatively charged than extracellular capsids. Further investigation revealed that extracellular capsids were composed of less empty capsids which helped to explain their more uniform charge distribution. LC-MS/MS of the various feed-streams unexpectedly showed that intracellular capsids had more asparagine N57 deamidation right out of the bioreactor than extracellular capsids and increased levels from day 3 to day 6 post-transfection. A linear correlation exists between N57 deamidation and capsids lost to the AEX strip, demonstrating the N57 deamidation was responsible for the increase in charge heterogeneity observed through AEX mass balance. Deamidation at N57 in the product was reduced by ~2x through AEX for all feed-streams tested, however, that step was not sufficient in completely removing deamidated species. Lastly, we found that potency, as measured by GFP expression and fluorescence, significantly dropped from day 3 to day 6 post-transfection for all feed-streams. We demonstrated that deamidation at N57 occurs within the bioreactor and is responsible for charge heterogeneity witnessed during the AEX step. While AEX is capable of removing some deamidated species generated through the bioreactor process, harvest time is a more significant parameter that can be controlled to decrease N57 deamidation and increase the potency of rAAV products. Finally, we show scale-up data demonstrating that it is possible to further reduce N57 deamidation and increase potency without significantly sacrificing titer by shortening the bioreactor duration to two days post-transfection.

292. Next Generation Adeno Helper Plasmids and Helper Stable Cell Lines for Efficient AAV Vector Production

Jiten Doshi[1,2,3], Emma Couto[1], Jillian Staiti[1], Luk H. Vandenberghe[1,2,3], Nerea Zabaleta[1,2,3]

[1]Mass Eye and Ear Infirmary, Boston, MA,[2]Harvard, Boston, MA,[3]Broad Institute of MIT and Harvard, Boston, MA

Recombinant adeno-associated viral vector (rAAV) has emerged as a preferred vector for therapeutic gene delivery due to advantageous features including non-pathogenicity, tunable tissue targeting, and durable transgene expression. The expanding number of AAV gene therapy products attaining regulatory approval and the substantial volume of ongoing investigational trials centered around AAV-mediated therapeutic approaches have led to a heightened demand for AAV vectors. Meeting this demand requires efficient, scalable production methods. The preferred method for AAV production involves transient transfection of three plasmids in HEK293 cells. These plasmids consist of packaging Rep and structural Cap genes, adenoviral helper genes, and an ITR-flanked transgene. Despite the method's advantages, there remains a notable unmet need for qualitative and quantitative improvements, especially in cost reduction. In this context, our investigation focused on the role of adenoviral helper genes in AAV production. Prior studies have recognized adenoviral E1A, E1B, E2A, E4orf6, and VA RNA I genes essential for production. Standard helper plasmids such as pDF6 and pALD-X80 harbor substantial additional adenoviral sequences, both coding and non-coding, which raise potential safety issues and inflate transfection costs. Here, we show that AAVs are produced with low efficiency as titers drop 7-fold when using only the essential genes in comparison to a standard helper, pDF6. Investigation of >15 adeno genes revealed that E2A, VA RNA I, and the previously overlooked 22k/33k genes are enough to restore viral vector titers equivalent to those obtained using the pDF6. Absence of E4orf6 gene did not impact production titers and hence proved dispensable. Armed with this knowledge, we engineered next generation helpers that were 5-7kb smaller yet achieved similar production titers across scales (up to 500mL), serotypes and transgenes to standard helpers. Moreover, stable cells were generated through random integration, incorporating both inducible E2A and 22k/33k genes, along with constitutive VA RNA I gene. The stable helper polyclonal cells exhibited similar viability and yielded titers 2-7 times lower than those achieved through the transient transfection method, pending the selection of monoclones with optimized production capabilities. In summary, we identified E1A/B, E2A, VA RNA I, previously unknown 22k/33k as the minimal adeno helper gene set for AAV production and generated shorter helper plasmids that improve safety and reduce cost yet match standard helpers in both qualitative and quantitative AAV production metrics. Additionally, our stable helper cell lines establish feasibility for streamlined AAV production requiring at least one less plasmid for transfection.

293. Development of a Powerful AAV Production Platform by Leveraging Comprehensive Design-of-Experiment & High-Throughput Automated Bioreactors

Érica A. Schulze[1], Christophe Chevé[2], Thomas Jeffery[3], Paul Giroud[4], Maina Kerbrat[5], Frederique Marmegalloo[5], Timo Schmidberger[1], Noushin Dianat[5], Emilie Audran[6], Veronique Blouin[2], Oumeya Adjali[2], Laurence Guianvarc'h[2]

[1]Sartorius Stedim Biotech GmbH, Göttingen, Germany,[2]Nantes Université, CHU de Nantes, INSERM, TaRGeT - Translational Research in Gene Therapy, Nantes, France,[3]Sartorius Stedim UK Ltd, London, United Kingdom,[4]Polyplus, IIIkirch, France,[5]Sartorius Stedim France SAS, Aubagne, France,[6]CAPACITES SAS, Nantes, France

Recombinant adeno-associated virus (rAAVs) vectors are a leading gene delivery platform. Seven rAAV-based gene therapies have been approved to date, and over 1000 clinical trials are underway worldwide. Despite advances in the clinic, rAAV vector manufacturing remains

challenging for therapeutic applications requiring high systemic doses due to the high productivity required and the high cost of goods (DNA as GMP starting material, affinity resin...). Efficient and scalable production of rAAV is critical to enable lower drug product cost and easier commercialization for gene therapy applications. Therefore, the aim of the project was to optimize a transient transfection platform of suspension HEK-293 for rAAV production using a Design-of-Experiment (DoE) approach to study the interaction of multiple factors (culture medium, pH, stirring,...) and its impact on CQAs. Experiments made use of DoE methodology in MODDE® to design, optimize, and predict process parameters. rAAVs were produced in Expi293F cells (Gibco®) with a three-plasmid system in a 24-way Ambr®15. At harvest, the encapsidated AAV genomes were quantified by qPCR and ddPCR and the total particles by ELISA. A Western Blot was performed to complete the analytical characterization, as well as cell culture media metabolite consumption was used for profile analysis. The impactful factors such as temperature, agitation, pH, culture medium, harvest time, and plasmid quantity were investigated using rAAV2-GFP as a representative vector to optimize virus production. The upstream process was developed in 3 phases: The first phase focused on cell culture media (HEK TF, ViP NB and NX) and initial process parameters, as pH and agitation. The second phase focused on the feeding strategy and different concentration ranges of recombinant Albumin (recombumin®) to boost AAV production. The third phase focused on optimizing the transfection step using FectoVIR®-AAV (Transfection cell density, DNA amount, FectoVIR-AAV:DNA ratio). Using this step wise approach supported by DoE to guide optimization of process parameters, we were able to significantly increase the productivity of the process in the Ambr®15. The optimized process was then scaled up into 2L bioreactors. The association of DoE with high throughput automated mini bioreactors should become a standard in the viral vector industry to speed up process optimization and to support process intensification needed to answer the ever-increasing necessity for higher productivity and quality of our production systems.

294. Short-Term and Large-Scale Production of High-Quality Recombinant Adeno-Associated Virus Using a Zonal Ultracentrifugation

Mikako Wada

Division of Molecular and Medical Genetics, Center for Gene and Cell Therapy, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

Background: High-yield purification of recombinant adeno-associated virus (rAAV) is required for the safe and efficient transduction in rAAV-based *in vivo* gene therapies because rAAV products contain full-genome (functional), intermediate, and empty rAAV particles as well as host-cell proteins. However, the conventional method for rAAV purification at the laboratory scale (up to 180 mL) is based on two-day ultracentrifugation with cesium chloride (CsCl) density-gradient protocol, limiting large-scale purification of functional rAAV for clinical usage. In addition, prolonged incubation in CsCl density-gradient ultracentrifugation reduces rAAV infectivity. In this presentation, we show a short-term purification method for large-scale rAAV production at high purity using a zonal ultracentrifuge. Methods: The rAAV-ZsGreen1 was harvested from culture supernatant without cell lysis 5 days after plasmid transfection. Large-volume rAAV-containing culture media (up to 1,000 mL) and escalating densities (2 or 4 steps) of CsCl solutions were separately placed in the zonal rotor, respectively. Following large-scale density-gradient ultracentrifugation of rAAV, the samples were fractionated automatically. These samples were evaluated for rAAV genome copies by quantitative PCR, rAAV capsid protein amounts by western blotting, and ZsGreen1 transduction efficiency by flow cytometry. The ratio of empty to full capsids was measured by mass photometry, analytical ultracentrifugation (AUC), and transmission electron microscopy (TEM) with phosphotungstic acid stain. The genomic DNA regions packaged in rAAV particles were detected by droplet digital PCR (ddPCR) using various primers and probes targeting the whole vector genome. Results: We performed 2-step CsCl density-gradient ultracentrifugation with a zonal rotor, allowing for a large volume (900-1000 mL) of rAAV purification as well as shorter ultracentrifugation for 4-5 hours with a steeper density-gradient in the area of rAAV fractions, as compared to the conventional method of 4-step density-gradient (300 mL) for 10 hours. rAAV capsid proteins were detected in 2 of the fractions, one of which also contained the peaks of rAAV capsid, genome copies, and ZsGreen1 transduction, demonstrating a separation between functional full-genome and non-functional empty rAAV particles. The intermediate particles would be included between these fractions. We determined full and empty ratios by mass photometry and a single AUC peak with separate sedimentation coefficients for fractions of full-genome (about 90S) and empty (about 60S) vectors, demonstrating a high-purity separation of rAAV. Interestingly, all genome regions were detected at similar levels in full-genome rAAV by ddPCR; however, ITR copies were 2.5-2.9 fold higher than the ZsGreen1 copies in the empty particles, suggesting that ITR fragments can be packaged in 'empty' particles. In addition, we purified large-volumes of rAAV (up to about $5x10^{14}$ v.g.) after removing small molecules and increasing concentration by simple pretreatment. Conclusion: We developed a 2-step density-gradient ultracentrifugation method with a zonal rotor in 4-5 hours, allowing for the large-scale purification of functional full-genome rAAV. We also improved the fractionation system for short-term purification. Our large-scale rAAV purification method would be applicable for considerable *in vivo* experimentation and clinical investigation. Furthermore, in respect to Good Manufacturing Practice (GMP) viral vector manufacturing, we improved the in-line system that should detect the rAAV during fractionation and shorten the time after zonal ultracentrifugation. We are also preparing for cleaning validation and regulatory compliance for GMP manufacturing of rAAV, to avoid cross-contamination.

295. Generation of a Stable High-Titer Production Cell Line for Therapeutic AAV Vectors

Stefan Seeber[1], Pedro A. Moura[2], Hendrik Knoetgen[3], Andreas Schaubmar[4], Nicole Schueler[1], Alina Schneider[1], Julia Hoelper[5], Nicole Faust[5], Ines Gil-Goncalves[5], Michael Muehle[5], Michelle Hussong[5], Sven Markert[4]

[1]Cell Technologies, Roche Diagnostics GmbH, Penzberg, Germany,[2]Spark Therapeutics, Philadelphia, PA,[3]Hoffmann La Roche, Basel, Switzerland,[4]Roche Diagnostics GmbH, Penzberg, Germany,[5]Cytiva, Cologne, Germany

AAV-vector based gene transfer is becoming routine in gene therapy. To meet the growing material demands, the use of stable cell lines for AAV-vector production is gaining attention in industry and science. Major advantages are scalability and high reproducibility paired with lower cost of goods compared to triple-transfection based processes. In this case study we describe the generation of a stable, high titer HEK293-based producer cell line for AAV-vectors using a therapeutically relevant transgene and an engineered capsid (both disclosed at the conference), which was performed in close collaboration between Roche, Spark and Cytiva (formerly Cevec Pharmaceuticals). This proof-of-concept study aimed to demonstrate the utility and effectiveness of stable ELEVECTA producer cell lines for manufacturing of therapeutic AAV-vectors. We will present the workflow for generating a pool and subsequently single cell clones, pool and clone characterization in ambr15 including the results of a 90-day stability study for selected clones.

AAV Vectors - Non-Human Primates and Large Animal Models

296. Neuron Regeneration Drives Functional Recovery from Severe Stroke Following Intracranial Administration of NXL-001 Gene Therapy in Non-Human Primates

Anna Kabanova[1], Lydia Wan[2], Gabriel Ramirez Garcia[2], Douglas J. Cook[2], Jie Xu[1], Andrew Grande[3], Chong Chen[1], Russ Addis[1]

[1]NeuExcell Therapeutics, Philadelphia, PA,[2]Queen's University, Kingston, ON, Canada,[3]University of Minnesota, Minneapolis, MN

Ischemic stroke is a leading cause of acquired adult disability with a high mortality rate worldwide. Stroke survivors often experience long-term disabilities due to neuronal cell loss caused by ischemic injury. Despite current advanced treatments that focus on the re-establishment of blood flow and neuroprotection, therapies that replenish the substantial neuronal loss and restore lost brain functions remain elusive. We developed a platform of neuro-regeneration in which direct in vivo overexpression of a single proneural transcription factor, NeuroD1, converts endogenous astrocytes into functional neurons. We now present an update on NXL-001, an AAV9 gene therapy delivering NeuroD1 to astrocytes via intracranial injection for the treatment of ischemic cortical stroke. To investigate NXL-001 treatment effect on neuronal regeneration and recovery of motor deficits in a clinically relevant NHP model of stroke, we induced transient cerebral ischemia in macaques by temporarily occluding the M2 branch of the middle cerebral artery. This model results in a large stroke with severe neurological and motor deficits as assessed by the Nonhuman Primate Stroke Score (NHPSS) and modified Rankin Score (mRS). Animals that received injections of NXL-001 in the peri-infarct area two weeks post-stroke showed significant functional improvement within 6-8 weeks post-treatment when compared to non-treated animals, with nearly complete recovery observed by 6 months post-treatment. Diffusion Tensor Imaging (DTI) of white matter projections demonstrated restoration of the cortico-spinal tract. Post-mortem histological analysis confirmed that newly formed neurons in the motor cortex sent axonal projections along the cortico-spinal tract, further validating the observed functional improvement. NXL-001 is on track to reach the clinic in 2024.

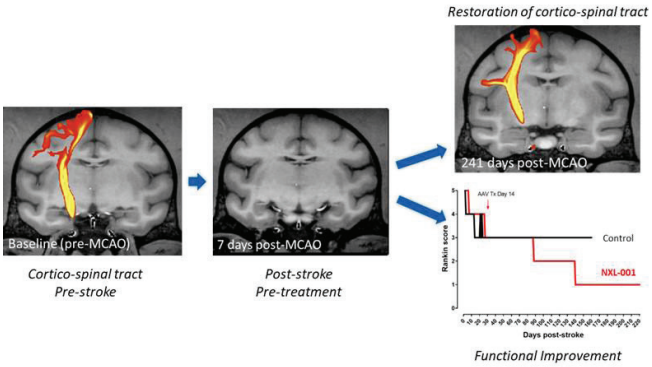


Figure: MRI Diffusion Tensor Imaging shows white matter tracts of the cortico-spinal tract (CST) at baseline and 7 days post middle cerebral artery occlusion (MCAO). NHPs treated at 2 weeks post-MCAO exhibited restoration of the CST and functional recovery (as measured by the modified Rankin score, with 5 = severely disabled and 0 = no disability).

297. AAV Intein Retinal Gene Therapy for Stargardt Disease is Effective and Safe in Large Animal Models

Ivana Trapani[1,2], Rita Ferla[3], Eugenio Pugni[1], Mariangela Lupo[1], Andrea Perota[4], Carlo Gesualdo[5], Settimio Rossi[5], Francesca Simonelli[5], Maria Laura Bacci[6], Cesare Galli[4], Ali Memon[3], Naveed Shams[3], Alberto Auricchio[1,2,3]

[1]Telethon Institute of Genetics and Medicine, Pozzuoli, Italy,[2]Dept. of Advanced Biomedical Sciences, University of Naples "Federico II", Naples, Italy,[3]AAVantgarde Bio, srl, Milan, Italy,[4]Laboratory of Reproductive Technologies, Avantea, Cremona, Italy,[5]Eye Clinic, Multidisciplinary Department of Medical, Surgical and Dental Sciences, University of Campania Luigi Vanvitelli, Naples, Italy,[6]Dept. of Veterinary Medical Sciences, University of Bologna, Ozzano dell'Emilia, Italy

Stargardt disease type 1 (STGD1) is the most common form of inherited macular degeneration, for which no therapeutic options are available. STGD1 is due to bi-allelic loss-of-function mutations in *ABCA4* which

has a large coding sequence that does not fit into adeno-associated viral (AAV) vectors, the most suitable vectors for retinal gene therapy. We have recently developed a gene therapy approach for STGD1 which relies on the use of two AAV vectors each encoding for one of the two halves of the ABCA4 protein flanked by split-inteins, that catalyze seamless joining of the two ABCA4 half proteins following AAV administration. Based on the finding that subretinal administration of AAV-*ABCA4*-intein vectors results in precise reconstitution of the full-length ABCA4 protein and in therapeutic efficacy in *Abca4*-/- mice, we set to evaluate both the efficiency and the safety of subretinal administration of AAV-*ABCA4*-intein vectors in relevant large animal models. Given the similarity between the swine and human retina, we have generated ABCA4 knock-out (KO) pigs, via *ABCA4* gene editing followed by somatic nuclear transfer. While KO pigs do not show obvious signs of photoreceptor degeneration up to 15 months of age, they accumulate lipofuscin in the retinal pigmented epithelium similarly to Abca4-deficient mice and STGD1 patients, and this was reduced upon subretinal delivery of AAV-*ABCA4* intein vectors. We then confirmed the safety of subretinal administrations of AAV intein ABCA4 vectors in a 13-week long toxicokinetic, ocular safety and expression study in non-human primates (NHP) which have the macula, similarly to humans. BaseScope analysis showed nearly total photoreceptor co-expression of mRNAs encoding both ABCA4-intein halves across an extended NHP retinal region. Overall, our data support both the efficacy and safety of AAV-*ABCA4*-intein vectors in relevant large animal models, providing important insights towards the clinical translation of this platform for gene therapy of STGD1.

298. Tissue De-Targeting Abrogates Hepatotoxicity and Complement-Related Thrombotic Complications Associated with High-Dose AAV Gene Therapies

Andrew D. Baik, Nicholas Giovannone, Nyanza Rothman, Chun Wai Yip, Poulami Samai, Leah Sabin, Laura Johnsen, Christos Kyratsous, Katherine Cygnar

Regeneron Pharmaceuticals, Tarrytown, NY

Many commonly used AAVs in gene therapies have extremely strong tropism to liver compared to other tissues. As a direct consequence, AAV gene therapies often require high doses (>1e14vg/kg) to achieve therapeutic efficacy in key peripheral tissues (*e.g.* muscle or brain). However, these doses have been associated with hepatotoxicity in humans and non-human primate models. In addition, excessive complement activation leading to thrombotic microangiopathy has also been observed in medium and high-dose systemic AAV clinical trials. Several factors have been suggested to be drivers of toxicity, such as activation of the alternative complement pathway by high capsid load in the bloodstream, activation of classical complement by anti-AAV antibodies, high capsid uptake, and transduction itself leading to cellular stress. However, the interactions of these factors remain poorly studied, especially in relevant toxicological models of AAVs. To parse the relative causes of AAV-induced liver and thrombotic complications, we conducted a three-week high-dose AAV toxicology research study in cynomolgus macaques comparing the impact of AAV capsid load, liver transduction, and mild pre-existing anti-AAV antibodies. To separate the contribution of AAV capsid load from transduction, we took advantage of an AAV9 capsid mutation, W503A. The W503A mutation disrupts capsid binding to galactose and profoundly de-targets many peripheral tissues and in particular, liver, which exhibits ~100 fold reduced transduction compared to wild-type (WT) AAV9. We dosed seronegative (nAb <1:5) and mild seropositive (1:10-1:20) macaques with 2e14vg/kg of either WT AAV9 or AAV9 W503A capsids containing CAG GFP genome (n=4 per group). As expected from previous reports, we found that macaques treated with WT AAV9 exhibited severe hepatotoxicity, with ALT elevations of ~50-100 fold 3 days post-AAV administration. Additionally, markers of complement activation (Bb, C3a, sC5b-9) peaked at Day 3, along with severe, but transient, thrombocytopenia (platelet counts <200,000/uL). Monkeys with the most severe thrombocytopenia displayed evidence of hemolysis and mild, transient impairment of kidney function. Notably, these findings were highly consistent between monkeys regardless of seropositivity, suggesting that either pre-existing immunity has a minor impact on overall toxicity at such high doses, or that these pre-existing titers were too low to have a meaningful effect. Conversely, animals that received AAV9 W503A exhibited almost complete absence of dose-related toxicities, with only mild ~2 fold ALT elevations, no changes in platelet counts, and significantly reduced markers of complement activation (Bb, C3a, sC5b-9). This data suggests that AAV exposure alone is insufficient to trigger excessive complement activation and transduction-related liver injury may be a more significant contributor to high-dose related AAV9 toxicities. We posit that improving AAV targeting, rather than viral load reduction alone, may significantly improve the safety profile of AAV gene therapies.

299. Toxicity Profile of Clade F Vectors Administered Intravenously in Nonhuman Primates

Juliette Hordeaux, Elizabeth L. Buza, George Buchlis, Sushobhana Bandyopadhyay, Alexandra L. Patterson, Amber Hamilton, Cecilia D. Dyer, Chelsea K. Wallace, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Gene therapy vectors based on clade F recombinant adeno-associated viruses (AAVs), such as AAV9, are the current tools of choice for delivering therapeutic transgenes to the central nervous system, skeletal muscles, or heart. Many programs using high doses of AAV have faced test-article-related serious adverse events, which, in most cases, were not predicted by nonclinical safety studies. We and others have reported the utility of nonhuman primate (NHP) models that recapitulate many hallmarks of known serious toxicity events such as liver failure, acute thrombocytopenia, and sub-acute transgene-dependent immune myocarditis. The goal of this work was to investigate severe toxicity events in NHPs following systemic AAV administration. We conducted a meta-analysis including all NHPs that received intravenous AAV at our institution with available electronic records. The cohort represents 448 NHPs, with 280 males and 168 females. The majority of NHPs were macaques, with 248 rhesus and 175 cynomolgus macaques. Marmosets (n=12), African green monkeys (n=4), and Squirrel monkeys (n=9) were used infrequently. The doses administered ranged from 2E12 genome copies (GC)/kg to 2.5E14 GC/kg. Most animals received low to moderate doses (2E12 GC/kg to 4.5E13 GC/kg, n=363), whereas 85 animals received doses ≥5E13 GC/kg (high dose). Among the 448 NHPs that received intravenous AAV, we observed severe test-article-

related toxicity leading to emergency necropsy in 13 animals (3% of the total, 15% of the high-dose cohort). Toxicity was acute (within the first two weeks) in 10/13 animals. All the acutely affected animals and 2/3 of the non-acutely affected animals had received high AAV doses (≥5E13 GC/kg). Among all the toxicity cases, the vector was either clade F or engineered clade F, which were the only serotypes assessed at high doses in our facility. The promoters were mostly ubiquitous (n=12) or neuron-specific (n=1). The transgenes were enhanced green fluorescent protein (n=6), therapeutic human codon-optimized transgenes (n=6), or a capsid library (n=1). The vectors were produced via transient transfection of adherent human embryonic kidney (HEK293) cells, and most lots were purified via AAVX affinity resin. Severe acute toxicity was characterized by a rapid course of transaminitis with thrombocytopenia three days after administration, often followed by generalized edema (capillary leakage), hyberbilirubinemia, and/or coagulopathy that culminated in an unscheduled necropsy. The average time point for humane euthanasia was 5.3 days post-administration. The liver was the organ with the highest levels of vector GCs and was the main organ affected by toxicity, as assessed by bloodwork and histopathology. To elucidate the factors characterizing the most severe events, we examined study endpoint livers from NHPs that succumbed to toxicity via histopathological and single-nucleus RNA sequencing analyses. Liver enzyme elevations corresponded to hepatocellular necrosis. Thrombocytopenia corresponded to sinusoidal platelet microthrombi and sinusoidal endothelial injury identified via immunohistology, single-nucleus RNA sequencing, and a blood biomarker. In conclusion, our retrospective meta-analysis of systemic AAV administration in NHPs indicates that doses between 2E12 GC/kg and 4.5E13 GC/kg were well tolerated in 99.7% of the animals (n=362/363), with none of the NHPs developing severe acute toxicity in this dose range. This data set provides key translational insights for AAV gene therapy and establishes a potential threshold for severe acute liver and endothelial toxicity in NHPs receiving an AAV dose ≥5E13 GC/kg.

300. AAV-ARSA Mediated Gene Replacement for the Treatment of Metachromatic Leukodystrophy

Shyam Ramachandran[1], Jeffery Ardinger[1], Jie Bu[1], MiAngela Ramos[1], Lilu Guo[2], Dhiman Ghosh[2], Mahmud Hossain[2], Swathi Ayloo[1], Erik Wischhof[1], Johanna Adams[1], Yuxia Luo[1], Emily Crosier[1], William McCarty[2], SHelley Nass[1], Catherine O'Riordan[1], Martin Goulet[1], Christian Mueller[1]

[1]Sanofi, Waltham, MA,[2]Sanofi, Cambridge, MA

Metachromatic leukodystrophy (MLD) is an autosomal recessive neurodegenerative disorder caused by mutations in the arylsulfatase A (*ARSA*) gene, resulting in lower sulfatase activity and the toxic accumulation of sulfatide in the central and peripheral nervous system. Children account for 70% of cases and become progressively and severely disabled with death occurring within 10 years of disease onset. The standard of care is focused on symptomatic management and psychological support, with a high unmet need in the patient community. We present an AAV-mediated gene replacement strategy to treat MLD and report therapeutic benefit in MLD mice, and meaningful ARSA expression and biodistribution in the CNS of non-human primates (NHPs). First, we report the discovery of a novel capsid AAV.GMU1, demonstrating superior biodistribution and transgene expression in the CNS of NHPs, compared to a gold-standard CNS-tropic capsid. Second, we demonstrate that AAV-treated MLD mice show persistent, normal levels of sulfatase activity in the brain and spinal cord and a concomitant reduction in toxic sulfatides. Treated mice also show a near complete absence of MLD-associated pathology and reversal of abnormal hearing phenotype, indicating that a single administration of AAV.GMU1-h*ARSA* is well tolerated, long lasting and highly effective in MLD mice. Lastly, we present a dose-range finding pharmacology and safety study in juvenile cynomolgus monkeys, treated with AAV.GMU1-h*ARSA*. NHPs showed dose-dependent vector, mRNA and protein biodistribution in the CNS, with therapeutically meaningful h*ARSA* expression in >90% of the brain. NHPs presented no clinical signs (functional or behavioral) for the duration of the study, indicating that a single administration of AAV.GMU1-h*ARSA* by iCM was well tolerated and results in therapeutically meaningful ARSA protein levels in the CNS. In summary, we propose AAV.GMU1-h*ARSA* mediated gene replacement as a clinically viable approach to achieve broad and therapeutic levels of ARSA in the CNS and PNS.

301. Applying Artificial Intelligence to Multi-Property Optimization of AAV Capsids for Neuronal Gene Delivery

Mugdha Deshpande, Daniel Bloodgood, Barbara Diaz-Rohrer, Kailyn Looby, Sarah Hilton, Sam Chen, Sam Sinai, Kathy S. Lin, Jamie C. Kwasnieski, Adrian Veres, Eric Kelsic

Dyno Therapeutics, Watertown, MA

Previously we reported an engineered AAV capsid that enables highly efficient transduction of neurons upon IV administration in adult cynomolgus and African green monkeys, with a pan-brain delivery profile broadly applicable for neurological gene therapies. Here we use two approaches combining artificial intelligence and *in vivo* NHP experiments to modify its sequence, significantly advancing the state of the art by improving neuronal delivery and liver detargeting. For efficient IV administration of neurological gene therapies, AAV capsids can be engineered to direct delivery to the CNS and away from the liver. As such, our engineered starting capsid is well-suited for neurological gene delivery, transducing up to 29% of all cells in specific brain regions, 14% of upper motor neurons, 56% of lower motor neurons and 5-20% of neurons across most CNS regions in adult cynomolgus monkeys with a 1e13 vg/kg IV dose, while detargeting the liver 10x vs AAV9, and with productivity similar to AAV9. With machine-learning models trained on *in vivo* NHP data, we evaluated billions of capsid variants for improved CNS delivery and liver de-targeting, filtering out poor producers. In contrast to random substitution and insertion libraries, the design strategy was fully synthetic, allowing sequence edits to be introduced at any location on the capsid, both within and outside of the surface exposed variable regions. Capsid variants were more than 4 edits away from the starting capsid sequence on average. ~100k capsid variants were synthesized, with 88% of capsids capable of successfully packaging vector genomes. Measuring *in vivo* delivery properties in two adult cynomolgus monkeys, top variants exhibited above 5x higher CNS delivery combined with 3x liver detargeting relative to

the starting capsid. CNS transduction was significantly improved pan-brain for transgenes expressed from both constitutive (Cbh) and neuronal (hSyn) promoters, including in deeper brain regions such as basal ganglia and brainstem. In a parallel library tested in two adult cynomolgus monkeys, we designed capsids to enable highly improved CNS transduction using a design strategy that differed dramatically from the approach described above. While in both cases billions of capsid sequences were evaluated *in silico*, here we employed extensive computational cross-validation and human curation, narrowing our selections to only 10 designed capsids that were synthesized and tested, yet still yielding capsids with dramatic delivery improvement. Relative to the starting capsid, the best capsid was 8x enhanced for CNS delivery relative to the starting capsid, with a pan-brain delivery profile. Within the same pooled study this capsid outperformed the best external engineered capsids tested, especially in delivery to deeper brain regions. Single-nuclei RNA-seq of prefrontal cortex, motor cortex and putamen showed increased numbers of transduced neurons, astrocytes and oligodendrocytes, with rates similar to those from bulk NGS. This efficient design of next-generation capsids with highly optimized properties, surpassing those of all previously tested capsids, demonstrates the rapidly advancing combined power of *in vivo* data and artificial intelligence, while unlocking new potential to treat neurodegenerative and neurometabolic diseases.

302. Improving the Expression of AAV-Delivered Anti-HIV Broadly Neutralizing Antibodies in Nonhuman Primates

Michael Kuipa[1], Peter Koroma[1], Isai Leguizamo[1], Natalie Correa[1], Priya Dhole[1], Matthew R. Gardner[1,2]

[1]Division of Microbiology and Immunology, Emory National Primate Research Center, Atlanta, GA,[2]Department of Medicine, Division of Infectious Diseases, Emory University, Atlanta, GA

Background: Long-term expression of anti-HIV-1 broadly neutralizing antibodies (bNAbs) using adeno-associated virus (AAV) vectors may prevent and treat HIV-1 infection. However, AAV vectors expressing bNAbs induce immune responses that inhibit bNAb expression *in vivo*, limiting the study of their efficacy in nonhuman primates. Programmed cell death protein (PD)-1 is an immune checkpoint protein on T cell surfaces which inhibits T-cell activity upon binding to programmed cell death protein ligand (PD-L)1. We hypothesized that co-expression of PD-L1 on cells transduced with AAV.bNAb maintains bNAb expression by suppressing host anti-bNAb antibodies and T-cell responses. The long-term goal of this research is to develop a system that achieves sustained AAV-expressed bNAb concentrations in nonhuman primates. Methods: To evaluate our hypothesis, we conducted a nonhuman primate study consisting of five groups of six rhesus macaques each that received the following vectors: 1) AAV9.PD-L1; 2) AAV9.10-1074; 3) AAV9.PD-L1 and AAV9.10-1074; 4) AAV9.3BNC117; and 5) AAV9.PD-L1 and AAV9.3BNC117. Serum antibody concentrations were tracked for over 20 weeks for all animals and determined by gp120 ELISA. Host immune responses were assessed by anti-drug antibody ELISA and by ELISpot assay to determine B and T cell reactivity, respectively. Additionally, we evaluated the effect of AAV9.PD-L1 on immunomodulatory gene expression in PBMCs by RNAseq. Beginning at 20 or 30 weeks post AAV9 administration, all animals were challenged biweekly via low-dose, intrarectal challenge using SHIV-AD8 challenge virus stock. Protection from SHIV challenge was determined by monitoring plasma viremia by qPCR. Results: All six macaques that received AAV9.10-1074 and AAV9.PD-L1 had sustained concentrations of 10-1074 throughout the study compared to four of six macaques that only received AAV.10-1074. Similarly, all six macaques that received AAV9.3BNC117 and AAV9.PD-L1 had sustained concentrations of 3BNC117 compared to three of six macaques that only received AAV9.3BNC117 at the time of this reporting. Macaques that had sustained bNAb expression for the duration of the study had lower anti-10-1074 or anti-3BNC117 antibodies and lower T cell reactivity to bNAbs compared to macaques that had declining bNAb expression. There were no differences in immunomodulatory gene expression in PBMCs between the groups. Lastly, all macaques that achieved sustained expression of 10-1074 resulted in significant protection from SHIV challenges. Macaques that received AAV9.3BNC117 are currently undergoing the challenge arm of the study. Conclusions: Co-expression of PD-L1 on cells transduced with AAV9.bNAbs improves the consistency of bNAb expression by suppressing host anti-bNAb responses in rhesus macaques. Thereby, we have developed a strategy to evaluate new AAV vectors in nonhuman primates without the interference of the host immune response.

Neurologic Diseases IV

303. Optimized Artificial miRNAs Delivered by rAAV9 Dramatically Improve Survival, Respiratory and Motor Functions of SOD1^{G93A}-ALS Mice

Fang Wan[1], Jinchen He[1], Hong Ma[1], Veena Kumanan[1], Jisun Lee[1], Debora PiresFerreira[1], Sha Zhu[2], Gabriela Toro[2], Lingzhi Zhao[2], Alisha Gruntman[1], Zuoshang Xu[3], Joan Shen[2], Guangping Gao[1], Jun Xie[1]

[1]Horae Gene Therapy Center, University of Massachusetts Chan Medical School, Worcester, MA,[2]NeuShen Therapeutics US, Inc., Lexington, MA,[3]Biochemistry & Molecular Pharm, University of Massachusetts Chan Medical School, Worcester, MA

Autosomal dominant mutations in superoxide dismutase 1 (*SOD1*) gene cause motor neuron degeneration and are linked to 10-20% of familial and 2% of sporadic cases of Amyotrophic Lateral Sclerosis (ALS), a fatal disease for which an effective treatment is urgently needed. While current treatments such as gene silencing using ASO or siRNA have shown limited clinical benefits with the need for repeated dosing, our study explores the use of Adeno-Associated Virus serotype 9 (AAV9) for the delivery of optimized artificial microRNAs (amiRs) targeting SOD1 in a single-doses treatment for ALS-SOD1. Our choice of a miR-33-based amiR scaffold in the design of SOD1-amiR is based on our previous studies demonstrating its gene silencing efficacy, reduced off-target activity, and fewer truncated AAV9 genomes, compared to conventional shRNAs and other scaffolds. We generated an AAV9 vector (AAV9-hSMN1-dual-amiR) using a promoter derived from the endogenous human survival motor neuron 1 (hSMN1) to drive the expression of two amiR-Sod1 embedded in the mouse and human miR-33 scaffold. The SOD1^{G93A} mice were injected by tail vein

at the dose of 1.0xe14 vg/kg at three different time points: day 60-68 (the asymptomatic stage), day 90 (non-motor symptomatic stage when mice start to lose body weight), and day 105-125 (the motor symptomatic stage). PBS-treated transgenic animals were used as controls. In comparison to a median survival of 117 days observed in PBS-injected $SOD1^{G93A}$ mice (n = 17), the median survival of the AAV9-hSMN1-dual-amiR treated mice (n = 17) was extended by 98.5 days when animals were injected at day 60-68.More importantly $SOD1^{G93A}$ mice treated at day 90 (n = 24) and day 105-125 (n = 29), showed a median survival extension of 39 and 22 days, respectively. We further investigated the $SOD1^{G93A}$ mice treated on day 60. *SOD1* levels measured on day 105 by qRT-PCR showed a substantial reduction in the brain stem (down by 46 ± 12%; $p< 0.0001$), the spinal cord (down by 35-45%; $P< 0.0001$) in $SOD1^{G93A}$ mice treated with AAV9-hSMN1-dual-amiR, compared to PBS-treated controls. Reduction of SOD1 expression in motor neurons was confirmed by immunofluorescent staining. *SOD1* levels in peripheral tissues such as liver, heart, quadriceps, and diaphragm from same animals were also reduced. Notably, diaphragm, crucial for respiratory function, showed a by ~90% reduction in SOD1 levels in mice treated with the AAV9-hSMN1-dual-amiR vector. To gauge respiratory function, ventilation was quantified using whole-body plethysmography on day 105, 45 days post-injection. When compared to age-matched litter mates, PBS-treated $SOD1^{G93A}$ mice showed hypercapnia in minute ventilation, peak expiration and inspiratory flows. In contrast, hSMN1-dual-amiR vector-treated $SOD1^{G93A}$ mice performed at the same level as the age-matched litter mates. Remarkably, neither rotarod nor grip tests showed any significant difference between the hSMN1-dual-amiR-treated group and age-matched healthy litter mates until day 190. Thus, a single intravenous injection of AAV9-hSMN1-dual-amiR vector significantly reduces SOD1 expression within CNS and non-CNS tissues, effectively preserves respiratory and motor functions, and extends survival by 98.5 days, 39 days, and 22 days when administered at the asymptomatic, non-motor symptomatic and motor symptomatic stages in $SOD1^{G93A}$ mice, respectively. These therapeutic outcomes, unprecedented in $SOD1^{G93A}$ mice, suggested the need for a clinical evaluation of this promising treatment strategy. G.G. and J.X. are Corresponding Authors

304. Reduction of Atxn2, a Therapeutic Target for Sporadic ALS, in Non-Human Primates Using a Novel, Intravenously Delivered AAV Capsid

Giridhar Murlidharan, Emily Grandell, Stephen Janack, Amanda Berry, Isabelle Guelin, Charles Gualtieri, Tyler Ironside, Elizabeth Scott, Cynthia Pryce, Bryan Mastis, Ramin Kamran Sami, Matt Edwards, John Reece-Hoyes, Cara West, Roberto Calcedo, Dharmendra Goswami, Sherry Cao, Lisa M. Stanek, Graham Lilley, Laura K. Richman, Charles F. Albright

Affinia Therapeutics, Waltham, MA

The lack of safe and effective strategies for gene delivery across the blood-brain barrier hinders development of therapeutic modalities such as RNAi for neurological disorders. We report the discovery of a novel adeno-associated virus (AAV) capsid ATC-134 that achieves widespread central nervous system (CNS) exposure and durable RNAi function after intravenous (IV)-dosing in non-human primates (NHPs). Polyglutamine (CAG) expansions of intermediate length (27-33 repeats) within the RNA-binding protein Ataxin-2 (Atxn2) gene are an epidemiological risk factor for amyotrophic lateral sclerosis (ALS), making Atxn2 reduction a target for sporadic ALS. Additionally, a growing body of literature links reduction of Atxn2 transcript to improved survival in preclinical models of ALS. To further explore this hypothesis, we built a miRNA-based therapeutic cargo against Atxn2 to assess safety-pharmacology of ATC-134 in NHPs. First, we used bioinformatics to identify favorable siRNA sequences and then screened these molecules, in human- and mouse-cell cultures to quantify Atxn2 knockdown (KD) in vitro. Based on these data, we embedded the most potent siRNAs into natural miRNA scaffolds and packaged the cargo as AAV PHP.eB vectors to conduct IV-pharmacology studies in human-transgenic (Tg) mouse model of Atxn2 [BAC-hATXN2-Q22]. We observed dose-responsive reduction of both human [~40-70% KD] and mouse [~28-61% KD] Atxn2 transcripts in AAV-miRNA treated Tg mouse CNS at 60-days post dosing. In-situ hybridization showed cytosolic Atxn2 mRNA reduction within Tg mouse spinal motor neurons. We then vectorized the validated Atxn2-miRNA into ATC-134 capsid and dosed adult cynomolgus macaques at low (2e13 vg/kg) or moderate (6.7e13 vg/kg) dose-levels in a study of 28 or 60-day duration. No adverse safety events have been observed to date. In situ hybridization labeling the miRNA-cargo showed transduction of up to 60% of spinal cord motor neurons in the ventral horn at 28-days post dosing. Pharmacological assessments demonstrated dose-responsive increase in vector genome biodistribution and transgene-mRNA expression resulting in Atxn2-KD of >50% in multiple CNS regions in the NHP. In summary, we showed reduction of Atxn2, a therapeutic target for ALS, in non-human primates using a novel, intravenously delivered AAV capsid at safe and tolerable doses.

305. Development of Tau Isoform-Specific Reduction Therapy

Bonnie C. Lloyd[1], Irvin T. Garza[2], Karina Alvarado[2], Krishanna Knight[2], Rachel M. Bailey[2]

[1]Graduate School of Biomedical Sciences, UT Southwestern Medical Center, Dallas, TX,[2]Center for Alzheimer's and Neurodegenerative Diseases, UT Southwestern Medical Center, Dallas, TX

The microtubule associated protein tau (MAPT/tau) binds and stabilizes microtubules. Tau undergoes alternative splicing of exon ten to yield three-repeat (3R) or four-repeat (4R) tau. Under pathological conditions, tau dissociates from microtubules, adopts prion-like conformations leading to protein aggregation and neurotoxicity. The progressive accumulation of tau is the pathological hallmark of a group of neurodegenerative disorders called tauopathies. Some tauopathies, such as Alzheimer's disease, have aggregates that consist of both 3R and 4R-tau while others, such as progressive supranuclear palsy (PSP), consist mainly of 4R-tau. Reducing tau protein in an isoform specific manner is a potential therapeutic approach for tauopathies. This study aimed to develop tau-specific artificial micro-RNA (miRNA) shuttles delivered to the brain using adeno-associated virus (AAV) to selectively reduce tau as a therapeutic approach for different classes of tauopathies. We hypothesize that treatment with isoform specific tau miRNA candidates will selectively degrade tau mRNA, decreasing tau protein levels and reducing tau pathology and neurodegeneration.

We developed artificial miRNA shuttles that target either all isoforms of human tau mRNA (hTaui) or only 4R tau isoforms (hTau4Ri) for degradation. The antisense guide-strands of our miRNA contain perfect complementarity with tau mRNA across a 22 nt stretch of sequence, thereby directing the transcript toward an RNAi degradation pathway. To identify a lead miRNA shuttle, we did a primary screen using a dual-luciferase assay. Knockdown efficiency and isoform specificity were tested for each shuttle and a mismatched control shuttle (Scr) in HEK293T cells. The top candidates were then assessed in a secondary screen to directly measure the mRNA and proteins levels of 2N4R and 2N3R tau when overexpressed in HEK293T cells via qPCR and western blot analysis, respectively. We identified lead candidates for hTaui that reduced 3R and 4R tau mRNA and protein levels and a lead candidate for hTau4Ri that selectively reduced 4R tau as compared to Scr. These were then packaged into AAV9. To verify target engagement and benefit of our tau miRNA *in vivo*, 2 mouse models were used: hTau knock-in mice and PS19 mice. Adult hTau mice express all 6 human tau isoforms in place of mouse tau, which we confirmed via western blot analysis using isoform specific antibodies. hTau mice were injected with AAV/Scr, AAV/hTaui or AAV/hTau4Ri to confirm tau isoform-specific knockdown of mRNA via qPCR and protein via ELISA analysis. As hTau mice do not exhibit a pathological phenotype, we used PS19 mice to test potential, which overexpress human 1N4R tau with the disease-causing P301S mutation. PS19 mice develop pathological tau seeding early that progresses to severe neurological abnormalities including hindlimb clasping, progressive paralysis, and decreased survival by 9-12 months of age. PS19 mice were intra-cisterna magna injected with AAV/Scr or AAV/hTaui and followed for a minimum of 3 months and up to 12 months of age. qPCR, ELISA, tau seeding assays, and histopathological analysis showed treatment with hTaui significantly reduced tau mRNA, protein, seeding, and pathology when compared to mice injected with the scrambled control. In addition, hTaui treatment increased survival and decreased disease associated phenotypes even when treated at an advanced disease stage. Similar efficacy studies with our hTau4Ri miRNA shuttle are ongoing and will be reported. Together, in cells and mice, we verified that the artificial tau miRNA we developed can selectively and efficiently reduce tau in an isoform-specific manner, supporting these as potential therapeutic approaches for tauopathies.

306. Towards an AAV-Based Gene Therapy Strategy for Hereditary Spastic Paraplegia Type 52 (SPG52)

Laura Rodriguez-Estevez[1,2,3], Bea Almolda[1,3,4], Irene Fernandez-Carasa[5,6], Alba Aguilar-Cifre[1,2], Ángel Edo[1,2,3], Ángela Sánchez-Osuna[1,2,3], Alejandro Brao[1,2], Núria Gaja-Capdevila[1,7,8], Sergi Verdés[1,2,3], Xavier Navarro[1,7,8], Assumpció Bosch[1,2,3,8], Antonella Consiglio[5,6,9], Miguel Chillón[1,2,3,10]

[1]Institute of Neuroscience, Universitat Autònoma de Barcelona (UAB), Cerdanyola Del Vallès, Spain,[2]Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès, Spain,[3]Vall d'Hebron Institut de Recerca (VHIR), Barcelona, Spain,[4]Unitat Histologia Mèdica, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès, Spain,[5]Institute of Biomedicine (IBUB), Universitat de Barcelona, Barcelona, Spain,[6]Department of Pathology and Experimental Therapeutics, Hospital Universitari Bellvitge-IDIBELL, Hospitalet de Llobregat, Spain,[7]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès, Spain,[8]Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas (CiberNed), Instituto de Salud Carlos III, Madrid, Spain,[9]Department of Molecular and Translational Medicine, Università degli Studi di Brescia, Brescia, Italy,[10]Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

Hereditary spastic paraplegia type 52 (SPG52) is an ultra-rare inherited neurological disorder caused by biallelic mutations in the AP4s1 gene, which encodes a subunit of the adaptor protein complex 4 (AP-4), whose function is believed to be key in the biogenesis of the autophagosome. The disease leads to lower-limb spasticity, weakness, global developmental delay, intellectual disability, and seizures and no effective treatment is available. Gene therapy aimed at restoring AP4s1 expression represents a rational therapeutic approach for SPG52. Fibroblasts and iPSC-derived cortical neurons from patient and control skin biopsies have been developed and characterized. These cortical neurons recapitulate an accumulation of the AP-4 complex main cargo ATG9A within the Trans-Golgi network. AP-4 complex-deficient cortical neurons also show abnormal neurite morphology and decreased activation of the neuronal network activity. We have overexpressed the AP4s1 subunit using AAV vectors and restored the formation of the complex, rescuing neuropathological phenotypes. Alongside our *in vitro* studies, we have successfully generated the first AP4s1 knock-out (KO) murine animal modeling SPG52. This animal model shows decreased body weight gain, impaired rotarod performance, a marked clasping phenotype, and a tendency towards diminished strength. Motor phenotypes were corroborated by neuromuscular deficits, reflected as decreased compound muscle action potential (CMAP) amplitudes of the hindlimb muscles. Nerve conduction tests also revealed a compromised sensory function. AP4s1 KO exhibited impaired short- and long-term memory during the Morris Water Maze test. Moreover, these animals manifested a more active exploratory behavior without signs of anxiety when performing Open Field tests. The thinning of the Corpus Callosum described in patients was also recapitulated, evidenced by magnetic resonance imaging, and subsequently validated through histological examination. Importantly, this model shows a significantly shortened lifespan

compared to healthy controls. To assess the phenotype rescue with our gene therapy approach, we systemically treated AP4s1 KO animals with neurotropic AAV vectors carrying a correct copy of the AP4s1 gene. Mice were injected postnatally or at 3 months of age and phenotype rescue was assessed through a range of motor and electrophysiological tests. The development of hiPSC-derived cortical neurons together with the AP4s1 KO model, both recapitulating SPG52 disease hallmarks, are crucial to elucidating the disease's pathogenesis. Our gene therapy approach paves the way for the treatment of SPG52 children.

307. Establishment of Gene Therapy Drug Development Assays Confirms Antiepileptic Activity of an AAV Encoding Neuropeptide Y (NPY) and Y2 Receptor (Y2R)

Pernilla Fagergren[1], Barbara Terzic[2], Esbjörn Melin[1], David Dobry[2], Juliet Eve Mengaziol[2], Benjamin Okyere[2], Lawrence Moon[2], Elizabeth Ramsburg[2], Karin Agerman[1]

[1]CombiGene AB, Stockholm, Sweden,[2]Spark Therapeutics, Philadelphia, PA

INTRODUCTION: Epilepsy affects approximately 50 million people worldwide; over 30% of patients are considered treatment resistant to currently available antiepileptic drugs. Neuropeptide Y (NPY) has been shown to inhibit excitatory synaptic transmission in human epileptic hippocampus, mediated via Y2 receptors. Hence, intrahippocampal gene-delivery of NPY and its receptor Y2 could provide a novel treatment for mesial temporal lobe epilepsy (TLE). **METHODS:** AAV consisted of a single stranded genome comprised of a CAG promoter, human NPY, IRES, human Y2R, WPRE and polyA. **RESULTS:** 1) Spontaneous neural activity was studied *in vitro*. Networks of primary rat cortical neurons were transduced *in vitro* using a lentiviral vector delivering a genetically encoded reporter of intracellular calcium ion concentrations (as a proxy for neural activity). Three days later, neurons were transduced with AAV.CAG.NPY-Y2R (5E5 or 5E6 vg per cell) or a negative control vector (AAV.CAG.FIX). Frequency and intensity of spontaneous neural activity was attenuated specifically by AAV.CAG.NPY-Y2R. 2) Acute epileptiform activity induced by 4-aminopyridine (4-AP) was studied in mouse hippocampal *ex vivo* slices. Slices of adult mouse hippocampus were recorded from *ex vivo* using multielectrode arrays in the absence or presence of 4-AP, a drug which induces epilepsy-like neural firing. Application of NPY peptide (1 μM) attenuated the 4-AP-induced increase in the number of spikes by ~80% and the number of bursts by ~95% (relative to vehicle) in the CA1 field of the hippocampus. 3) Next, mouse *ex vivo* slices were studied following bilateral intrahippocampal injections of AAV.CAG.NPY-Y2R (6E9, 1.9E10 or 6E10 vg/mouse) four weeks prior to recordings. 4-AP-induced neural activity in CA1 was attenuated by AAV.CAG.NPY-Y2R versus control. Subsequent bioanalysis of slices confirmed the overexpression of NPY protein in AAV.CAG.NPY-Y2R treated animals over control. 4) Effects of AAV.CAG.NPY-Y2R on epileptic activity *in vivo* were studied using a rapid kindling model. Anesthetized adult rats were injected bilaterally into the hippocampus with either vehicle or AAV.CAG.NPY-Y2R (1.2E10 or 1.2E11 vg/rat). Ten days after treatment, stimulating and recording electrodes were implanted for subsequent rapid kindling and electroencephalography (EEG) analysis. At 38 days, hippocampal and cortical EEG were recorded during electrical kindling of ventral hippocampus, and seizure intensity was quantified using the Racine Scale. This rapid kindling paradigm involved finding the amount of current (μA) needed to generate a hippocampal afterdischarge ('threshold') and then giving one 400 μA stimulus every five minutes (total of 40). Treatment with AAV.CAG.NPY-Y2R increased the threshold and reduced both seizure intensity and hippocampal EEG afterdischarge duration versus control. **CONCLUSIONS:** Anti-seizure activities of exogenous NPY peptide and AAV.CAG.NPY-Y2R were demonstrated using different models. Spontaneous neural activity was attenuated by AAV.CAG.NPY-Y2R in primary rat cortical cultures. Bath application of NPY peptide or AAV-mediated overexpression of NPY and Y2R both markedly attenuated activity evoked by 4-AP in *ex vivo* slices of mouse hippocampus. Seizure intensity and seizure duration measured by EEG were both reduced in the rat, *in vivo* rapid kindling model of epilepsy. These results validate the feasibility of NPY-Y2 receptor gene therapy as a therapeutic opportunity in focal epilepsies and the potential to use the in vitro and in vivo assays as a screening cascade. **P.F. and B.T. are joint first authors; E.R. and K.A. are joint senior authors.**

308. Long-Term Safety and Feasibility of a Cas13-Based RNA Editing Therapy for *MECP2* Duplication Syndrome in Humanized *MECP2* Transgenic Mice

Dong Yang, Mengsi Duan, Xiaoqing Wu, Meixian Wu, Shaoran Wang, Xuan Yao, Alvin Luk, Hui Yang, Linyu Shi

HuidaGene Therapeutics Co., Ltd., Shanghai, China

Background: Methyl-CpG binding protein 2 (*MECP2*) is a dosage-sensitive, X-linked gene critical for neurodevelopment whose gain of function causes *MECP2* duplication syndrome (MDS). This devastating disorder is characterized by intellectual disability, motor dysfunction, infantile hypotonia, epilepsy, respiratory tract infections, and premature death with no curative therapy. We developed a translational strategy using our novel, CRISPR high-fidelity Cas13Y (hfCas13Y) to reduce the MeCP2 protein levels and reverse disease phenotypes. **Methods**: We generated hfCas13Y and *MECP2*-targeting gRNAs (g*MECP2*) packaging in a single AAV vector (AAV-hfCas13Y-g*MECP2*) and then evaluated the editing efficacy in the brain of the *MECP2* transgenic (*MECP2*-TG) mouse model after neonatal intracerebroventricular (ICV) injection. The motor function, anxiety behavior, society impairment, and the restoration of fear learning deficits using the fear conditioning test of *MECP2*-TG mice were tested at 8 and 26 weeks following ICV injection. The long-term effects (48 weeks post-injection) and safety of AAV-hfCas13Y-g*MECP2* were further investigated in *MECP2*-TG mice via RNA-seq and RNA integrity analysis in cortex tissues 4 and 48 weeks after ICV injection of AAV-hfCas13Y-g*MECP2*, respectively. **Results**: We have demonstrated AAV-hfCas13Y-g*MECP2* editing efficacy in reducing MeCP2 protein levels in the humanized *MECP2*-TG mouse model at 8 and 26 weeks after ICV injection. The motor function, anxiety behavior, and society impairment of *MECP2*-TG mice were rescued by AAV-hfCas13Y-g*MECP2*. The fear conditioning tests revealed that 8-week-old *MECP2*-TG mice displayed an abnormal increase in fear learning in context paradigms, a hippocampal-dependent behavior, but not significant compared to wild-type (WT) mice (Fig. 1A). In contrast,

26-week-old *MECP2*-TG mice exhibited a significant elevation (Fig. 1B). Notably, the abnormal contextual learning was reduced by the AAV-hfCas13Y-g*MECP2* at both 8 weeks (Fig. 1A) and 26 weeks (Fig. 1B) post-treatment. In addition, *MECP2*-TG mice injected with the AAV-hfCas13Y-g*MECP2* had a significant increase in median lifespan (226 days) compared to that of PBS-injected littermates (156 days) (Fig. 1C, P<0.01). Among 90.9% (10 out of 11) of *MECP2*-TG mice treated with AAV-hfCas13Y-g*MECP2* surviving over 220 days, 45.5% (5 out of 11) of those *MECP2*-TG treated mice survived even over 300 days. Conversely, only 10% (1 out of 10) *MECP2*-TG mice treated with PBS lived past 220 days (Fig. 1C). Furthermore, RNA-seq analysis showed that abnormal gene expression in *MECP2*-TG male mice was largely restored (Fig. 1D). To comprehensively detect the potential collateral effects accompanying targeted RNA degradation by the AAV-hfCas13Y-g*MECP2* system, we analyzed total RNA integrity in cortex tissues of *MECP2*-TG mice after ICV injection. Cortical RNA integrity remained unaltered compared to PBS-injected controls at both time points, suggesting that the hfCas13Y-g*MECP2* system lacked detectable collateral activity *in vivo*. Furthermore, H&E staining showed no injury in the brain, spine cord, liver, and kidney of *MECP2*-TG mice 48 weeks after treatment. **Conclusions**: Our findings demonstrate the long-term efficacy and safety of AAV-hfCas13Y-g*MECP2* treatment in *MECP2*-TG mice, providing a promising CRISPR/RNA therapeutic strategy for MDS treatment. Recently, this program has granted orphan drug and rare pediatric disease designations by the U.S. FDA.

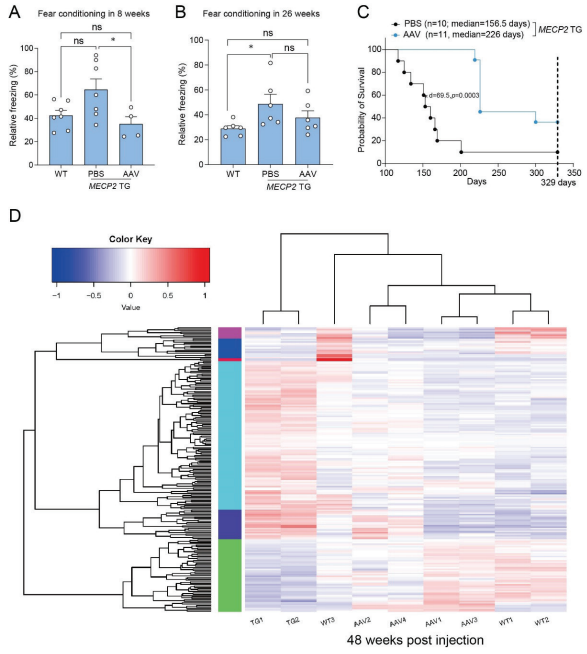


Figure 1. The long-term effect of AAV-hfCas13Y-gMECP2 in *MECP2* TG mice

309. AI Enabled ASO Design Can Lead to Rapid Initiation of Treatment for an Ultra-Rare Disorder Leading to Allele Selective Knockdown of a Toxic Protein and Consequent Clinical Improvement

David P. Dimmock[1], Lynne Bird[2], Amy Bouck[1], Allen Castro[1], Jason Ferrone[1], Jennifer Foley[3], Louis Gonzalez[1], Christopher Hart[1], Maureen Hart[1], Steven Kuntz[1], Mahati Krishna[4], Matthew Levy[5], Swagatam Mukhopadhyay[1], Timothy Nicholson-Shaw[1], Luke Ofria[1], Sankha Pattanayak[1], Amanda Reyes[1], George Samaan[1], Jeyaprakashnarayanan Seenisamy[4], Yiwei She[6], Megan Swim[1], Ruben Valas[1], Sara Yanke[1], Nicole G. Coufal[2]

[1]Creyon Bio, Carlsbad, CA,[2]UCSD, San Diego, CA,[3]Rady Children's Hospital, San Diego, San Diego, CA,[4]Creyon Bio, Bengaluru, India,[5]Creyon Bio, Durham, NC,[6]TNPO2 Foundation, New York, NY

Backgound: The subject suffers from a de novo heterozygous pathogenic variant causing a missense change in Transportin-2 (TNPO2) resulting in severe developmental delay and refractory epilepsy. TNPO2 is a nucleocytoplasmic transport receptor that facilitates transport of cellular cargo between the nucleus and cytoplasm (Kimura and Imamoto 2014). De novomissense variants in TNPO2 cause human developmental defects (Goodman et al. 2021). Data from Drosophila find that overexpression of this variant causes severe toxicity when compared to overexpression of the wild-type protein consistent with a dominant negative phenotype (Goodman et al. 2021). Human biobank data suggests that heterozygous loss of this gene is not associated with significant health or developmental problems. Further work by collaborators has demonstrated that knocking down this disease-causing variant leads to correction of a poor growth phenotype in patient derived brain organoids. However, TNPO2 is essential for cellular function and both loss of expression and overexpression are toxic. Consequently, therapy must selectively remove the toxic allele while maintaining function of the normal copy.The introduction of endogenous 'foreign' nucleic acids including both gene therapy vectors and antisense oligonucleotides (ASOs) are associated with immune and cytoplasmic DNA sensing mediated inflammatory reactions. Inflammatory reactions to ASOs have led to the failure of multiple drugs in early phase trials and interruption of dosing in individualized patient therapies.Creyon Bio implements a custom designed learning dataset paired with artificial intelligence (AI) to identify ASO sequence/chemistry collusions that are safe, thereby enabling the rapid design of allele selective ASOs. **Methods:** Proprietary tools were utilized to design 96 3-10-3 "Locked Nucleic acid gapmer" oligonucleotides against the disease causing variant. Eighty eight of these were demonstrated to be safe utilizing in vivo and in vitro model systems. Multiple candidates demonstrated allele selective knockdown in patient derived cell lines and the correction of an in-vitro phenotype. Based on selection criteria, the safest and most effective compounds were scaled up for GMP manufacturing, quality assessment and compounding. After the completion of GLP toxicology studies and after FDA permission, the index case was treated via intrathecal delivery with escalating doses of the allele selective ASO. **Results:** AI-informed design enabled initial approval to dose within 1 year of project initiation. Dose escalation up

to 40mg has been tolerated with no increase in inflammatory markers in the CSF or adverse changes on brain MRI. Subsequent to the second dose the child demonstrated a dramatic reduction in seizure frequency. Following the third dose he regained developmental milestones that were lost and developed new skills. This included previously regressed skills such as rolling and sustained attention and novel skills such as tripod sitting and responsive babbling. These clinical improvements were lost 6-8 weeks into the first (planned) extended (3 month) dose interval and were regained within 1 week of the subsequent 40mg dose. **Conclusion:** Allele selective knockdown of a toxic TNPO2 protein, using an AI-informed ASO design, leads to clinical improvement in a single case.

AAV Capsid Engineering: Multilevel Approaches for Enhanced AAV Delivery

310. Identification of AAV Capsid with Improved Tropism to Macular Retina Through Multi-Species Screening in Mice, Rabbits, Pigs, and Monkeys

Shengjiang Liu, Haifeng Chen, Xiaoming Gong, Sameera Peraramelli, Ningguang Luo, Alan Chen, Jinzhong Li, Eduardo Santillan, Derrick Huynh, Shuyi Li, Ying Li, Kevin Lin, Minghua Dai, Jianwu Chen, Zhe Yu, June Song, Son Thai Nguyen, John Zhang, Li Ou

Avirmax, Inc., Hayward, CA

Adeno-associated virus vector is the main *in vivo* gene delivery method. To improve tissue tropism, capsid engineering through rational design, directed evolution, and artificial intelligence has attracted much attention and has been performed, resulting in the usage of engineered capsids in clinical trials. However, the lack of cross-species translatability of novel capsids limits its broader application and creates significant challenges for clinical development. In this study, a novel capsid (AAV2.N54) with improved tropism to deep regions of retinas was identified through multi-species screening in mice, pigs, rabbits, and monkeys. In screening studies, fundus images, immunohistochemistry, and confocal scanning laser ophthalmoscopy were performed to assess the tropism of 18 candidate capsids and controls after intravitreal administration. Across all four species, AAV2.N54 showed improved tropism over wildtype AAV2 and a previously described engineered capsid AAV2.7m8, evidenced by efficient delivery to the macular retina and the ability to detarget from the ciliary body. AAV2.N54 also had a solid safety profile with minimal inflammation, similar to controls. The transgene expression cassette for treating wet AMD was optimized through in vitro screening and measurement of protein levels by ELISA. Then, AAV2.N54-Aflibercept was assessed in disease models of mice and rabbits. In the laser-induced choroidal neovascularization (LCNV) mouse model, fluorescein angiography and flatmount analysis showed that AAV2.N54-Aflibercept prophylactically reduced retina lesions, with improved efficacy than the commercial protein drug Eylea. In the DL-AAA rabbit disease model, AAV2.N54-Aflibercept demonstrated remarkable therapeutic efficacy in reducing retina leakage. In addition, a GLP toxicity study in NHPs was performed, demonstrating the safety profile of intravitreal dosing of AAV2.N54-Aflibercept at the dose up to $5.7x10^{11}$ vg/eye. Based on these results, GMP production has been finished, and an investigational new drug (IND) application will be submitted. In conclusion, an engineered capsid with improved delivery to the macular retina and cross-species translatability was identified, and a novel gene therapy for treating wet AMD was developed.

Capsid	Species	CB	GCL	IPL	INL	OPL	ONL	IS	OS	RPE	C	ONH	S
AAV2	Mouse	ND	+++	++	+	+	+	-	-	-	-	-	-
	NHP	+++	+++	-	-	-	-	-	-	-	-	-	-
N54	Mouse	ND	+++	+++	+	+	+	+	-	-	-	-	-
	Pig	ND	+++	+++	+++	+++	+++	++	++	+	-	-	-
	NHP	-	++	+	++	++	+++	+++	+++	++	-	-	-
	Rabbit	-	+++	+++	++	++	++	++	++	+	-	+++	-
7m8	Mouse	+++	+++	+	+	+	+	+	-	-	-	-	-
	Pig	ND	+++	+++	+++	+++	++	++	++	+	-	-	-
	NHP	++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-

Figure 1. Graphical representation of GFP expression from N54 and controls across all species.
GFP expression was scored across all retinal and extra-retinal layers and across both antibody cocktails, for a survey of ocular GFP expression across all eyes enrolled in cryosection analysis. Dark green cells (+++) indicate where GFP expression was the highest; Medium green cells (++) indicate where there was moderate GFP expression; Light green cells (+) indicate where there was weak GFP expression; Red cells (-) indicate where GFP was absent. Abbreviations: GCL/ILM – ganglion cell layer/Inner limiting membrane; IPL – inner plexiform layer; INL – inner nuclear layer; OPL – outer plexiform layer; ONL – outer nuclear layer; IS – inner segment; OS – outer segment; RPE – retinal pigmented epithelium; C – choroid; S – sclera; M – muscle; ONH – optic nerve head.

311. AAV Capsid Selection at Spatial and Single-Cell Resolution in Non-Human Primate Retina

Ashley Robbins[1,2], Yonghong Chen[2], Brian Lewandowski[2], Dominika Houserova[2], Paul T. Ranum[2], Luis Tecedor[2], Beverly L. Davidson[2,3]

[1]Neuroscience Graduate Group, University of Pennsylvania, Philadelphia, PA,[2]Raymond G. Perelman Center for Cellular and Molecular Therapeutics, Children's Hospital of Philadelphia, Philadelphia, PA,[3]Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

The development of novel adeno-associated viruses (AAVs) with tissue and cell-type-specific tropism is crucial to meet the need for efficient delivery of gene therapies. AAV selection methodologies have given rise to a new class of engineered AAV vectors capable of improved gene delivery at lower doses and with greater specificity than wild-type AAVs. However, a challenge remains in accurately resolving transduction status within heterogeneous cell populations and across multiple tissues. Existing approaches for identifying cell-type-specific tropism of engineered AAVs through single-cell RNA sequencing are acknowledged to be either low-throughput and/or cost-prohibitive. This limitation is, in part, due to their reliance on droplet-based single-cell or nuclei partitioning. Moreover, the use of fluorescent transgenes for detection raises concerns about potential toxicity, particularly in neuronal populations. Our lab has overcome these constraints by establishing an efficient approach for extensively multiplexed profiling of AAV tropism in specific cell types and regions. Our approach utilizes a modified version of the combinatorial indexing technique for sequencing single-cell transcriptomes (SPLiT-seq), first described by Rosenberg et al. in 2018, to simultaneously capture transcriptome and AAV transduction information at single-cell resolution. This

strategy offers numerous benefits in terms of scalability. A single experiment can profile ~150,000 cells or nuclei derived from hundreds of biological samples and enables the simultaneous assessment of tens to hundreds of AAV capsid variants. Importantly, our approach doesn't necessitate specialized equipment and is applicable across various tissues and species. To illustrate the utility of AAV SPLiTseq in defining the cell-type-specific tropism of clinically translatable capsids we performed a mixed pool capsid screen with 90 PM-AAVs from two parental serotypes delivered intravitreal to the retina of non-human primates (NHP). The cellular diversity of the NHP and human retina significantly varies spatially, and the degree of damage or sparing of the foveal cones differs across human retinal diseases. To ensure adequate coverage of therapeutically relevant retinal cell types, we isolated nuclei from both peripheral and foveal regions for single-cell sequencing. Capsid transduction was assessed at single-cell resolution using AAV SPLiTseq and more broadly using bulk RNA and DNA sequencing. This allowed for integrated analysis across these complementary screening modalities. Top-performing capsid variants were identified by comparative analysis of bulk and single-cell data and further evaluated for performance and cell-type tropism. In summary, we demonstrate the successful implementation of AAV SPLiT-seq for regional and cell-type-specific tropism to identify clinically relevant PM-AAVs for gene therapy delivery.

312. Identification of Novel AAV3B and AAV8 Variants with Superior Retina Transduction Profiles Following Suprachoroidal Delivery in Multiple Large Animal Models

April R. Giles, Elad Firnberg, Sigmund K. S. Tejada, Samantha A. Yost, Jessica D. Gumerson, Levi Winter, Ryan Kruse, Sungyeon Cho, Trang Nguyen, Christopher B. Tully, Bezawit Ejigu, Mi Shi, Samitha Venu, Ryan N. Clarke, Brendan N. Lilley, Ye Liu, Andrew C. Mercer, Olivier F. Danos

REGENXBIO Inc., Rockville, MD

Recent clinical advancements delivering adeno-associated viral (AAV) vectors into the suprachoroidal space (SCS) have established the feasibility and value of this in-office route of administration in ocular gene therapy. The identification of engineered capsids optimized for SCS delivery that both efficiently transduce ocular tissue in a targeted fashion and display favorable off-target transduction profiles would enable widespread use of this route of administration. We designed and produced three high diversity peptide insertion libraries (up to 10^8 variants per library), each encoding AAV *cap* under the control of the CMV promoter with randomized 7-mer insertions at VR-IV of AAV3B and VR-IV or VR-VIII of AAV8, and delivered them intraocularly to non-human primates (NHP, cynomolgus macaques). Leveraging our directed evolution platform NAVIGATE (Novel AAV Vector Intelligent Guided Adaptation Through Evolution), we identified 2-4k novel variants enriched in retina and RPE-choroid from each high diversity library, which we then re-packaged and administered to NHP via SCS delivery. We then selected 63 variants (41 AAV3B.VR-IV, 12 AAV8.VR-IV, and 10 AAV8.VR-VIII) for final library down-selection. Each variant (as well as parental controls AAV3B and AAV8) was produced individually with genomes encoding a barcoded CAG.tdTomato cassette, pooled, and delivered to NHP, again by SCS delivery at a dose of 3e12 GC/eye. All variants expressed transgene mRNA in retina more efficiently than parental serotypes as measured by average RNA relative abundance across multiple samples per eye. AAV3B.VR-IV insertions and AAV8.VR-IV/AAV8.VR-VIII insertion variants were up to >30-fold and up to >50-fold improved, respectively, when compared to the parental serotypes. Most AAV8-based insertion variants also expressed >20-fold more transgene mRNA in RPE-choroid compared to AAV8, with the top variant producing in excess of 200-fold more mRNA than AAV8. We also identified numerous variants with advantageous off-target transduction profiles. For example, the majority of AAV8.VR-IV and AAV8.VR-VIII variants that showed a greater than 10-fold increase in retinal transduction did not transduce liver, heart, or kidney to a greater degree than AAV8. We subsequently evaluated these variants in additional *in vivo* and *in vitro* model systems. We assessed the 63-member library and a subset of single vectors via SCS delivery in Yucatan minipigs. In a library setting, the improvement of AAV3B variants over AAV3B appeared to be species-limited. Findings from the AAV8 insertion variants, however, were largely translatable from NHP to minipigs, with top hits achieving improvement relative to AAV8 of >60-fold and >100-fold in RPE-choroid and retina, respectively. Variants evaluated as single vectors achieved up to 150-fold higher secreted transgene protein expression in the aqueous humor as compared to the AAV8 control. Transduction of iCell RPE cells and an ARPE-AAVR cell line with either the 63-member library or a subset of top hits as single vectors established that most variants exhibit superior transduction efficiency compared to AAV8 with top variants achieving >20-fold improvement in both the library and single vector settings. These results validate the utility of the NAVIGATE platform and its ability to identify AAV variants that significantly outperform parental serotypes and that have the potential to be developed as gene therapy treatments in ophthalmology.

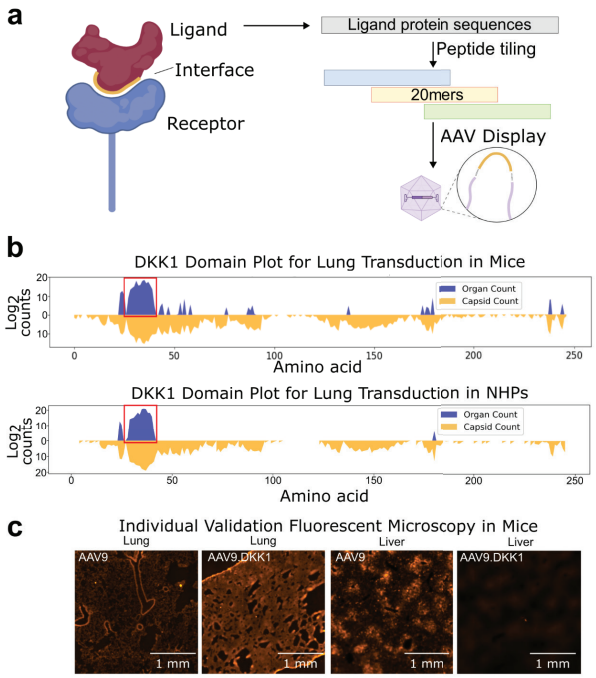
313. Reprogramming Adeno-Associated Virus Tropism via Displayed Peptides Tiling Receptor-Ligands in Mice and Non-Human Primates

Andrew Portell[1], Kyle Ford[1], Duy An Le[1], Alice F. Tarantal[2], Prashant Mali[1]

[1]Bioengineering, University of California San Diego, La Jolla, CA,[2]University of California, Davis, Davis, CA

Adeno-associated viruses (AAVs) are common gene therapy vectors, however, their effectiveness is hindered by poor target tissue transduction and off-target delivery. Hypothesizing that naturally occurring receptor-ligand interactions could be repurposed to engineer AAV tropism, we fragmented all annotated protein ligands known to bind human receptors into tiling 20-mer peptides and displayed these onto the surface loops of AAV5 and AAV9 capsids at two surface-exposed sites (Figure a). The resulting four capsid libraries, comprising >1 million AAV variants, were screened across 9 tissues in C57BL/6 mice. Tracking variant abundance, we identified >15,000 variants that efficiently transduced at least one mouse organ. We individually validated 21 AAV variants with 74.3% of the organ tropism predictions accurately reproducing, confirming overall screen efficacy. We next performed mutagenesis on the top-performing displayed peptides and screened the resulting millions of AAV variants in C57BL/6 mice

and non-human primates. As our displayed peptides are systematically tiled from human ligands, we observed strong species concordance in transduction patterns with certain peptides also displaying consistent activity across mice strains, capsid insertion contexts, and capsid serotypes (Figure b-c). Comprehensive tiling and deep mutagenesis also enabled a thorough examination of homologous peptide activity with an ability to map functional domains across the entire amino acid sequence of the proteins from which the peptides were derived (Figure b). Interestingly, we observed functional peptides tended to be derived from specific domains on ligands which enabled the prediction of putative AAV-receptor interactions, which we successfully validated by targeted genetic perturbations. We anticipate this comprehensive ligand peptide tiling and display approach will enable the future engineering of tropism across diverse viral, viral-like, and non-viral delivery platforms, and shed light on basic receptor-ligand biology.



314. Improvement of Liver Tropic AAV Capsids: Neutralizing Antibody-Evading Variants of sL65 Which Demonstrate Cross-Species Transduction

Jing Liao[1], Shiliang Hu[1], Nannan Jia[1], Joy Wang[1], Krishna Chintaluri[1], Yinxing Chen[1], June Deng[1], Kirsten Lapoint[1], Megan Ericson[1], Isabel Alberta[1], Noel Walsh[1], William Lee[1], Delaney Dowdell[1], Nick DiGioia[1], Lauren M. Drouin[1], Gregory Preston[1], Ma Qicheng[1], Wei Ding[1], Marti Cabanes Creus[2], Leszek Lisowski[2], Matthias Hebben[1], Mariana Nacht[1]

[1]Alexion Genomic Medicine, Astra Zeneca Rare Disease, Lexington, MA,[2]Children's Medical Research Institute, Sydney, Australia

Antibodies against AAVs are a major cause of patient exclusion in gene therapy trials and prevent redosing of treated patients. We previously reported the generation of the novel human liver tropic capsid sL65, isolated in humanized mice and validated in non human primates (NHPs). The sL65 capsid shares a limited number of epitopes with capsids commonly used in clinical trials. However, its variable region I (VR I) is homologous to AAV2, AAV3B and AAV LK03, making sL65 vulnerable to certain antibodies directed against these serotypes. To enhance antibody evasion, we created around 15,000 variants of sL65 through VR I region mutations and screened them in humanized mice with and without neutralization by human immunoglobulins (IVIG). Remarkably, we identified candidates that evaded pre-existing neutralizing antibodies (NAb) while maintaining potency in human hepatocytes from FRG mice. These engineered capsids also exhibited enhanced transduction efficiency in various human cell lines, including HuH7 and RC32 Hela cells. Interestingly, several capsid variants showed increased transduction efficiency in mouse hepatocytes, indicating a potential improvement in cross-species translatability. Next, we barcoded key capsid variants and administered pooled capsids in NHPs following systemic administration. mRNA expression was quantified by next-generation sequencing (NGS) of the barcodes associated with each capsid variant. Confirmation was obtained that the transduction efficiency in NHP livers with NAb evading capsids was maintained, aligning with results observed in humanized mice studies. To explore the possibility of redosing by serotype switching, we performed an *in vitro* RC32 cell based NAb assay utilizing serum from NHPs previously dosed with different liver tropic capsids, including AAV8, AAV DJ, AAV LK03, and sL65. As hypothesized, we observed that, *in vitro*, the sL65 variants were less impacted by neutralizing antibodies from sera of NHPs dosed with AAV LK03 compared to the other capsids tested. In summary, the targeted engineering of sL65 VR I led to decreased capsid neutralization by pre existing anti AAV neutralizing antibodies while maintaining high transduction in human hepatocytes, mouse and NHP livers. This enhances cross species translatability, while offering the potential to treat a broader patient population and opening the possibility to re retreat patients who previously received gene therapy with an unrelated serotype.

315. Enhanced Precision for *In Vivo* Gene Delivery into Lymphocytes Through Mono- and Bispecific DART-AAVs

Luca J. Zinser, Samuel A. Theuerkauf, Fabian John, Muhammed Burak Demircan, Christian J. Buchholz

Gene Therapy and Molecular Biotechnology, Paul-Ehrlich-Institut, Langen, Germany

One of the major obstacles for *in vivo* gene therapy are vectors mediating highly selective gene transfer into a defined population of therapy-relevant cells. Currently, only a limited number of presently approved *in vivo* gene therapies tackle this challenge through the localized administration of non-targeted AAVs. However, numerous genetic and acquired diseases, including HIV, distinctly affect certain tissues or even a particular subset of cells. In this study, we present mono- and bispecific DARPin-targeted AAVs (DART-AAVs) addressing the lymphocyte markers CD8, CD4, CD56 or CD32a. By combining two DARPins in a specific manner, the bispecific DART-AAVs exhibit binding behavior that follows an AND-gate-like pattern for both surface markers. For CD56 a DARPin was selected *de novo* via ribosome display. Two DARPin candidates demonstrated

favorable binding characteristics to CD56 in a cellular binding assay conducted with primary human NK cells and NK92 cells. The most promising CD56 DARPin, along with previously established DARPins targeting CD8, CD4, and CD32a, was integrated into surface-exposed loops of the AAV2 capsid — either individually or in pairs. Both, the mono- and bispecific DART-AAVs preserved their structural integrity, as confirmed through high-resolution cryo-electron microscopy. Remarkably, bispecific vectors, in contrast to their monospecific counterparts, exhibited a clear preference e.g. for CD4/CD32a, CD4/CD8 and CD8/CD56 double-positive cells. This preference was consistently observed in cell mixtures as well as *in vivo* after systemic vector administration into a mouse model harboring double-positive cells in bone marrow. Even when double-positive cells constitute only a small fraction of the overall cell population such as the CD4/CD32a HIV reservoir cells, the CD4/CD8 T-cells and CD8/CD56 NKT-cells these vectors reliably targeted such cells in several settings, including healthy donor blood. The preference in transduction of double positive cells was up to 30-fold compared to single positive cells. Cell trafficking studies revealed an increased cell entry rate for bispecific over monospecific AAVs. Our results suggest a novel avenue for receptor targeting by enabling gene transfer into cell subtypes defined by two cell surface markers.

316. Engineered Adeno Associated Virus Capsids for Packaging Proteins

Ryan Sorensen[1], Mareike Daniela Hoffmann[2], Ajay Extross[2], Yungui He[2], Daniel Schmidt[2]

[1]Department of Biochemistry, Molecular Biology, & Biophysics, University of Minnesota, Minneapolis, MN,[2]Department of Genetics, Cell Biology, & Development, University of Minnesota, Minneapolis, MN

Adeno Associated Virus (AAV) is widely used in gene therapy as it is nonpathogenic and can efficiently deliver a therapeutic DNA payload to target tissue. Efficient nuclear delivery of DNA to a wide range of cell types is the consequence of evolved properties of the AAV capsid that overcome significant biological and cellular barriers (i.e., extravasation from the blood stream, escape from the endosome, and transport to the nucleus). While AAV is currently an excellent delivery vector for DNA, the possibility of packaging non-DNA payloads has not yet been explored. With the aim of expanding the payload options of AAV beyond DNA and to improve the utility of AAV as a delivery vector we engineered an AAV capsid capable of packaging protein. In prior work, we performed a domain insertion screen of AAV-DJ VP1 that, in addition to identifying new domain insertion positions on the outside of the capsid for cell-specific targeting, revealed positions on the inside of the capsid permissive to insertions. We utilize these permissive positions on the interior of the capsid to insert a nanobody, acting as a binding scaffold, which mediates packaging of a target protein. Production of engineered AAV packaging protein is performed by adapting widely used helper-free production in HEK293AAV cells wherein the plasmid normally encoding the transgene to be packaged into AAV is replaced by a plasmid encoding the target protein and lacking ITRs. The capsid protein containing the nanobody insertion is provided on a separate plasmid and is supplemented by the remaining wild-type capsid proteins required for capsid assembly. The expressed target protein binds to the nanobody inserted into the capsid protein and becomes packaged within AAV during the capsid assembly process. First, we demonstrate that our engineered AAV capsids containing either a GFP nanobody or an ALFA nanobody inserted into VP1, chosen based on our previous insertional data, are capable of packaging their cognate ligand (GFP or ALFA tagged GFP protein). Additionally, the low copy number of VP1 in assembled capsids was predicted to promote incorporation of the engineered VP1 into assembled capsids and packaging of the target protein. Western blot analysis of iodixanol purified AAV packaging protein demonstrated two permissive positions that package the target protein with differing efficacy. Next, we increased the efficiency of protein packaging by adding a nuclear localization signal to the target protein leading to its enrichment in the nucleus where capsid assembly occurs. Moreover, we demonstrate that through insertion of the nanobody into VP3, which has a higher rate of incorporation into the capsid than VP1, we can further enhance the efficiency of protein packaging. Finally, we determined the bulk ratio of target protein packaged per capsid using quantitative mass spectrometry. In conclusion, this work demonstrates the first instance of packaging protein inside of AAV and opens the possibility for the use of AAV to transiently deliver theragnostic agents and research tools in the form of proteins.

Physical Delivery and Novel Approaches

317. Single-Particle Spectroscopic Hydrodynamic Chromatography Reveals Heterogeneous RNA Loading and Size Correlations in Lipid Nanoparticles

Sixuan Li[1], Yizong Hu[2], Jinghan Lin[3], Kuangwen Hsieh[1], Fangchi Shao[4], Lai Wei[1], Tine Curk[3], Hai-Quan Mao[3], Tza-Huei Wang[1]

[1]Mechanical Engineering, Johns Hopkins University, Baltimore, MD,[2]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA,[3]Materials Science and Engineering, Johns Hopkins University, Baltimore, MD,[4]Biomedical Engineering, Johns Hopkins University, Baltimore, MD

Introduction: Lipid nanoparticles (LNP) have emerged as pivotal delivery vehicles for RNA therapeutics. Previous research and developments usually assume that LNPs are homogeneous in population, loading density, and composition. Such intuitive yet unverified perspectives remain due to the lack of suitable tools to characterize these physicochemical properties at the single-nanoparticle level. Here, we explore integrating spectroscopy and chromatography as a generalizable strategy to dissect the complexities of multi-component LNP assembly. We characterize a benchmark siRNA LNP formulation with a size distribution ranging from 30 to 200 nm and reveal the existence of seven distinct LNP species, variations in RNA packing densities spanning multiple orders of magnitude among similarly sized LNPs, and size-dependent loading-size correlations. **Methods:** We used Cylindrical Illumination Confocal Spectroscopy (CICS), a technique recently applied for LNP characterization[1] to examine the LNPs with fluorescently labeled siRNA, DSPC lipids, and PEG lipids. The LNPs were injected continuously or in a sample plug for Single Nanoparticle Free Solution Hydrodynamic Separation

(SN-FSHS)[2] in a microfluidic channel. A commonly referenced benchmark LNP formulation with DLin-MC3-DMA as the ionizable lipid was formulated by rapid mixing in a T-junction at pH 4.0 and dialyzed against PBS at pH 7.4. **Results:** The platform can report 4-dimensional LNP parameters (**Fig. 1a**), including the fluorescence of labeled siRNA, PEGylated lipid, and helper lipid detected by the single particle fluorescence (i.e., CICS), plus the size measured by the hydrodynamic chromatography (i.e., SN-FSHS). The 3-color fluorescence was used to distinguish 7 subpopulations within the LNP sample, based on the compositional features (**Fig. 1b**). We showed that SN-FSHS can measure LNP size in an unbiased manner and clearly differentiate two populations within a bimodal mixture of LNPs, while the DLS results are masked by the larger LNP population (**Fig. 1c**). By analyzing the LNP content and size at single particle level, we revealed the heterogenous loading-size correlation across LNP size ranges from 30 to 200 nm for 3 tested LNP formulations prepared with different mol% of PEGylated lipid (**Fig. 1d**), that would be otherwise masked by the bulk average method. **Conclusions:** The SN-FSHS-CICS platform provides the first integrated characterization tool for the simultaneous assessment of population distribution in composition and loading capacity of RNA in LNP formulations. This analysis can provide valuable insights into the heterogeneity of LNP preparations and permit systematic investigations in understanding critical physicochemical parameters in LNP engineering. The SN-FSHS-CICS platform can guide formulation optimization and manufacturing processes to achieve greater compositional homogeneity and further improve the performances of LNP delivery systems. **References:** (1) Nat. Comm. 2022, 13 (1): 5561. (2) J. Am. Chem. Soc. 2016, 138 (1): 319.

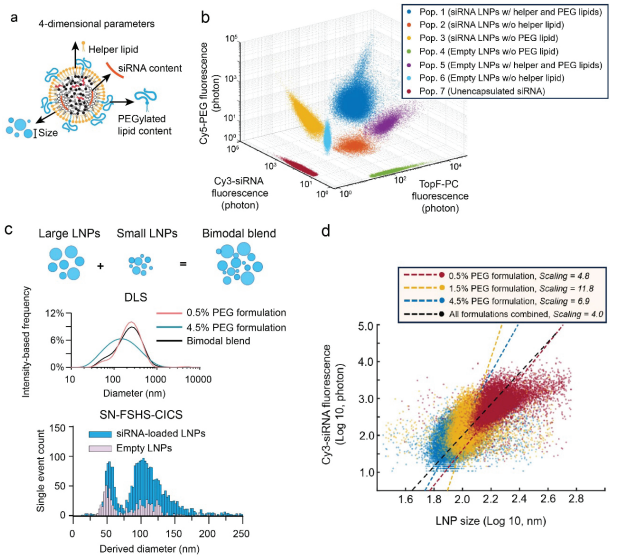


Fig. 1. (a) Four-dimensional parameters reported by SN-FSHS-CICS platform that enables the following single-nanoparticle size and loading (including siRNA, helper lipid and PEG lipid) correlation analysis. (b) 3D scatter plot of the subpopulations within a benchmark siRNA LNP formulation classified based on the compositional features. Each dot represents one particle/molecule. (c) The hydrodynamic size of siRNA LNPs with bimodal mixture of 0.5 mol% and 4.5 mol% PEGylated lipid formulation, measured by DLS and SN-FSHS-CICS. (d) Correlation between siRNA loading and LNP size by SN-FSHS-CICS single particle measurement, overlaying all three formulations including 0.5%, 1.5% and 4.5% PEGylated lipid. n = 11,068, 15,191, and 8,637 LNPs analyzed for 0.5%, 1.5%, and 4.5% PEG formulations, respectively.

318. Engineered *Staphylococcus Epidermidis* as a Protein Delivery System for Treating Skin Diseases

Travis M. Whitfill[1], Roger Leger[1], Dorival Martins[1], Azim Munivar[2], Julia Oh[3], Sara Mootien[1]

[1]Azitra Inc., Branford, CT,[2]Atman Therapetuics, New York, NY,[3]The Jackson Laboratory, Farmington, CT

The topical delivery of large molecules through the skin surface has been a challenge for treating skin diseases, as human skin barrier function generally prevents large molecules from penetrating intact skin. Here, we present data for a novel platform using *S. epidermidis* (SE), a safe human skin commensal, as a chassis for delivering proteins throughout the epidermis. This system has important advantages over other methods of drug delivery, as it can establish residence on a patient's skin and continuously deliver therapeutic proteins *in situ*. We first developed genetic tools for using SE as a delivery system using GFP as a reporter, including plasmids for genetic manipulation, promoters to optimize protein expression, and secretion signals to export recombinant proteins from SE (**Fig 2B**). Engineered GFP-secreting SE secretes more GFP in culture with a secretion signal vs. no secretion signal (**Fig 2C**) and colonizes mouse skin after a single application **(Fig 2D)**. *In vivo* two-photon and light microscopy in mice showed engineered SE colonizes mouse skin down to ≥80μm and is found in hair follicles (**Fig 2E-I**).

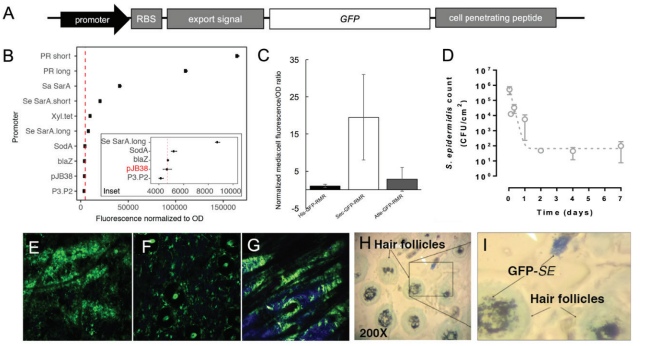


Figure 1. Features of a novel, SE-based protein delivery system using GFP. **(A)** Construct design comprised of a promoter, RBS, export signal. **(B)** Characterization of a selection of promoters for tunable control of protein expression (GFP used as reporter. **(C)** Characterization of export signals for protein export out of SE (GFP used as reporter) using fluorescence:OD measurements as a ratio of media to cells, normalized by the control (His-GFP-RMR). **(D)** Colonization of GFP-secreting *Staphylococcus epidermidis* onto mouse skin (tails) (n=3). 10^9 CFU were applied to 1 cm^2 of skin, and skin was recultured to determine CFU count over 7 days. **(E-G)** *In vivo* two-photon microscopy of mouse skin three days following treatment of GFP-expressing *S. epidermidis* (green). 25 μm **(E)** and 50 μm **(F)** depth of unshaved mouse ear skin, and **(G)** 80 mμ depth on shaved dorsal skin. **(H)** Light microscopy of dorsal skin of mice following SE-GFP application magnified in **(I).**

We focused on a disease case, Netherton syndrome (NS), a rare genetic disease caused by the loss of the lympho-epithelial Kazal-type-related inhibitor (LEKTI), which causes unregulated activity of kallikrein (KLK) proteases. ATR12-351 is a topical ointment containing an auxotrophic strain of SE, SE351, engineered to secrete domain 6 of human LEKTI protein (rhLEKTI-D6) to restore normal KLK activity levels in NS patients. A series of *in vitro* and *ex vivo* studies was performed to demonstrate proof-of-concept of SE351, an auxotrophic strain of SE, and its drug product, ATR12-351, as an rhLEKTI-D6 delivery chassis to treat NS (**Fig 3A**). *In vitro* studies of an NS model using human skin extracts (HSE) supplemented with recombinant KLK5 (7-fold protease activity excess over normal human skin) were used to measure the effect of SE351 on trypsin-like activity. SE351 supernatant rescued protease activity to normal skin baseline levels ($p<0.01$) (**Fig 3B**). We then used *ex vivo* tape stripped human skin to measure rhLEKTI-D6 penetration from ATR12-351 vs. vehicle

or topical rhLEKTI-D6 application. ATR12-351 delivered 6.1 µg of rhLEKTI-D6 throughout the epidermis in significantly higher amounts vs. topical protein alone (6.µg vs. 2.3 µg, p=0.008) (**Figs 3C-F**). Post application (t=24 hours) of ATR12-351 or vehicle, rhLEKTI-D6 activity was found in significantly elevated levels throughout all 30 tape-stripped skin layers (6.1 µg vs. 0.5 µg, p<0.01). The skin penetration of rhLEKTI-D6 to deeper layers in the skin was more prominent vs. topical application of rhLEKTI-D6.

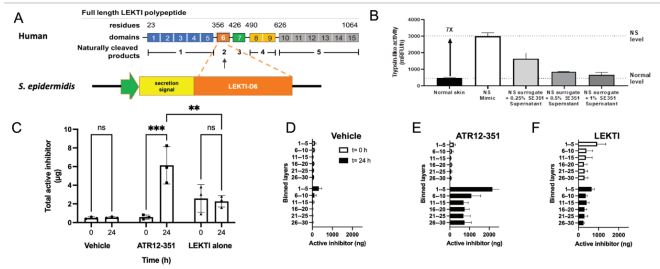


Figure 2. Design of a human recombinant LEKTI-secreting strain of SE (SE351). **(A)** Design of the gene sequence that was chromosomally integrated into *S. epidermidis*. **(B)** *In vitro* studies using tape stripped skin from healthy volunteers spiked with KLK5 to mimic Netherton syndrome. Samples were then added with ascending doses of culture supernatant of SE351. **(C)** Total active LEKTI-like inhibitor levels (µg) from *ex vivo* human skin treated with placebo, ATR12-351 or formulated LEKTI-D6 over 24 h. The total LEKTI-like activity was obtained by adding the µg amounts through layers 1-30 of human skin treated with (●) placebo, (■) ATR12-351 at 10^7 CFU/cm² or (▲) formulated LEKTI-D6 at 300-400 ng/cm² at t = 0 h and t = 24 h of exposure at 30°C. Data are means ± SD (N = 3). Statistical analysis in (**C**) was carried out using two-way ANOVA. NS: no statistical difference; ***: p < 0.001; **: p < 0.01. **(D-F)** Amounts of active LEKTI-like inhibitors in layers extracted from tape strips samples from *ex vivo* human skin treated with (**D**) placebo, (**E**) ATR12-351 at 10^7 CFU/cm² or (**F**) formulated LEKTI-D6. The sample collection proceeded immediately post skin application (t = 0 h, white bars) or after 24 h incubation at 30°C (t = 24 h, black bars). Data are means ± SD (N = 3).

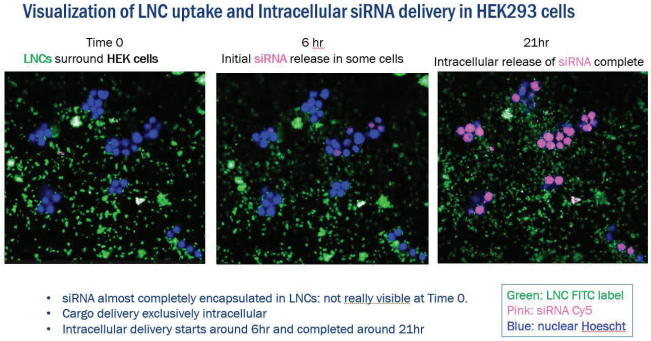
Here, we demonstrated proof-of-concept of a novel cutaneous protein delivery system using a live, engineered bacterial chassis composed of SE. We showed that engineered SE colonizes mouse skin, secretes functional rhLEKTI-D6 in an in vitro NS model, and delivers the desired LEKTI activity deep into the layers of the epidermis in ex vivo human skin. A clinical study of ATR12-351 in adult patients with NS has been initiated (NCT06137157).

319. Lipid Nanocrystal Delivery of siRNA: Dynamics of Uptake in Innate Immune Cells in Human Blood and Visualization of Small Oligonucleotide Delivery in Cell Culture

Hui Liu[1], Miriam Mikhael[1], Amra Tabakovic[1], Anne-Rika Holtzhausen[2], Caroline Beltran[2], Partha Samadder[1], Tzong-Jen Sheu[1], Thomas Hoover[1], Natalie Strickland[2], James J. Ferguson[1]

[1]Matinas BioPharma, Bedminster, NJ,[2]Synexa Life Sciences, Cape Town, South Africa

Background: Lipid nanocrystals (LNCs) are highly stable nanoparticles that self-assemble when phosphatidylserine and calcium are combined. Their unique multi-layer structure permits incorporation of cargo between layers, and they are able to deliver small molecules and small oligonucleotides to cells. Outside cells, normal high calcium levels maintain their crystalline structure, while in tissues they are avidly taken up by certain cells, where much lower calcium levels in the cytosol disrupt their structure and release cargo. We examined the dynamics of LNC uptake and cargo delivery ex vivo in human blood and in cell culture. **Methods:** Initial flow cytometry studies in human whole blood were performed with Cy5.5-labeled empty LNCs (20 and 80 ug/mL) incubated for 3 hours and 24 hours, and examined uptake in neutrophils, monocytes, dendritic cells, and T, B and NK cells, including stimulation with PHA and SEB. Subsequent studies in dual-labeled cargo-carrying LNCs (LNC-FITC, siRNA-Cy5) examined both the LNC uptake and cargo delivery in the same cells. Additional studies were performed to look in more detail at classical, non-classical and intermediate monocyte subsets. Dynamic live cell imaging studies were conducted in HEK293 cells with the same dual-labeled LNCs. 10^4 HEK293 cells were seeded into an imaging chamber system and allowed to attach and rest for 24 hours. Dual-labeled LNCs were added and cells imaged over 24 hours. For higher-resolution mechanistic imaging cells were stained overnight using LAMP1 in blocking buffer with AF555 secondary antibody used for detection; nuclei were stained with Hoescht 33342. Slides were imaged immediately using a LSM confocal microscope. **Results:** Empty LNCs were avidly taken up by innate immune cells (neutrophils, monocytes and mDCs) and B cells, with greater uptake at higher concentrations. Dual-labeled cargo-carrying LNCs showed similar uptake patterns; with siRNA cargo delivery distinct from LNC uptake. Stimulating cells did not noticeably affect uptake or delivery. Additional in vitro dynamic cell imaging corroborated LNC uptake and delivery of siRNA cargo into the cytosol and provided visual confirmation that LNCs encapsulate and protect cargo until intracellular release (**Figure 1**). The preparation protocols for higher-resolution simultaneous endosome/lysosome imaging proved to be incompatible with the current LNC/fluorophore formulation, and details of endosomal escape could not be ascertained. With LAMP1 imaging there was a prominent increase in endosome formation, peaking at 9 hrs; large structures were visible in all cells, strongly suggesting uptake via endocytosis. Both LNCs and cargo were present in the cytosol with cargo outside of endosomes; additional uptake by fusion of LNCs with cell membranes remains a possibility. **Conclusions:** LNCs, with and without cargo, are avidly taken up by innate immune cells in human blood, especially professional phagocytes. Delivery of fluorescently labeled siRNA can be dynamically visualized, with marked endocytotic uptake. LNCs hold considerable promise for intracellular delivery of therapeutics beyond the liver. Future work will examine relative uptake by endocytosis vs fusion, as well as exploring additional details of endosomal escape.



320. Improving Nonviral Gene Expression in T Cells via Inhibition of the Restrictosome

Eric Warga, Jacob Elmer

Chemical and Biological Engineering, Villanova University, Villanova, PA

Gene delivery to T cells has made it possible to successfully treat many serious blood cancers with CAR-T cell therapy. Gene delivery to other cell types enables effective treatment of other conditions, like hemophilia A (Roctavian) and B (Hemgenix), β-thalassemia (Zynteglo), and sickle cell disease (Casgevy). However, the viral vectors used to deliver these genes are associated with high treatment costs,

which could be alleviated by using nonviral vectors. The reason viral vectors are used is for their greater efficiency, which may be attributed to the expression of genes like ICP0 that specifically combat the host cell innate immune response. The goal of this study was to provide a similar inhibition of the innate immune response for plasmid DNA and determine if this inhibition improves nonviral gene expression, particularly in T cells. **Our research has identified ND10 bodies (also called restrictosomes) are problematic components of the innate immune response that repress nonviral gene expression. In this study we show that inhibiting the restrictosome improves nonviral gene expression in T cells.** Figure 1 is a simple diagram of the main proteins of the restrictosome and how they function.

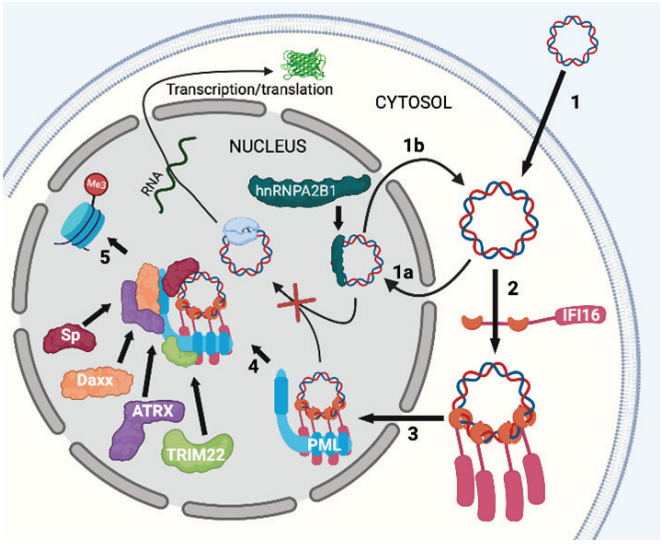


Figure 1. Overview of predicted restrictosome action on plasmid DNA. (1) Plasmid DNA enters cell. (1a) Plasmid DNA unbound by IFI16 enters nucleus. (1b) hnRNPA2B1 transports free plasmid back into cytosol. (2) At least 4 IFI16 monomers bind to plasmid. (3) Plasmid enters nucleus and PML is recruited. (4) PML recruits other complex members Sp100/110/140, Daxx, ATRX, and TRIM22. (5) pDNA is converted to heterochromatin, making transcription impossible. In the first part of this study, we compared the transcriptomes of T cells (e.g., Jurkat and Primary T cells) to HEK-293T cells to determine if the significant difference in transfection efficiency (with Lipofectamine LTX) between these cell types can be correlated to differences in the expression levels of host cell genes involved in the innate immune response. These experiments revealed that HEK-293T cells, which can be transfected to nearly 100% efficiency, lack several components of the restrictosome complex (e.g., IFI16, Sp100, Sp110, Sp140, and TRIM22), as shown in Table 1. Furthermore, an experiment where IFI16 was overexpressed in HEK-293T cells and followed by transfection with a GFP plasmid showed that the average transfection efficiency was reduced to 33.2% in the presence of IFI16 (n=18).

Table 1. Expression of selected genes in transcripts per million reads (TPM). n.d. = not detected.

Gene	Pan-T Cells	HEK-293T
IFI16	280	0.01
PML	65	20
Sp100	80	1.7
Sp110	62	0.23
Sp140	140	n.d.
ATRX	21	13
Daxx	87	110
hnRNPA2B1	730	800
TRIM22	48	0.02

Because the overexpression of IFI16 alone reduced transfection efficiency in HEK-293T cells, we knocked out IFI16 in Jurkat T cells and compared lipofection in the KO cells to lipofection in wild-type cells with GFP as the reporter. While knocking out IFI16 did not have any effect, we knocked out **every restrictosome component at once** and observed that the %GFP$^+$ cells increased by **2.5-fold on average**, measured by flow cytometry (n=12). This demonstrates that the inhibition of the restrictosome is a promising way of improving the efficiency of nonviral gene expression.

321. Megakaryocyte-Derived Extracellular Vesicles Deliver Gene Therapy Cargo That Targets Bone Marrow and Evades the Liver *In Vivo*

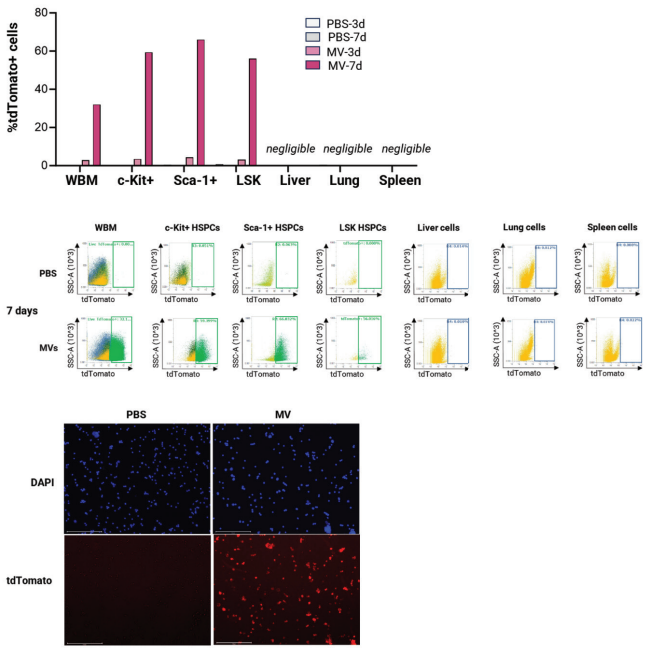
Iain R. Thompson, Navdeep Malik, Joe R. Inigo, Pragati Jain, Jenny T. Korunes-Miller, Diana Hernandez Hernandez, David M. Raiser, Sadettin S. Ozturk, Laura R. Goldberg, Jonathan N. Thon

STRM.BIO, Cambridge, MA

Limitations of current gene therapy tools are well documented, including toxicity, off-target effects, and inability to repeat dose. We are presenting a novel, non-viral based delivery platform, in which megakaryocyte-derived microvesicles (MVs) preferentially target bone marrow *in vivo* and are safe for repeat dosing. Here we demonstrate that MVs loaded with Cre recombinase mRNA, when IV injected into Ai14 Cre-LoxP reporter mice, completely bypass the liver and target bone marrow cells, preferentially editing the hematopoietic stem and progenitor cells (HSPCs). Similarly, when MVs loaded with luciferase mRNA were injected IV into NSG mice, luciferase expression was observed in bone marrow but not in the liver. Bone marrow targeted delivery of gene therapy drug substances that bypass the liver is a significant breakthrough, unachievable to-date by current delivery systems. Primary human CD34+ hHSPCs were differentiated into megakaryocytes *in vitro*. MVs were harvested and loaded with mRNA, encoding either Cre recombinase or luciferase, followed by removal of free cargo. For *in vivo* cargo-mediated protein expression studies, loaded MVs were IV-injected into mouse tail veins (Ai14 or NSG). For *in vitro* studies, HEK293 LoxP-GFP-LoxP-RFP cells were co-cultured with vehicle control or MVs loaded with Cre mRNA for 24-90 hr, followed by microscopy to determine relative fluorescence of GFP and RFP intensities. For Cre-LoxP reporter mice studies, tissues were

collected 3 and 7 days after IV injection, with MV payload delivery quantified by flow cytometry for % cells positive for tdTomato in bone marrow, liver, lung, and spleen, and by microscopy of bone marrow cells from mice injected with vehicle control or loaded MkEVs. For *in vivo* luciferase studies, luciferase expression was quantified by whole body IVIS 24hrs post IV injection. In order to define the cellular specificity and editing efficiency of MVs following IV administration, we employed the Cre-LoxP reporter system. First, we validated our MV loading and potency in HEK293 LoxP reporter cells *in vitro*. We consistently observed successful activation of RFP signal in cells treated with Cre mRNA loaded MVs. When MVs loaded with Cre recombinase mRNA or vehicle control were injected intravenously into Ai14 mice, we observed that tdTomato was specifically expressed in bone marrow cells, with preferential targeting of the HSPC compartment evident at day 7. tdTomato expression was elevated significantly in MV treated mice: 32%, 59%, 66%, 56% vs negligible in control mice, in live bone marrow cells; c-Kit$^+$; Sca-1$^+$; and LSKs, respectively (day 7). Strikingly, liver, lung, and spleen tdTomato levels were negligible and not significantly different between control and MV injected mice (day 3 and 7). NSG mice were injected with LNPs encapsulating HiExpress LUC (control) or MVs loaded with LUC2 mRNA. For LNP injected mice, whole body IVIS imaging revealed predominant signal in the liver. In stark contrast, luciferase expression was detected only in the bone marrow in MV injected animals, completely bypassing the liver. Taken together, these data establish an advanced gene therapy platform that can bypass the liver completely to target bone marrow cells, following IV administration, potentially eliminating the need for current *ex vivo* approaches to treat rare blood disorders.

Figure 1: MVs Deliver Cre recombinase mRNA Exclusively to Bone Marrow in Mice



Graphical representation of tdTomato positive cells identified by flow cytometry **(A)**. Representative flow cytometry plots of cells analyzed 7 days post injection of vehicle (PBS) or Cre-recombinase mRNA loaded MVs **(B)**. Microscopy of dispersed Ai14 mouse whole bone marrow cells after treatment with vehicle (PBS) or Cre-recombinase mRNA loaded MVs **(C)**. MVs, megakaryocyte-derived extracellular vesicles; WBM, whole bone marrow; HSPCs, hematopoietic stem and progenitor cells; LSK, lineage negative/Sca-1+/c-Kit+ hematopoietic stem and progenitor cells.

322. Ultrasound-Facilitated Brain Genome Editing

Yeh-Hsing Lao[1], Robin Ji[2], Naoto Yoshinaga[3], Kathy Snow[4], Chun-Wei Chi[1], Stephen Murray[4], Elisa Konofagou[2], Kam W. Leong[2]

[1]University at Buffalo, The State University of New York, Buffalo, NY,[2]Columbia University, New York, NY,[3]RIKEN, Wako, Japan,[4]The Jackson Laboratory, Bar Harbor, ME

CRISPR-mediated genome editing provides new opportunities to advance molecular medicine. Efficient delivery of CRISPR elements is critical to fully realizing the potential of this technology. CRISPR delivery to the brain is especially challenging because of the blood-brain barrier (BBB), which only permits the passage of certain small molecules. Despite significant efforts to develop new adeno-associated virus (AAV) serotypes with brain tropism and brain-targeting nanoparticles, brain genome editing through systemic administration remains inefficient or limited to certain animal models. Local intracranial administration is still the gold standard for CRISPR delivery to the brain. However, for diseases that may require multiple doses, this invasive administration route is not ideal. Here we present a focused ultrasound (FUS) delivery approach that enables somatic genome editing in the brain in a noninvasive manner. When co-administered with microbubbles, the confined acoustic field can elevate BBB permeability via microbubble cavitation-induced mechanical stress, thereby enhancing penetration of CRISPR element-encoding

AAV or nonviral vectors into the FUS-targeted region. In this study, we first optimized the FUS conditions for AAV delivery followed by screening of a library of DNA-barcoded nonviral polyplexes for brain genome editing. With our inhouse-optimized AAV vector, we demonstrated >25% gene editing efficiency in the target neurons in two different mouse models, while we also identified a potential polyplex composition for this application through our screening. Taken together, we confirm the feasibility of FUS for genome editing in the brain with both viral and nonviral vectors; our findings could potentially add to the armamentarium of somatic genome editing strategies for treating intractable genetic disorders in the brain.

323. Exploring mRNA Expression Dynamics in Mammalian Cells through Live Imaging of Single-Cells

Judith Anna Müller[1], Gerlinde Schwake[1], Sophia Rudorf[1], Joachim Rädler[2]

[1]Faculty of Physics, Ludwig-Maximilians-University, München, Germany,[2]Ludwig-Maximilians-Universität München, München, Germany

Introduction: A lot of effort has been directed towards improving lipid nanoparticle (LNP) mediated mRNA delivery and translation efficiency. However, there remains little control over expression level as dose dependent uptake dominates efficiency. As a consequence, heterogenous biodistribution can lead to local overdosing with possible detrimental effects. To address this issue, mechanisms of intrinsic expression control are required. Here, we investigate incoherent feed-forward loops (icFFLs) as well as variations of ribosome density as two approaches to control the kinetic time course and overall expression level of mRNA mediated gene expression. We use time resolved microscopy of reporter gene expression at the single-cell level to study the modulation of expression dynamics. Firstly, icFFLs are frequently occurring motives of transcriptional gene regulation when transcription factors are co-expressed with downregulating micro-RNAs. As a result, gene expression response is faster, less noisy and the steady state level better controlled. We incorporate this motif into transient LNP based gene expression by encapsulating eGFP-mRNA together with siRNA targeting the same eGFP-mRNA (Fig. 1a). The codelivery of these opposing components demonstrates characteristic features of icFFLs with an expression output determined by the ratio of mRNA and siRNA.

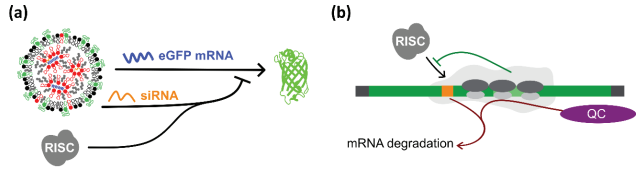


Figure 1: Approaches to control mRNA expression dynamics: (a) Schematic representation of the icFFL model; (b) Modulating mRNA stability via slow-codon windows on the ORF leads to quality control (QC) related degradation with simultaneous protection from RNAi.

Secondly, we employ the mRNA's codon bias within the coding region of mRNA to modulate ribosome movement by insertion of slow-translating codon windows. We then examine the effects of this synonymous recoding on the translation kinetics with a particular focus on the implications for mRNA lifetime. **Methods:** Live imaging on single cell arrays (LISCA) is used to follow many single-cell gene expression time courses in high throughput. Micropatterned substrates with cell adhesive squares (Fig. 2a) facilitate time resolved single-cell live-imaging (Fig. 2b). GFP serves as a fluorescence reporter, enabling quantitative time-resolved measurement of protein expression (Fig. 2c). We describe mRNA translation with biochemical rate equations (Fig. 2d) to assess expression rates, mRNA stabilities (Fig. 2e) and information about timing (Fig. 2f).

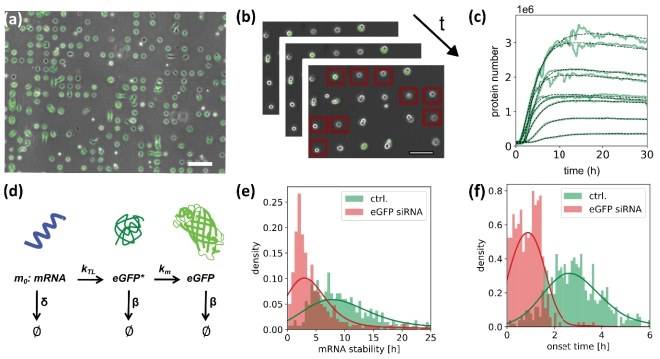


Figure 2: LISCA platform: (a) Exemplary overview of single cells transfected with eGFP mRNA, (b) Long-term imaging under physiological conditions and automated single cell tracking (scale bar corresponds to 400µm), (c) Exemplary fluorescence trajectories extracted with in-house software (green) fitted (dashed black) to (d) Three-stage maturation model for mRNA translation, (e) mRNA stability distribution in presence (red) and absence (green) of targeting siRNA, (f) corresponding onset time distribution.

Results: Codelivery of eGFP-mRNA with siRNA in LNP transfection experiments demonstrates reduced noise in single-cell protein expression levels and control over timing, dependent of the ratio between mRNA and siRNA. Additionally, we find a power law decrease in the total protein expression levels dependent on siRNA/mRNA ratio. The data show typical features of icFFLs gene regulation and implement self-regulation in transient mRNA transfections. Furthermore, we found that insertion of slow codon windows in the open reading frame of GFP mRNA enables tuning of its stability. We report a position dependence of slow-codon windows on mRNA lifetime. This effect inverts dependent on the presence or absence of targeting siRNA. Both approaches represent potential toolboxes towards intrinsically regulated gene expression for future combinatorial LNP based mRNA therapies.

Targeted Gene and Cell Therapy I

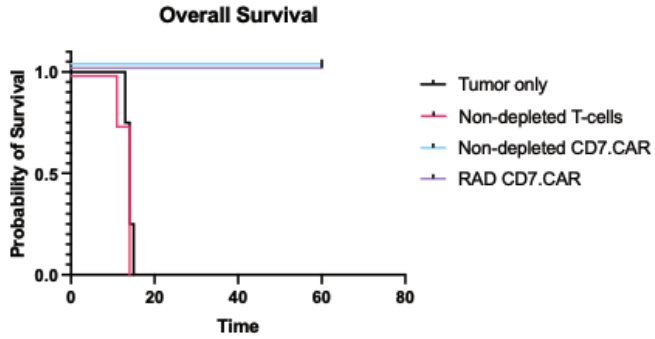
324. Healthy Donor-Derived Unedited CD7. CAR T-Cells for Improved Therapy of T-Cell Malignancies

Daniil Shmidt[1], Chiou-Tsun Tsai[1], Norihiro Watanabe[1], Maksim Mamonkin[1,2,3]

[1]Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX,[2]Texas Children's Hospital, Houston, TX,[3]Houston Methodist Hospital, Houston, TX

Patients with treatment-refractory or relapsed T-ALL and T-cell lymphoma have limited therapeutic options and poor prognosis. Development of CAR T-cell strategies targeting prominent surface antigens in T-cell neoplasms, such as CD7, requires mitigating unwanted targeting of normal T-cells that express the same antigens using gene editing or additional transgenes. Additional challenges include high levels of circulating leukemia in patients with treatment-resistant disease, which increases the risk of accidental CAR transduction, and low quantity and poor fitness of normal T-cells following multiple

rounds of lymphotoxic therapies. Treatment with allogenic CAR T cells manufactured from third-party donors overcome the latter challenges but produce short-term activity in HLA-mismatched patients and often induce fatal infectious complications due to the use of high-intensity lymphodepletion required to delay immune rejection. We have recently developed an alternative strategy to manufacture unedited CD7. CAR T cells where CAR T-cell fratricide is minimized by reversible pharmacologic inhibition. In the ongoing Phase I clinical trial at Baylor College of Medicine (NCT03690011), autologous unedited CD7.CAR T cells expand and select for fratricide-resistant CD7-negative CART cells with long-term functional persistence producing MRD-negative remissions in patients with recalcitrant T-cell malignancies. However, manufacturing of autologous CD7.CAR T-cell products for many patients with advanced disease has been difficult due to high levels of circulating disease and low quantity and quality of normal T cells. To overcome these limitations, we explored manufacturing unedited CD7. CAR T cells from haploidentical donors, such as parents or siblings. Potentially alloreactive T cells are removed using CD45RA depletion (RAD); importantly, this depletion preserves fratricide-resistant CD7-negative T cells, most of which are CD45RA-negative. Indeed, we show unedited CD7.CAR T cells generated from RAD T cells expand post-transduction in the presence of pharmacologic inhibitors dasatinib and ibrutinib and produce robust cytotoxicity in vitro against CD7+ malignant T cells (99.4% cytotoxicity over 3 days at 1:1 effector:target ratio), on par with control CD7.CAR T cells generated from non-depleted T cells. Critically, we observed strong anti-leukemic activity of RAD CD7.CAR T cells in a mouse xenograft model of human T-ALL resulting in complete and durable eradication of leukemia in most experimental animals and a significantly prolonged survival compared to control mice ($p<0.001$). Notably, despite an enrichment for effector/memory cells in the RAD CD7.CAR T-cell pre-infusion product, about half of long-persisting CD7.CAR T cells (60 days post-infusion) acquire a central memory phenotype. Anti-leukemic activity of RAD CD7. CAR T cells was similar to non-depleted control CD7.CAR T cells thus supporting feasibility and efficacy of donor-derived RAD CD7.CAR T cells for clinical translation. We surmise this method would expedite the treatment of patients with rapidly progressing disease while utilizing healthy donor T cells without the risk of malignant cell transduction. A clinical trial with donor-derived RAD CD7.CAR T cells for patients with refractory or relapsed T-ALL and T-cell lymphoma will open at Baylor College of Medicine in 2024.



325. Protection of CD33 Modified HSC Progeny from CD33-Directed CAR T Cells in Nonhuman Primates

Nicholas E. Petty[1,2], Stefan Radtke[1], Emily Fields[1], Olivier Humbert[1], George S. Laszlo[1], Sam Fiorenza[1], Mallory Llewellyn[1], Justin Thomas[1,2], Zach Burger[1], Seunga Choo[1], Greta Kanestrom[1], Haiying Zhu[3], Keith R. Jerome[3,4], Cameron J. Turtle[1], Roland B. Walter[1,3], Hans-Peter Kiem[1,3]

[1]Translational Science & Therapeutics, Fred Hutchinson Cancer Center, Seattle, WA,[2]Medical Scientist Training Program, University of Washington, Seattle, WA,[3]Laboratory Medicine & Pathology, University of Washington, Seattle, WA,[4]Vaccine & Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA

Acute myeloid leukemia (AML) is a devastating hematologic malignancy. Current work towards developing new anti-AML therapies is stymied by the shared antigen profile of leukemic cells with healthy myeloid cells. An example of this challenge is CD33, which is overexpressed on some AMLs but also widely expressed on mature and maturing myeloid cells. To overcome this challenge our group has investigated gene editing technologies to modify CD33 on hematopoietic stem cells (HSCs). This has been explored in conjunction with therapeutics such as CD33-directed chimeric antigen receptor T cells (CAR33), a combination that is now under investigation in the allogeneic cell transplant setting. However, the kinetics of CAR33 activity and protection conferred by CD33 editing have not yet been studied in an autologous large animal model. Here we report the development of a novel CAR33 construct and demonstrate its activity in vitro and in vivo. We further investigate the impact of CAR33 in two nonhuman primates (NHPs) that had previously received CD33-modified autologous HSC transplantations (HSCTs). We generated a second-generation CAR construct based on the antibody structure of a known CD33-binder. CAR functionality and specificity were validated in vitro using both chromium release and Xcelligence real-time cell analysis assays. CAR33 T cells were then infused into two animals previously transplanted with CD33-modified HSCs using CRISPR/Cas9 or base editor (BE). After CAR infusion, both animals were monitored for cytokine release syndrome (CRS), changes in immune composition in the peripheral blood (PB) and bone marrow (BM), and representation of edited cells within the PB and BM. The CAR33 construct showed specific, dose-dependent killing of $CD33^+$ cells in both in vitro assays. The two animals treated with CAR33 showed variable responses, with one animal displaying symptoms of CRS (lethargy, elevated temperature, elevated IL-6) and one animal remaining asymptomatic. CAR33 expanded in both animals as demonstrated by complete blood count and flow cytometric analysis of lymphocyte and T cell compartments in the PB, respectively. T cell expansion coincided with depletion of $CD33^+$ cells, primarily within the granulocyte compartment, as well as enrichment of CD33-modified peripheral blood mononuclear cells as assessed by digital droplet PCR and next generation sequencing. CAR expansion was transient, with T cell preponderance and CAR marking peaking at 7 days post-infusion and waning to pre-infusion levels after 14 days. Initial depletion of $CD33^+$ granulocytes reverted to baseline 1-2 months post-infusion, coinciding with a return to pre-treatment levels of editing. Here we report the first study of CAR33 in NHPs with CD33-edited HSCs.

We also demonstrate that BE rather than CRISPR/Cas9 editing CD33 confers similar protection from CAR33, a critical transition in mitigating the use of technology associated with double strand breaks in DNA and large-scale genomic recombination events. CD33-edited cells in the PB were selectively spared by our CAR33 T cells, leading to transient enrichment of edited cells within the PB compartment. Taken together, we demonstrate safety of CAR33, protection conferred to CD33-edited cells, and validated BE as an alternative, DSB-independent strategy to edit CD33. This work offers further support for the use of CAR33 in conjunction with CD33-modified HSCT for the treatment of AML.

326. Chimeric Antigen Receptor Discovery Through T Cell Display

Joseph J. Muldoon[1,2], Chang Liu[1,2], Charlotte H. Wang[1,2], Zhongmei Li[2], Akul Naik[3], Matthew A. Nix[3], Arun P. Wiita[3,4,5,6], Justin Eyquem[1,2,7,8,9]

[1]Department of Medicine, UCSF, San Francisco, CA,[2]Gladstone–UCSF Institute of Genomic Immunology, San Francisco, CA,[3]Department of Laboratory Medicine, UCSF, San Francisco, CA,[4]Department of Bioengineering and Therapeutic Sciences, UCSF, San Francisco, CA,[5]Helen Diller Family Comprehensive Cancer Center, UCSF, San Francisco, CA,[6]Chan Zuckerberg Biohub San Francisco, San Francisco, CA,[7]Parker Institute for Cancer Immunotherapy, UCSF, San Francisco, CA,[8]Department of Microbiology and Immunology, UCSF, San Francisco, CA,[9]Institute for Human Genetics, UCSF, San Francisco, CA

A standard approach for chimeric antigen receptor (CAR) discovery is to select for binders that are high affinity using phage or yeast display, characterize them on receptors in vitro, and assess a subset in vivo. However, this process is low-throughput and based on metrics that do not predict CAR efficacy. To accelerate discovery and select directly for anti-tumor potency, we developed T cell display, in which binders are functionally screened in primary cells. Specifically, CARs are integrated as a pooled knock-in library at the T cell receptor alpha constant (*TRAC*) locus, which confers monoallelic and homogeneous expression. To investigate this new approach, a pool of 25 nanobodies (VHHs) targeting the B cell leukemia antigen CD72 was cloned into a CAR backbone. Libraries were packaged as adeno-associated virus homology-directed repair templates and integrated via CRISPR/Cas editing (>75% knock-in). T cells were subjected to in vitro assays and to an in vivo challenge with or without NALM6 leukemia followed by bone marrow harvest. Binders were amplified from genomic DNA and sequenced to identify those that promote antigen-dependent expansion. Arrayed validation against NALM6 leukemia and JEKO1 lymphoma indicated that higher-ranked CARs indeed led to more expansion, better survival, and lower tumor burden. Notably, in vitro assays for cytokine production ($R^2 < 0.1$) and proliferation ($R^2 < 0.4$) did not correlate well with these outcomes. Therefore, in vivo T cell display accurately identified potent receptors. Affinity measurement is another low-throughput process. To address this, we took advantage of the scalability of T cell display to develop a method using recombinant antigen, flow-sorting, and sequencing for pooled measurement. A formula that weights the MFI of sorting bins by library member abundances was developed to calculate an affinity score. Comparison of these scores with other assays indicated that antigen escape occurred with a high-affinity binder, the best CAR was moderate affinity, and the highest cytokine production was from a moderate-affinity one. Furthermore, although CARs that did poorly in vivo were low affinity, those that did well were not necessary high affinity (overall $R^2 = 0.23$). These results support the utility of screening in the *TRAC* CAR-T cell context to map various receptor characteristics. We next examined whether a top VHH could be ported onto the HLA-independent TCR (HIT), which with an scFv improves antigen sensitivity over a conventional CAR. We modified the original HIT design and explored VHH placement on the alpha and beta chains. VHH on both chains compared to either alone synergistically increased antigen binding, which might be because CD72 is a homodimer. Importantly, VHH-HIT exhibited improved sensitivity vs. CAR against low-antigen-expressing cells in vitro; in vivo studies are ongoing. Thus, a VHH identified on a CAR can function on a HIT and retain its intended advantage. In all, T cell display identifies the best binders. We have also generated data showing this method is extensible to scFv binders and much larger libraries (~1,000). This novel method for binder discovery can streamline the development of immune cell therapies for cancer.

327. "Off-the-Shelf" Combination CAdVEC and CAR-NK Cell Immunotherapy for Pancreatic Ductal Adenocarcinoma

Greyson W. Biegert[1], Amanda Roswell Shaw[1], Caroline E. Porter[1], Daisuke Morita[2], Masataka Suzuki[1]

[1]Baylor College of Medicine, Houston, TX,[2]Shinshu University, Matsumoto, Nagano, Japan

To overcome the hostile, immunosuppressive tumor microenvironment (TME) and unreliable tumor-associated antigen expression in solid tumors, our laboratory exploits the distinct properties of local oncolytic adeno-immunotherapy (CAdVEC) and systemic CAR-modified immune effector cells. Currently in clinical testing, CAd*Trio* (expressing IL-12p70, anti-PD-L1 minibody, and HSV-tk safety switch) enhances both adoptively transferred CAR-T cells and endogenous NK cell anti-tumor activities in preclinical animal models, including pancreatic ductal adenocarcinoma (PDAC); a class of tumors that are difficult to treat and often characterized by limited immune cell activity within the TME. Our clinical data indicate that local CAd*Trio* treatment induces significant anti-tumor effects and is safe in combination with systemic autologous CAR-T cells (NCT03740256). In light of this success, quality autologous T-cell products are difficult to generate consistently and have high production costs. To circumvent these challenges, adoptive NK cell therapy is frequently investigated as a readily available "off-the-shelf" alternative. Since CAd*Trio* transgenes improve NK cell expansion and anti-tumor effects, we hypothesized that CAd*Trio* combined with adoptive CAR-NK cell therapy could provide sustained systemic tumor targeting as an "off-the-shelf" combination immunotherapy. CAR-NK cell treatment alone has minimal anti-tumor effects against PDAC in immunodeficient mouse models. However, the addition of CAd*Trio* treatment significantly improves CAR-NK cell persistence and anti-tumor effects, resulting in increased survival. To understand the underlying mechanism, we analyzed RNAseq data that indicates CAdTrio-derived transgenes activate memory-like NK cell developmental pathways. Since CAR-NK cells kill PDAC cells through natural ligand/receptor interactions in the absence of CAR target antigen, unlike CAR-T cells, we tested the efficacy of CAR-NK cells combined with CAd*Trio* in a heterogeneous

PDAC PDX tumor model. We observed that CAR-NK and CAd*Trio* combination treatment consistently controlled tumor growth superior to single-agent therapy. In humanized mouse models, this "off-the-shelf" combination immunotherapy is well tolerated and significantly controls PDAC tumor growth. Currently, we are investigating the contribution of the endogenous immune system in the context of this combination immunotherapy. Overall, our data indicates local CAd*Trio* and systemic CAR-NK cell combination immunotherapy may be an effective immunotherapy for treatment-resistant PDAC and potentially other immunologically "cold" solid tumors.

328. Selective Support of CAR-T Cell Therapies by Cis-Targeted IL2

Sara Sleiman[1], Tyler Hansen[1], Hannah Thomas[2], Jack Swain[2], Raimon Duran-Struuck[2], Nathan D. Mathewson[3], Feng Shen[1], Kelly D. Moynihan[3], Paul Bessette[3], Chris Kimberlin[3], Meghana Sukthankar[3], Terrence Park[3], Andy Yeung[3], Ivana Djuretic[3], Saar Gill[1,4,5]

[1]Center for Cellular Immunotherapies, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA,[2]Department of Pathobiology, University of Pennsylvania School of Veterinary Medicine, Philadelphia, PA,[3]Asher Biotherapeutics, South San Francisco, CA,[4]Division of Hematology-Oncology, Department of Medicine, Perelman School of Medicine at the University, Philadelphia, PA,[5]Abramson Cancer Center, University of Pennsylvania, Philadelphia, PA

INTRODUCTION In vivo behavior of CART cells has shown a strong correlation with improved outcomes, which has spurred an interest in developing approaches to enhance the expansion and persistence of infused CART cells. Although one proposed method combines CART cell therapy with exogenous cytokines such as the IL2 family, the pleiotropic nature of these cytokines can activate other immune cells, leading either to fatal toxicities or weakening of the immune response. Even engineered IL2 variants with attenuated binding to the IL2 receptor remain non-selective for CART cells. Other methods using orthogonal cytokine/cytokine receptors require further genetic modification of T cells. We have developed CAR-T-specific IL2 fusion molecules that selectively activate CART cells by recognizing an extracellular tag to address these challenges. METHODS Cis-targeted cytokine is comprised of a targeting antibody directed against an exogenous tag expressed on the CAR-T surface (truncated non-signaling epidermal growth factor receptor [EGFRt]) and an IL2 mutein with attenuated binding to IL2Rα and IL2Rβ subunits (EGFRt-IL2). The targeting arm of the fusion molecule enhances the functional avidity of the attenuated cytokines, resulting in potent yet selective activation of the targeted cells. RESULTS In vitro, the EGFRt-IL2 fusion molecule was able to rescue the proliferation of a small population of CART cells taken from the peripheral blood of a lymphoma patient 7 days after the patient received an infusion of a commercial CART19-targeting CAR-T cell that also expresses EGFRt. In vivo activity was demonstrated in NSG mice bearing an acute lymphoblastic leukemia (ALL) NALM-6 cell line treated with a suboptimal dose (0.1 x106) of healthy donor EGFRt-CART19 cells with or without the IL2 mutein. EGFRt-IL2 converted the suboptimal dose of CAR-T cells to a curative therapy (CR 5/5), compared to CAR-T cells alone (CR 0/5). The enhanced anti-tumor activity was subsequently confirmed in a model in which the CART cells were generated from a lymphoma patient. Response to treatment was accompanied by CART cell expansion resulting in superior survival in the mutein-treated mice ($p = 0.0015$). CONCLUSION In summary, EGFRt-IL2 enhances in vivo anti-tumor activity and survival in xenografted mice and enhances the activity of lymphoma patient CAR T cells. Given the unique ability to selectively expand EGFRt-expressing CAR-T cells, EGFRt-IL2 may have the potential to reduce the need for lymphodepletion prior to CART cell infusion. Temporal control of the cis-targeted cytokines directed by anti-tag antibodies represents a promising approach to enhance CART cell therapies.

329. ADI-270: An Armored Allogeneic Anti-CD70 CAR γδ T Cell Therapy Designed for Multiple Solid and Hematological Cancer Indications

Yvan Chanthery, Gauri Lamture, Nadine Jahchan, Melinda Au, Alexander G. Teague, Morgan Smith-Boeck, Michael Salum, Yogendra Verma, Ramandeep Kaur, Smitha Rao Yelaguli Gundurao, Jie Zhang, Amy Doan, Jyothi Sethuraman, Ngoc T. Hoang, Hayden Tessman, Philip Storm, Soojin Han, Swapna Panuganti, Christopher J. Rold, Marissa Herrman, Arun Bhat, Kevin P. Nishimoto, Shon Green, Blake T. Aftab

Adicet Bio, Redwood City, CA

Background: CD70 is a compelling target for CAR T cell therapy, given its elevated expression across multiple solid and hematologic malignancies, with expression in normal tissues limited to a subset of activated lymphocytes. γδ T cells combine innate and adaptive immunity and their capability to infiltrate solid tumors significantly correlates with clinical benefit. We have previously described ADI-270, an allogeneic CAR γδ T cell product engineered with a next-generation CAR to target CD70 using its natural receptor (CD27) as the binding moiety and armored with a dominant negative form of the TGFβ receptor (dnTGFβRII) that provides functional resilience to the immunosuppressive tumor microenvironment (TME) found in many solid tumors. We have shown that the design of ADI-270 provides enhanced functionality and potency in CD70+ expressing tumors such as clear cell renal cell carcinoma (ccRCC) models, where we observed robust infiltration and persistence, resulting in significant preclinical efficacy. Here we expand on previous data, evaluating the kinetics of ADI-270 activity in the ccRCC model and its efficacy against additional tumor indications, including models designed to mimic the low and/or heterogeneous antigen expression profile expected in most cancers. **Methods:** CD70 expression profiling by flow cytometry and immunohistochemistry (IHC) on tumor microarrays (TMAs) was used to identify tumor indications for which ADI-270 represents a potential therapeutic. Healthy donor PBMCs were used to activate, expand, and engineer Vδ1 T cells to express the armored anti-CD70 CAR construct. Functional characterization of ADI-270 against cancer cells with varying levels of CD70 expression was determined using cell-based cytotoxicity assays. Human tumor xenografts in NSG mice were used to evaluate the in vivo efficacy of ADI-270 against solid and hematological cancers including ccRCC, NSCLC, and T cell lymphoma. Tumors and blood were collected from A498 xenograft

models for pharmacodynamic analyses using IHC, multiplex IF, and Luminex. In addition, cytotoxicity assays against mixed CD70 +/- expressing cells were used to inform functional comparisons between ADI-270 and multiple clinically relevant benchmarks representing scFV-based anti-CD70 CAR approaches. **Results:** Elevated CD70 expression was confirmed in various solid and hematological tumor indications, with frequency being highest in ccRCC samples. ADI-270 exhibited potent in vitro cytotoxicity against a range of cancer cell lines expressing various levels of CD70. CAR-mediated killing of CD70(+) tumor cell lines by ADI-270 promoted enhanced γδ T cell activity, including innate and adaptive activity against CD70(-) tumor cells. ADI-270 significantly inhibited tumor growth in CD70+ ccRCC (A498), NSCLC (H1975) and T cell lymphoma (Hut78) human tumor xenograft models in NSG mice. In A498 tumor xenografts, robust ADI-270 tumor infiltration, proliferation, and effector function were observed at the earliest assessment time point (Day 3), leading to potent efficacy and eradication of CD70+ tumor cells. **Conclusion:** These results demonstrate the activity of ADI-270 in multiple cancers, which includes innate anti-tumor immunity, potent next-generation CAR targeting, and resilience to attenuation by TGFβ. Based on the functional characterization demonstrated to date, further clinical evaluation of ADI-270 is warranted in ccRCC and additional CD70+ cancers.

330. Discovery of Novel CARs for Solid Tumors Using Senti REVEAL™, a Massively Parallel Technology Platform Comprising Pooled Screening, Machine Learning, and Lab Automation

Tony Hua, Marcus Gainer, Brian Garrison, Kanya Rajangam, Timothy K. Lu, Nicholas W. Frankel
Senti Biosciences, South San Francisco, CA

Introduction. CAR-T and CAR-NK therapies have limited efficacy in solid tumors due to multiple factors including the immunosuppressive tumor microenvironment and the limited potency of the signaling domains of current approved CAR therapies. More potent CAR signaling domains may address this limitation by activating different pathways (or combinations) that increase robustness to the immunosuppressive tumor microenvironment. FDA-approved CAR therapies for liquid tumors tend to use a limited set of intracellular domains, highlighting a need for new signaling domains for solid tumors. Conventional approaches of screening and optimizing CAR designs are manual labor intensive, low throughput, and can suffer from operator and batch error. **Methods**. We developed a massively parallel technology platform, REVEAL™ (Research Engine for Validation of Engineered Asset Libraries), comprising (1) pooled screens of CAR libraries of 10,000s to 100,000s of signaling domains, (2) machine learning models to predict performance, and (3) parallel clonal validation of 100s to 1,000s of candidates using a purpose-built automated liquid handler. Here we used CAR-NK cells, but we anticipate that this framework can be used for other types of effector cells such as CAR-T. We designed libraries of CARs with a fixed extracellular domain targeting CEA, an antigen expressed on a range of solid tumors including colorectal cancer, and over 50,000 combinatorial signaling domains consisting of arrays of subdomains derived from native receptors. High-performing CARs in the libraries were discovered using a sort-seq approach: cells expressing CARs driving robust degranulation (CD107a+) were sorted, and CARs enriched in these cells were identified via NGS. We used these data to train machine learning models mapping CAR structure (i.e., the position and identity of constituent subdomains) to performance. In addition to reproducing experimental CAR performance, these models were used to predict CAR structures not present in the dataset due to cloning or coverage constraints. The highest performing library CARs and model-predicted CARs were then clonally validated in parallel using a liquid handler-based workflow that automated viral production, NK transduction, co-culture assay setup, and imaging-based readout of target killing. **Results**. Our library screens and modeling indicate, and our clonal validations confirm, that signaling domains enriched for TRAF-binding motifs have high performance in the context of anti-CEA CAR-NK cells. Among highest performing CARs, however, TRAF domains are typically accompanied by other functional motifs, constituting a more diverse signaling array than would be found in a single native immune receptor. Successful CARs often comprised multiple canonical immune signaling types, i.e., signal 1 (CD3z), signal 2 (one or more costimulatory motifs) and signal 3 (cytokine signaling), demonstrating that a variety of signaling features can be successfully packaged into a single compact CAR. Additionally, these CARs contained sequences from a wide variety of sources outside of canonical CAR domains such as CD3z, CD28, 4-1BB, indicating the untapped potential for designing new CARs for solid tumors. **Conclusion**. The REVEAL™ framework described here is capable of extensive optimization of CAR structures, and could potentially be applied to development of potent CAR cell therapies targeting solid tumors and hematologic malignancies beyond B cell cancers.

Oligonucleotide Therapeutics

331. Prenatal Intra-Amniotic Administration of an Antisense Oligonucleotide Achieves Widespread Biodistribution with Therapeutic Levels in Fetal Lambs

Beltran Borges[1], Maria T. Clarke[1], Akos Herzeg[1], Joshua Ross[2], Jae Hong Park[2], Daniel Ramos[2], Lingling Kong[2], Nalin Gupta[1], Stephanie Klein[3], John Matson[3], Frank Bennett[3], Charlotte Sumner[2], Tippi C. MacKenzie[1]
[1]University of California San Francisco, San Francisco, CA,[2]Johns Hopkins University, Baltimore, MD,[3]Ionis Pharmaceuticals, Carlsbad, CA

Introduction: The antisense oligonucleotide (ASO) nusinersen improves outcomes of patients with spinal muscular atrophy (SMA). Despite neonatal treatment, patients with severe genotypes still have suboptimal outcomes due to prenatal disease. We hypothesized that intra-amniotic (IA) injection of ASO may penetrate the CNS prior to formation of the blood-brain barrier. We tested the safety and efficacy of IA delivery of a therapeutic ASO in fetal SMA mice and fetal lambs. **Methods:** An SMN2-targeting ASO was injected IA in E13.5 fetal wild-type (WT) and Δ7-SMA mice and full-length SMN2 mRNA, survival, weights, motor behavior, and motor neuron number were assessed. We next injected the same ASO in fetal lambs (using both

IA and intra-cisterna magna (IC) injections [the latter as a positive control for CNS delivery]) and measured biodistribution using a dual-probe hybridization-electrochemiluminescent (ECL) assay and immunohistochemistry (IHC) and evaluated safety parameters including tissue histopathology, serum blood counts, coagulation panels and liver and kidney function tests. **Results:** *In mice,* SMA pups treated prenatally + postnatally showed improved weight gain, behavior, and survival compared to those treated postnatally alone. SMA mice treated prenatally also showed increased motor neuron numbers in lumbar spinal cord (SC) segments compared to postnatally treated SMA mice that were comparable to WT. *In sheep,* we injected 7 fetal lambs IA [137mg (n=2); 500mg (n=3); 1000mg (n=2)) and 3 fetal lambs intra-cranially (30 mg) at E75 (term=150 d). Survival to harvest at E96-E104 was 100% in all groups, with no growth restriction. We detected broad biodistribution of the ASO by hybridization-ECL in numerous fetal tissues after IA injection, with therapeutic levels (>1-10 μg ASO/g tissue) seen in the SC and skeletal muscle at 500-1000 mg doses. Supra-therapeutic levels were detected in liver and kidney after IA injection but there were no concerning histopathological changes in any fetal organ or derangements in serum chemistries. ASO was also detected by IHC, with more robust staining in liver, kidney, and lungs compared to SC. Maternal exposure to the ASO was only detected in the uterus of some dams, without spread to other organs or alterations in serum chemistries. **Conclusion:** Intra-amniotic ASO delivery ameliorates disease in SMA mice and appears safe for both fetus and mother in a large animal model. Levels of fetal CNS penetration are relevant for SMA while distribution to fetal lung and skeletal muscle suggest therapeutic utility in other disease settings. Biodistribution in the placenta and fetal respiratory and gastrointestinal tracts following IA administration suggest routes of entry into the fetal circulation. Accumulation in kidney and liver in lambs, likely due to recirculation after renal excretion, could potentially be mitigated through interval amniotic fluid exchange. Prenatal IA ASO therapy is a promising strategy to provide early, minimally invasive disease-modifying therapy without the risks of permanent genetic alterations associated with viral gene therapy or gene editing.

332. Targeting NLRP3 Splicing Variants with Antisense Oligonucleotides to Control Pathological Inflammation

Roni Klein[1], Janset Onyuru[2], Estela Viera[1], Lijo John[3], Rahul Vijay[3], Hal M. Hoffman[2], Michelle L. Hastings[4]

[1]Center for Genetic Diseases, School of Graduate and Postdoctoral Studies, Rosalind Franklin University of Medicine and Science, North Chicago, IL,[2]Department of Pediatrics, University of California, San Diego, CA,[3]Center for Cancer Cell Biology, Immunology and Infection, Department of Microbiology and Immunology, Rosalind Franklin University of Medicine and Science, North Chicago, IL,[4]Department of Pharmacology, University of Michigan, Ann Arbor, MI

Introduction: The innate immune system defends against pathogenic invaders and sterile insults. NLRP3 inflammasome activation, a key component of this defense mechanism, leads to IL-1β, IL-18 release and pyroptosis (inflammatory cell death). Aberrant NLRP3 activation is implicated in autoinflammatory, metabolic, and neurodegenerative diseases. To date, there are no approved NLRP3 inhibitors in the clinic. Alternative splicing of NLRP3 generates various mature RNA isoforms with distinct function. We hypothesize that manipulating alternative splicing using antisense oligonucleotides (ASOs) can be used to modulate NLRP3 activity and provide a means to treat NLRP3-driven inflammatory diseases. **Methods:** To identify NLRP3 spliced isoforms that lack the ability to activate inflammatory processes, we systematically evaluated the effects of NLRP3-targeted splice-switching ASOs on inflammasome activation in vitro in human THP-1 cells and mouse iBMDMs. We tested one promising ASO from our in vitro studies in a mouse model of acute inflammation following an intraperitoneal LPS injection and cryopyrin associated periodic syndrome (CAPS) myeloid-specific constitutive NLRP3 hyperactivation. **Results:** ASOs induced alternative splicing of NLRP3, knocked down NLRP3 protein expression, and inhibited IL-1β and IL-18 release in human THP-1 cells and mouse iBMDMs. Treatment with our most active ASO significantly reduced IL-1β and IL-6 levels in the plasma of LPS-injected mice. In $Nlrp3^{D301N+/LysMCre+}$ CAPS model, ASO treated mice exhibited improved survival and reduced IL-1β and IL-6 levels in the plasma compared to dPBS control littermates. **Conclusions:** This study demonstrates that altering NLRP3 splicing can modulate protein activity, revealing the importance of specific NLRP3 domains in inflammasome activation and identifying potential therapeutic targets for mitigating pathological activation of the inflammasome. To this end, splice-switching ASOs can specifically induce inactive spliced isoforms of NLRP3 to effectively inhibit the NLRP3 inflammasome. ASO treatment is efficacious in mouse models of acute inflammation and CAPS, providing evidence for a novel approach for treating NLRP3-mediated inflammation.

333. First in Class ASO Targeting *IGHMBP2* Cryptic Splice Variant: Efficacy and Safety

Sandra Paulina Smieszek, Bart Przychodzen, Christina Tyner, Caroline Johnson, Christos M. Polymeropoulos, Gunther Birznieks, Mihael H. Polymeropoulos

Vanda Pharmaceuticals Inc., Washington, DC

Charcot-Marie-Tooth disease Type 2S (CMT2S) is a rare autosomal recessive Charcot-Marie-Tooth disease subtype. Rare variants in immunoglobulin mu-binding protein 2 (*IGHMBP2*) have been shown to cause CMT2S by resulting in abnormal RNA processing leading to alpha-motor neuron degeneration. A patient was reported with pathogenic variants in *IGHMBP2*. Whole genome sequencing (WGS) revealed a paternally inherited cryptic splice site variant (non-coding variant (c.1235+894 C>A) deep in intron 8). The resulting transcript undergoes nonsense-mediated decay (NMD), resulting in IGHMBP2 haploinsufficiency. Our objective was to target this cryptic splice site, rescuing IGHMBP2 protein levels with a novel antisense oligonucleotide (ASO). We obtained the patient's fibroblast cell line and confirmed the variant with WGS and the existence of NMD. A 19mer ASO was designed to target deep in intron 8 (c.1235+894 C>A), around sequence CACTTCCAC(A)GGGGGAAGA. Fibroblast cells underwent ASO treatment (1μM) and 48-hour incubation. Induced pluripotent stem cells (iPSCs), iPSC-motor neurons (iPSC-MNs), and skeletal myofibers (SkM) stocks were generated through expansion and differentiation of the CMT2S patient's fibroblasts. We characterized the physiology of CMT2S-MNs through patch-clamp electrophysiology and examined subsequent neuromuscular junction (NMJ) defects. Derived MNs were then incubated with ASO, and treatment effects were quantified using Hesperos Inc.'s *in vitro* NMJ

system platform. Upon patient fibroblast treatment with ASO, we observed a significant IGHMBP2 protein level increase (~50-70%) in treated samples compared to control (untreated) samples (WB antibody Sigma SAB2106426). qPCR results confirmed an increased ratio of restored wild-type transcript to cryptic exon-containing transcript (~1.8-fold). RNA sequencing further confirmed an increase of *IGHMBP2* expression of 1.3$\log_2$-fold (adjusted p-value < 0.002) following treatment. Preclinical data support this ASO as a potential treatment for restoring IGHMBP2 protein levels, with limited off-target effects *in silico*. Electrophysiological studies revealed hyperexcitability and spontaneous firing of CMT2S-MNs, resembling an amyotrophic lateral sclerosis (ALS) phenotype, and a reduced resting membrane potential, capacitance, and cell body area, as compared to WT-MNs. Analysis of the CMT2S NMJ system revealed a higher fatigue index (FI) compared to the WT NMJ system. Following muscle contraction, the CMT2S NMJ system showed quick fatigue, revealing a tetanus followed by decay phenotype. We examined the effects of ASO treatment and demonstrate rescue of NMJ functioning, as captured by a decrease in fatigue index. NMJ tetanus characterization following treatment shows this rescue with an increase in classical tetanus and a decrease in decay and sporadic responses. To evaluate the potential toxicity of the ASO, a 3-month rat toxicity study was conducted. Transient clinical observations were noted following administration; however, findings were resolved within 24 hours. There were no neurobehavioral changes noted at the 24-hour post-dose evaluation. The no-adverse-event-level (NOAEL) was set at the highest dosage. An increased number of autosomal recessive CMT2S cases caused by *IGHMBP2* pathogenic variants are being reported. The N-of-1 precision medicine approach may prove instrumental to the design of treatments for this highly diverse genetic disorder. Patient-specific phenotypic analysis of MNs and subsequent NMJ systems further confirms the genetic diversity of this disorder. This N-of-1 case exemplifies the shifting boundary between rapid WGS-based clinical diagnoses and research capabilities allowing for the design of personalized ASO-based treatments, serving as a framework for others with ASO-amenable mutations in *IGHMBP2*.

334. Potent, Durable mRNA Knockdown in Extrahepatic Tissues Using siRNAs with Novel Phosphoryl Guanidine Backbone Variants

Wei Liu, Naoki Iwamoto, Subramanian Marappan, Himali Shah, Snehlata Tripathi, Erin Purcell-Estabrook, Khoa Luu, Anthony Lamattina, Qianli Pan, Fangjun Liu, Frank Favaloro, Arindom Chatterjee, Tomomi Kawamoto, Genliang Lu, Jake Metterville, Priyanka Prakasha, Hailin Yang, Yuan Yin, Lola Owen, Hui Yu, Michael Byrne, Pachamuthu Kandasamy, Chandra Vargeese

Wave Life Sciences, Cambridge, MA

Conjugation of *N*-Acetylgalactosamine (GalNAc) to small interfering RNA (siRNA), which enhances their receptor-driven uptake by hepatocytes, has driven the successful *in vivo* knockdown of liver mRNAs. By contrast, the development of siRNA chemical modifications that result in potent, durable knockdown in extrahepatic tissues remains a major challenge for the field. Previously, we showed that incorporating phosphoryl guanidine (PN) backbone linkages in GalNAc-siRNAs can improve the potency, tissue exposure, and durability of knockdown in mouse liver *in vivo*. We have recently developed a new stereopure siRNA design that, when incorporated with chemically modified PN linkages called "PN variants," results in potent, durable knockdown in extrahepatic tissues *in vivo*. To initially understand the impact of the new stereopure siRNA design, we developed GalNAc-siRNAs with PN linkages directed against the liver target transthyretin (*Ttr*) in mice. siRNAs with the new design enhanced knockdown durability, supporting >50% knockdown of serum Ttr protein levels up to 50-days post-dose, compared to the benchmark siRNA, which sustained >50% knockdown for only ~27-days post-dose. Using this more durable siRNA format, we decided to test the impact of introducing PN variants on knockdown by non-GalNAc-siRNAs in both hepatic and extrahepatic tissues, including the central nervous system (CNS), adipose tissue, kidney, and heart and skeletal muscle. In the CNS, we developed a stereopure siRNA format, incorporating the new design and PN variants, that targets mouse *Amyloid precursor protein* (*App*). These siRNAs delivered potent knockdown, resulting in 70-90% *App* mRNA reduction across 6 regions of the mouse CNS, including the cortex and striatum, for up to 16 weeks after a single intracerebroventricular (ICV) dose. Consistent with these data, marked reductions in App protein levels were observed by immunohistochemistry across the mouse brain at 16 weeks post-ICV injection. For systemically delivered siRNAs, incorporation of PN variants can support siRNA target engagement in multiple peripheral tissues of interest and can be used to tune relative potency among specific tissues, such as liver versus kidney. By incorporating select PN variants, we designed siRNAs that could knockdown *Superoxide dismutase 1* (*Sod1*) mRNA in desired combinations of metabolically important tissues, such as both liver and adipose tissue. PN variants can also improve the potency of non-GalNAc siRNAs in the liver, which may be useful when simultaneous RNA suppression of a gene in both hepatic and extrahepatic tissues is desired. Incorporating one type of PN variant in a non-GalNAc-siRNA resulted in >80% knockdown of liver *Sod1* mRNA up to 14-days post single dose, compared to ~25% knockdown in liver by non-GalNAc-siRNAs containing PN linkages. In sum, our novel siRNA designs that include PN variant backbone modifications support potent and durable knockdown of both hepatic and extrahepatic targets, including the brain cortex and striatum. In addition, these data support the potential to tune siRNA targeting toward tissues of interest through chemical modifications to backbone linkages.

335. miRNA Site-Blocking Oligonucleotides as a Novel Therapeutic Strategy for STXBP1 Encephalopathy

Alex J. Felix[1,2], Jennine M. Dawicki-McKenna[1,2], Elisa A. Waxman[2,3], Congsheng Cheng[3], Alexey Bogush[1,2], Lea V. Dungan[2,3], Deborah L. French[2,3], Beverly L. Davidson[2,3], Benjamin L. Prosser[1,2]

[1]University of Pennsylvania, Philadelphia, PA,[2]Center for Epilepsy and NeuroDevelopmental Disorders (ENDD), Philadelphia, PA,[3]Children's Hospital of Philadelphia, Philadelphia, PA

STXBP1 encephalopathy is a rare neurodevelopmental disorder caused by heterozygous mutations in the *STXBP1* gene. STXBP1 plays an essential role in neurotransmitter release by regulating pre-synaptic vesicle exocytosis. Loss of function mutations in *STXBP1* impair

neurotransmission, resulting in epilepsy, intellectual disability and motor and behavioral impairments. In this case, the upregulation of the remaining functional *STXBP1* allele may be therapeutic. Since most human mRNAs are at least partly repressed by miRNAs, disrupting the interaction between miRNAs and *STXBP1* mRNA could upregulate STXBP1 expression. Here, we performed comprehensive *in silico* analyses and *in cellulo* miRNA sequencing to identify multiple miRNAs that are both predicted to bind to STXBP1-3'UTR and highly expressed in STXBP1 patient iPSC-neurons. Further, our CLIP-seq data from human brain tissue showed prominent binding of AGO2 to the same region of the STXBP1-3'UTR in which multiple miRNA candidates are predicted to bind, suggesting miRNA-mediated regulation of *STXBP1* mRNA in human brain. In STXBP1 patient iPSC-neurons, blocking miRNA candidates using antagomirs led to STXBP1 mRNA upregulation, thus identifying miRNAs that are repressing STXBP1 in the disease context. As a therapeutic approach, we designed Steric Blocking Oligonucleotides (SBOs) targeting candidate miRNA binding sites in STXBP1-3'UTR and screened them in NGN2-inducible neurons, identifying SBOs that increased STXBP1 mRNA abundance. Also, we showed that several "lead" SBOs were capable of augmenting STXBP1 mRNA abundance in a dose-dependent manner in STXBP1 patient iPSC-neurons, and demonstrated that these compounds act by specifically disrupting the binding of the candidate miRNAs to STXBP1-3'UTR. Finally, we have engineered and characterized the first STXBP1 humanized mouse model in which we replaced the mouse *Stxbp1 locus* with the entire human *STXBP1* gene. We are currently generating a STXBP1 humanized haploinsufficient mouse model, which represents an ideal platform for therapeutic testing and functional rescue assessment *in vivo*. Overall, these studies reveal several miRNA site-blocking oligonucleotides as promising therapeutic candidates for the treatment of STXBP1-related disorders.

336. Modulation of Somatic Repeat Expansion with Small Interfering RNAs

Jillian Belgrad[1], Daniel O'Reilly[1], Ellen Sapp[2], Ashley Summers[1], Eric Luu[1], Nozomi Yamada[1], Hassan Fakih[1], Raymond Furgal[1], Nicholas Gaston[1], Sarah Allen[1], Allison Maebius[1], Katherine Gross[1], Dimas Echeverria[1], Neil Aronin[1], Marian DiFiglia[2], Anastasia Khvorova[1]

[1]UMass Chan Medical School, Worcester, MA,[2]Massachusetts General Hospital, Mass General Institute for Neurodegenerative Disease (MIND), Charleston, MA

Huntington's disease (HD) is a neurodegenerative disorder affecting 7/100,000 people with no treatment available. HD is caused by CAG trinucleotide repeats in exon 1 of the huntingtin gene. Symptoms including chorea, hemiballism, and depression present at >37 CAG repeats, with the age of disease onset decreasing as CAG repeat length increases. The somatic CAG expansion in striatal medium spiny neurons primarily contributes to age-driven HD neurodegeneration. Human GWAS have associated multiple mismatch repair (MMR) pathway components in HD progression. MMR mediates the expansion of trinucleotide repeats by facilitating strand cleavage of slipped loop intermediates during transcription. Blockage of somatic expansion slows HD progression in mice. Thus, MMR-driven somatic expansion is a therapeutically targetable driver of HD. Potent and selective CNS RNAi is now possible with di-valent siRNA. Here, we have screened, identified, and validated potent siRNA silencing key MMR proteins. Using these di-valent siRNA, we performed a systematic evaluation of the impact of RNAi-based silencing of single MMR components on somatic repeat expansion *in vivo* in the Q111 mouse model of HD. We have so far identified that RNAi-based interventional lowering of MSH3, PMS1, and MLH1 each blocks somatic expansion. This work allows a mechanistic understanding of the key players facilitating somatic expansion in HD and represents a therapeutic category of repeat-expansion blocking agents. These siRNAs can be used alone or in combination to manipulate somatic expansion as a potential HD intervention. Further, the work presented here informs novel, RNAi-based treatment options for HD and similar trinucleotide repeat disorders.

337. Therapeutic Oligonucleotides Induce Acute Toxicity in CNS, Preventable by the Addition of Divalent Cations to Formulation

Rachael Miller[1], Joseph Paquette[1], Dimas Echeverria-Moreno[1], Ken Yamada[1], Ellen Sapp[2], Nicholas McHugh[1], Brianna Bramato[1], Nazomi Yamada[1], Alexandra Barker[1], Julia Alterman[1], Christelle Anaclet[3], Marian DiFiglia[2], Anastasia Khvorova[1], Neil Aronin[1]

[1]UMass Chan Medical School, Worcester, MA,[2]Mass General Institute for Neurodegenerative Disease, Charlestown, MA,[3]University of California Davis, Davis, CA

Oligonucleotide therapeutics (ASOs and siRNAs) have become widely explored for the modulation of gene expression in the central nervous system (CNS). Huntington's Disease (HD), a neurodegenerative disorder caused by a dominant genetic mutation resulting in the production of toxic mRNA and protein, is an optimal target for oligonucleotide therapeutics. Previous studies show that intracerebroventricular (ICV) injections of siRNAs targeting huntingtin (HTT) protein effectively spread throughout small and large brains and reduce total HTT. Administration of highly concentrated compounds carrying a negative charge to the CNS can induce acute toxicity. We hypothesized that these negatively charged regions promote cation exchange, subsequently chelating divalent cations from the cerebral spinal fluid (CSF) and intracellular stores, which results in abnormal behavioral phenotypes. Electroencephalogram (EEG) and electromyography (EMG) electrophysiology studies in awake mice confirmed these behavioral observations were seizures. Additionally, *in vitro* ion chromatography studies revealed that each phosphodiester/phosphorothioate (PO/PS) in the oligonucleotide backbone has the capacity to bind divalent cations at molar equivalents. To alleviate this acute toxicity, we developed a method of buffering siRNAs with precise divalent cation concentrations in artificial CSF (aCSF) buffer prior to injection, to prevent this cation exchange and subsequent neurotoxic effects. Here, we demonstrate that adding divalent cations to the injected formulation can mitigate this type of toxicity in a dose-dependent fashion *in vivo*, without impacting distribution or efficacy. This new formulation enables the safe delivery of oligonucleotides at high doses directly into the CNS, which broadens the opportunities for treating patients with HD and other neurodegenerative diseases with these therapeutic modalities. The data presented here defines the

importance of evaluating the safety of therapeutic oligonucleotides for CNS administration and defines the path toward better clinical translation.

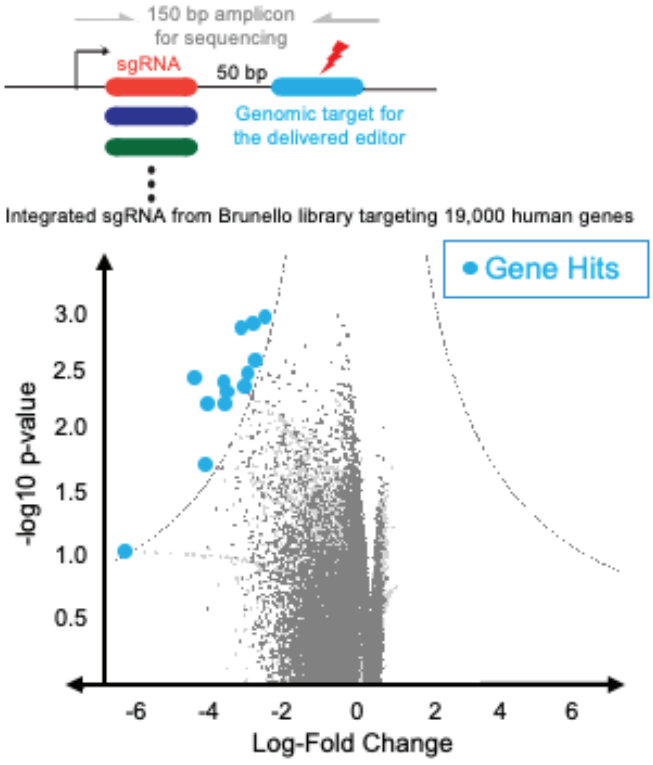
Nonviral Methods for Delivering Nucleic Acids

338. A Novel High-Throughput Screen to Identify Factors Controlling CRISPR-Mediated Non-Viral Genome Editing

Shivani Saxena[1], Min Zhu[2], Divya Sinha[2], Amr Abdeen[2], David Gamm[2], Shaoqin Gong[2], Marcela Martinez[2], Srikumar Sengupta[2], Krishanu Saha[2]

[1]University of Wisconsin-Madison, Middleton, WI,[2]University of Wisconsin-Madison, Madison, WI

Background: After administering a genome editor, the delivery of genome editors is limited by a multi-step process, involving cellular uptake, trafficking, and nuclear import of the vector and its payload. These processes vary widely across cell types and differ depending on the nature of the vector, whether it is a lipid nanoparticle or a different synthetic material. To better understand these delivery limitations within human cells, we developed a novel genome-wide CRISPR screening strategy to identify genes modulating cellular uptake and gene editing efficiency. **Methods:** Our assay interrogates the cellular processes controlling delivery and stimulating non-homologous end-joining (NHEJ) in human embryonic kidney (HEK293) cells. We transduced HEK293 with a pooled lentivirus sgRNA library comprising of 19,000 target genes (Brunello library), prior to delivering non-viral genome editors. Using a low multiplicity of infection, each of the 19,000 genes in the library was knocked out within a specific subset of cells, allowing us to test the influence of nearly every human gene in a single experiment. To facilitate screening, the lentiviral vector genomically integrated into HEK293 cells included the perturbation identifier (i.e., sgRNA sequences) and the target for NHEJ-based editing within a single PCR-based amplicon (*Figure*). We delivered Cas9 ribonucleoprotein (RNP) payloads using lipofectamine 2000, to generate double-stranded DNA break within the integrated screening vector (Figure A).Next, we performed next-generation sequencing to quantify indel frequency distribution and sgRNA identity within the same amplicon from genomic DNA collected from the perturbed cell population. The library was sequenced on Illumina NovaSeq 6000 system (paired-end 150 bp reads). Our coverage was >2000 reads per sgRNA in the library. **Results:** We used MAGeCK analysis to assign 'Phenotype,' defined as the enrichment of a particular sgRNA in an edited pool of reads. This enrichment phenotype is evidence that the activity of the corresponding gene suppresses NHEJ-mediated Cas9 editing. Based on this enrichment analysis (*Figure*), we identified 27 top genes involved in trafficking pathways that facilitate enhanced cellular uptake. Upon the knockout of these top genes, the NHEJ editing rate in HEK293 cells increased dramatically. Identified genes were further validated in separate arrayed screen experiments, successfully verifying the reproducibility of our novel screening technique. Knocking out the top hits uncovered in our screen led to a 10-fold increase) in the gene editing efficiency of Cas9 mRNA delivered via lipid-based nanoparticles (e.g., moving from 6% to 60% editing efficiency). Six of the genes also led to a 5-fold increase in Cas9 RNP editing efficiency using lipofectamine delivery. **Conclusion:** We report a novel genome-wide screen that examines the impact of 19,000 human genes on the delivery and outcomes of genome editors. Distinguished by its potential for large-scale screening, the screen allowed the genome-wide exploration of delivery mechanisms that can be further manipulated for efficient trafficking of CRISPR-Cas9 machinery. We anticipate this high-throughput technique could facilitate the systematic engineering of novel nonviral genome editing delivery strategies.



339. Development of Envelope NanoBodies for Guided *In Vivo* Cell Engineering

Jesus Beltran[1], Barbara S. Perez[1], Jonathan Gunn[1], Dan S. Kaufman[2]

[1]University of California, San Diego, San Diego, CA,[2]University of California - San Diego, La Jolla, CA

Cell and gene therapy technologies continue to rapidly advance. However, despite their exciting potential, most current therapies remain confined to *ex vivo* applications, grappling with challenges in achieving precise and efficient *in vivo* delivery. To address these challenges, we introduce Enveloped NanoBodies (ENaBs), an innovative genetic delivery platform designed for targeted and efficient *in vivo* cell engineering. ENaBs incorporate an engineered version of the GAG protein, complemented with an engineered version of baboon envelope fusogen and designed ankyrin repeat proteins (DARPins), to enable efficient and precisely targeted cellular delivery. We designed our ENaBs to carry cell-lineage specific mRNA-expressing chimeric

antigen receptors (CARs), tailored to each immune cell line, including T cells, NK cells and macrophages. When tested in human immune cells, ENaBs were able to specifically target each cell line of interest, reducing the number of bystander cells and reprograming the immune cells to target and kill ovarian cancer cells, increasing the killing capacity of T cells (from 15% to 80%) and NK cells (from 35% to 95%), as well as the phagocytic activity of macrophages (from 20% to 60%) *ex vivo*. When testing the ENaBs capacity to *in vivo* engineer human immune cells, a single injection of ENaBs generated high levels of *in vivo* engineered cells, ranging from 25% engineered T lymphocytes by anti-CD3 targeted ENaBs to 30% engineered NK cells using anti-NKp46 darpins and 40% engineered macrophages using anti-CD14 darpin-targeted ENaBs. Notably, naked ENaBs also engineered high levels of macrophages, achieving up to 45% cellular editing, likely due to the phagocytic activity of these cells. When analyzing the cellular specificity achieved by each class of ENaB *in vivo*, all of them showed enhanced levels of cellular specificity compared to naked ENaBs, reducing the cellular off-targets, and significantly increasing cellular engineering levels, particularly for NK cells. We then tested the ENaBs ability to *in vivo* reprogram endogenous immune cells to target and kill ovarian cancer tumors. Here, a single injection of each cell-specific targeted ENaB mediated improved anti-tumor activity compared to untreated (human PBMCs alone) mice. Interestingly, the mice treated with the macrophage-targeted ENaBs (CAR-Mac group) had the longest remissions, reducing the tumor mass by 97% at day 15 post-treatment (66% survival at day 60) compared to the untreated tumor group that had a median survival of 27 days. NK cell-targeted ENaB-treated mice also had improved anti-tumor activity with a median of survival of 56 days, reducing the tumor mass by 52% at day 15 post-treatment, and the T cell-targeted mice had a median of survival of 43 days, reducing the tumor mass 84% at day 15 post-treatment. These results demonstrate the ability of our novel ENaB system to mediate complex cell-engineering *in vivo*, reprograming different immune cells to specifically target and kill difficult to treat solid tumors. This new approach introduces an efficient and versatile modular platform that enables specific, targeted *in vivo* engineering of human cells for wide-ranging research and therapeutic applications.

340. An Optimized PBAE Nanoparticle Formulation Allows for Targeted Gene Delivery to the Lungs Following Systemic Injection

Erin Kavanagh[1], Stephany Tzeng[1], Anya Joynt[2], Gregory Newby[3], Neeraj Sharma[3], Garry Cutting[3], Jordan Green[1]

[1]Biomedical Engineering, Johns Hopkins School of Medicine, Baltimore, MD,[2]UMass Chan Medical School, Worcester, MA,[3]Genetic Medicine, Johns Hopkins School of Medicine, Baltimore, MD

Current treatments for cystic fibrosis (CF) primarily focus on modulator drug therapies designed to correct malfunctioning CFTR protein. However, these modulators are ineffective for the 10% of CF individuals with variants that do not allow protein production. Gene editing of these variants would allow production of CFTR protein and restoration of function. Here, we demonstrate poly(β-amino ester) (PBAE) nanoparticle (NP) formulations allow for efficient delivery of GFP mRNA to primary human nasal epithelial (HNE) and bronchial epithelial (HBE) cells from healthy and CF individuals. Optimized PBAE endcap formulations systemically administered *in vivo* allow for high expression of fLuc mRNA within the lungs, with E63 demonstrating high levels of editing in an Ai9 reporter mouse model across bronchial, epithelial, and endothelial cell populations. Multiple administrations with and without a targeting ligand proved both efficacious and non-toxic. PBAEs were synthesized via Michael addition, fabricated into NPs by electrostatic self-assembly with mRNA, and evaluated for gene delivery by flow cytometry. mRNA-encoded GFP served as a reporter to evaluate transfection efficiency, cell viability and mean fluorescence intensity across 3 dosages (150, 75, 32.5 ng mRNA), 4 polymer-to-mRNA weight to weight ratios (60, 40, 30, 20) and 4 polymers. Primary HBE and HBE cells from CF and healthy donors were seeded in 96-well plates then transfected with NPs before undergoing microscopy and flow 24 hours post transfection. NPs carrying Cre mRNA were delivered intravenously to Ai9 mice at three intervals (1x, 3x, 9x). Lungs were dissociated and stained with antibodies before undergoing analysis. NPs achieved 80-90% GFP transfection in primary HNEs from a CF and healthy individual, with ~70% transfection of primary HBEs from a CF individual and ~50% transfection of those from a healthy donor (**Figure 1a**) at 32.5 and 72ng doses. Viability with these two doses was consistently between 70-85%. Systemic delivery to the Ai9 mouse model showed consistent increase in transfected cells across all lung cells as the number of injections increased, from 0.8 to 1.9 to 9% at 1x, 3x and 9x dosing respectively. Additionally, the number of epithelial cells transfected consistently increased across dosing, with the number of endothelial and epithelial transfected cells ranging from 15-40% and 15-30% respectively (**Figure 1b**). We hereby demonstrate systemic delivery of optimized PBAE NP formulation E63 allows for over 90% transfection rate in healthy primary HNEs *in vitro* while maintaining at least 75% viability. This same polymer achieves 20-30% transfection of epithelial and bronchial cells with systemic delivery in mice. *In vitro*, electroporation of modified CRISPR-Cas9 base editor mRNA to primary HNEs carrying 3120+1G>A/G480S achieves 81% correction of the 3120+1 G>A allele. This nonviral transfection results in CFTR function within or above the range of WT values observed in WT HNEs. This gene editing approach is promising for new, durable CF treatment. In ongoing work, we are investigating targeted cell types during *in vivo* delivery to a CF mouse model.

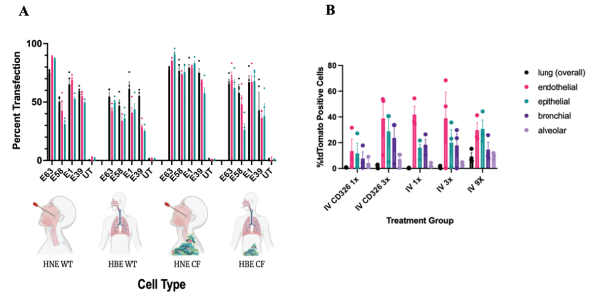


Figure 1: (A) Representative GFP transfection from PBAE NPs screens in primary HBE and HNEs from WT, CF individuals across 4 polymers and 3 dosages at a 30 weight-to-weight ratio. Data shown as mean ± SEM (N=4 biological replicates). **(B)** Flow cytometry in Ai9 mice after systemic delivery of 5 μg Cre mRNA with or without a targeting ligand (CD326) and number of injections (1x, 3x, 9x) displaying relevant tdTomato+ lung cell types.

341. High-Throughput Screening Platform Identifies Nanoparticles Capable of Delivering 19.5 kbp Plasmid to Neuronal Cells *In Vitro* and *In Vivo*

Cherry Gupta[1], Ashlee Colbert[1], Caleb Hillrich[1], Emma Schmitz[1], Molly Kaufman[1], Allison Secard[1], Bradley William[2], Hui Liu[2], Deeann Wallis[2], Robert Kesterson[3], Andrea McCue[1], Michael Riedl[1], Danielle Huk[1], Smith Brian[1], Joanna Hoy[1], Miguel Pedrozo[1], Nick Andrioff[1]

[1]Battelle, Columbus, OH,[2]University of Alabama, Birmingham, AL,[3]Pennington Biomedical Research Center, Baton Rouge, LA

Reconstituting functional proteins via exogenous cDNA delivery to treat genetic diseases such as Neurofibromatosis 1 (NF-1) presents a promising treatment modality which, due to its wide applicability, has high potential for near-term clinical translation. This is currently blocked because of the lack of suitable delivery vectors that can encapsulate the large NF1 cDNA. To overcome these challenges, we used self-assembled cationic polymeric nanoparticles (PNP) to encapsulate and deliver a *hNF1* plasmid (19.5 kbp). *Polymer synthesis and characterization*: Diblock copolymers were synthesized using reversible addition-fragmentation chain-transfer (RAFT) polymerization process, self-assembled into cationic PNPs and functionalized with different targeting groups. The resultant library of ~500 PNPs was tagged with unique DNA barcodes to enable high-throughput *in vivo* screening and loaded with the *hNF1* plasmid (19.5 kbp). The loaded PNPs were tested *in vitro* in the hTERT NF1 -/-(null) ipNF97.4 SC line (derived from parental NF1 +/+ (wildtype) SC line ipNF97.4). Plasmid loading efficiency was characterized using a RiboGreen dye exclusion assay, transfection efficiency (TE) was quantified by flow cytometry, and cytotoxicity was quantified using a live-dead stain. Our results demonstrate that a subset of the PNP library demonstrated high DNA encapsulation and ability to transfect the SCs better than the rest. The cell viability was >80% for all tested PNPs. In parallel, >250 of these barcode tagged PNPs were pooled together and tested for bio-distribution in wild-type mice via intracerebroventricular injection (ICV) injections. DNA barcodes were extracted, sequenced, and analyzed to assess relative tissue specific PNP abundance. Multiple DNA barcodes corresponding to unique PNPs were found to be significantly enriched in the brain and one barcode was found to be significantly enriched in the sciatic nerve. This early parallel screening is unique to our approach and tailored to eliminate the risk of having delivery vectors that work *in vitro* but not *in vivo*. Further, the combinatorial high-throughput platform used here increased the probability of quickly finding potential candidates thereby reducing the risk of failure inherent in a rational design approach. *These results provide a basis for future optimization of these delivery vehicles that can transfect this challenging cell target. PNP mediated delivery and expression of NF1 protein***.** Based on the high-throughput screenings described above, 19 PNPs were down-selected and tested in hTERT NF1 SCs (97.4 NF1 -/-) and MCF7 NF1 -/- mammary epithelial breast cancer cells. Preliminary data indicates that PNP mediated delivery of the *hNF1* results in detectable NF1 protein expression in both cell lines. *PNP mediated delivery and expression in mouse and rat cortex***:** A single PNP, selected based on TE was loaded with the *hNF1* minigene plasmid and dosed in wild-type mice via ICV injection. No adverse toxicity related events were observed. Enhanced GFP fluorescence was observed in the mouse cortex. The same plasmid-loaded PNP was then administered to wild-type Sprague Dawley rats via ICV injection to assess repeatability and investigate cell-specific expression. Multiple GFP positive neural cells were seen in cortex, but no GFP positive astrocytes were detected. These results are the *first ever demonstration of delivering the large 19.5 kbp hNF1 minigene plasmid in vivo and point to an exciting discovery for non-viral gene delivery to neuronal cells.*

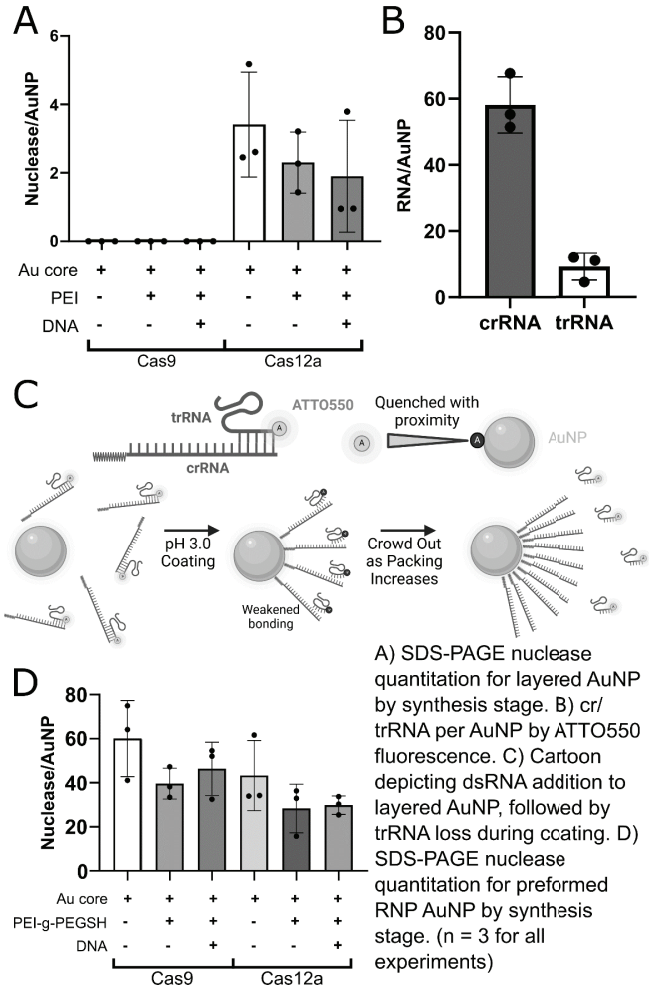
342. Mechanistic Investigation of Cas9 Affinity for CRISPR-AuNP Utilizing Direct RNA Surface Assembly

Daniel D. Lane, Karthikeya Gottimukkala, Rachel Cunningham, Youngseo Jwa, Molly Ellena Cassidy, Jennifer E. Adair

Fred Hutchinson Cancer Center, Seattle, WA

State of the art for hematopoietic stem cell (HSC) genetic editing (GE) occurs *ex vivo,* requiring advanced facilities with treatment costs in the millions (e.g. $2.2 million for Casgevy). A reduction in cost and equipment is essential to global dissemination of GE therapy. We previously described a layered nanoformulation to deliver CRISPR *in vitro* without electroporation (CRISPR-AuNP)[1] to reduce cost of therapy, among other advantages. Formulating CRISPR-AuNP with Cas12a and Cas9 ribonucleoproteins (RNP) resulted in *in vitro* HSC GE levels of 7.65% and 10.42%, respectively, by TIDE. While CRISPR-AuNP showed matching Cas12a/9 GE levels, RNP electroporation shows clear advantages for Cas9, with higher indel formation (47.6%) vs Cas12a (15.18%) at the B2M locus by Miseq (guides cut within 2 bp). This resulted in B2M surface expression knockdown of 10% (Cas 9) vs 0.24% (Cas12a) (5 guides, n = 3), by flow cytometry. Consensus comparing Cas12a/9 matches this conclusion, so we believed the lower Cas9 activity was structural to the AuNP. Investigation of protein binding by SDS-PAGE, after stripping all non-covalent nuclease-Au interactions, found that Cas9 binding was undetectable and Cas12a bound 4 RNP complexes per AuNP. Furthermore, DNA cutting activity of Cas9 CRISPR-AuNP was undetectable while Cas12a CRISPR-AuNP showed substantial activity after β-mercaptoethanol (BME) release from the Au surface. As CRISPR-AuNPs are stabilized through Au-thiolated gRNA interactions, we first tested whether gRNA differences between Cas12a/9 alters binding. Cas9 gRNA consists of two separate, complementary RNA components (crRNA/trRNA) that are duplexed (dsRNA) to become active, with only the crRNA containing the anchor thiol. Cas12a consists of a single thiolated gRNA. dsRNA of Cas9 relies on 10 complementary base pairs, resulting in low coordination energy (melting temperature = 27 °C). Using labeled trRNA, we found 10 trRNA molecules retained on the AuNP, vs 60 crRNA-thiol after dsRNA loading. Kinetic fluorescent recovery shows Cas9 dsRNA to be poorly stable, with buffer conditions alone driving 100% loss of bound trRNA within 12 hrs, compared to a <1% loss of Cas12a's gRNA. To increase active RNP attachment, we loaded preformed Cas9 RNP onto the Au surface, with the protein interaction stabilizing the dsRNA. We investigated RNP cutting activity at differing loading pHs, showing an IC50 of 3.01± 0.03 for Cas12a and 3.23 ± 0.10 for Cas9. At the pH without decrease in Cas9 cutting activity, the nuclease loading increased to 60 RNP/AuNP for Cas9 and 43 RNP for Cas12a, with confirmed cutting activity after stripping non-covalent interactions by dialysis. This AuNP formulation has reduced charge density and requires an

alteration of the polymer coating. The ideal endosomal escape polymer identified for nanoparticle stability includes a thiol anchor to covalently attach to the AuNP (PEI-g-PEG-SH), overcoming the reduced charge's decreased polymer affinity. However, confocal microscopy revealed endosomal entrapment of these 2nd generation CRISPR-AuNP in K562 endosomes, suggesting polymer optimization is required to achieve GE activity in living cells. Future studies in primary HSC and other cell types will permit fine tuning of nanoparticle characteristics to achieve the desired GE which the increased RNP loading will allow.



A) SDS-PAGE nuclease quantitation for layered AuNP by synthesis stage. B) cr/trRNA per AuNP by ATTO550 fluorescence. C) Cartoon depicting dsRNA addition to layered AuNP, followed by trRNA loss during ccating. D) SDS-PAGE nuclease quantitation for preformed RNP AuNP by synthesis stage. (n = 3 for all experiments)

1. Shahbazi R, et al. Targeted homology-directed repair in blood stem and progenitor cells with CRISPR nanoformulations. Nat Mater. 2019;18:1124-1132.

343. Peptide-Based Nanoplatform for Cas9 RNP Delivery and Gene Editing

Joseph V. Lavalla[1], Alyson Schwartz[1], Audrey Wessinger[1], Jessica Larsen[2], Angela Alexander-Bryant[1]
[1]Bioengineering, Clemson University, Clemson, SC,[2]Chemical Engineering, Clemson University, Clemson, SC

Introduction: Gene editing using CRISPR/Cas9 for the treatment of genetic diseases has rapidly advanced to become an integral part of the biotechnology field over the past decade. Delivering Cas9 via RNPs has demonstrated higher editing efficiencies and lower off-target effects than either plasmids or mRNA. However, due to their xenogeneic nature and charge properties, RNPs alone cannot be internalized into cells and require a delivery vehicle. Viruses, lipids, and polymers have been used as vehicles for Cas9 RNPs, though limitations of safety and efficiency in delivering the gene-editing cargo into cells have posed a large hurdle to overcome. Peptides have emerged as a promising alternative for transporting bioactive nucleic acids into cells due to their high tunability, stability, and biocompatibility. We have previously demonstrated that a novel fusogenic peptide, DIV3W, can electrostatically complex with and protect RNA, is cytocompatible, and mediates efficient endosomal escape of bioactive RNAs. This proof-of-concept study aims to evaluate the DIV3W peptide for delivery of RNPs to induce efficient gene knockout. **Methods:** RNP:DIV3W complexes were synthesized by combining gRNA and Cas9 at equimolar ratios, followed by complexation with various molar concentrations of DIV3W peptide. Complexation of the RNP with the DIV3W peptide was analyzed using agarose gel electrophoresis. To assess the cytocompatibility of the RNP:peptide complexes, RNP:DIV3W complexes were synthesized and incubated with HEK293 cells at increasing molar ratio complexes for 48 hours, followed by an MTS assay to determine RNP:peptide cytotoxicity. RNP:DIV3W complexes were additionally delivered to HEK293-GFP cells to knockout GFP expression. Changes in fluorescence after 96 hours were analyzed using flow cytometry. **Results and Discussion:** We previously observed that molar ratios greater than 1:1:20 (Cas9:gRNA:DIV3W) resulted in the complete complexation of RNPs and, therefore, a successful delivery vehicle capable of protecting the RNP from premature degradation. MTS results confirmed RNP:DIV3W complexes were not cytotoxic to HEK293 cells, with viability greater than 90% for molar ratios up to 1:1:80 (Figure 1). Preliminary knockout studies have demonstrated successful GFP editing, with up to 33% knockout after RNP:DIV3W treatment (Figure 2). **Conclusion:** The DIV3W peptide can self-assemble with and completely complex free RNPs and are cytocompatible with HEK293 cells. The RNP:DIV3W complexes can escape the endosome prior to lysosomal degradation and traverse the nuclear membrane, as shown through successful gene editions. These results demonstrate the early potential of peptide-based delivery systems for Cas9 RNPs and warrant further investigation. Future work of this project includes characterization of the size and charge of the RNP:DIV3W complexes, evaluation of cellular internalization, and quantification of indel mutations.

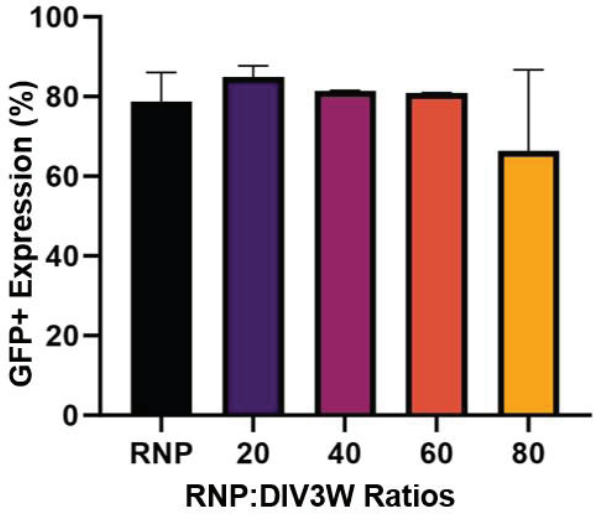


Figure 2: HEK293 cells were treated with RNPs complexed at increasing ratios with DIV3W. GFP expression was analyzed using flow cytometry after 96 hours. Data are mean ± SEM

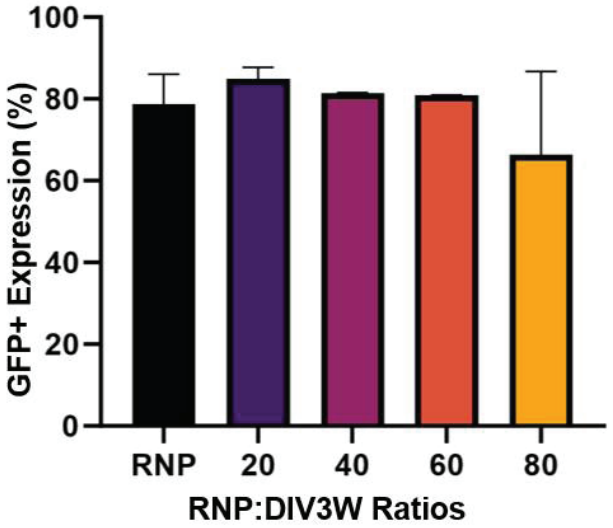


Figure 2: HEK293 cells were treated with RNPs complexed at increasing ratios with DIV3W. GFP expression was analyzed using flow cytometry after 96 hours.

344. Novel Non-Viral Delivery Vehicles That Distribute to the Sciatic Nerve and Brain Identified via Iterative Design-Build-Test-Learn Polymer Nanoparticle Discovery Platform

Kenneth R. Sims[1], Emma Schmitz[1], Caleb Hillrich[1], Ashlee Colbert[1], Michael Riedl[2], Andrew Matas[2], Adele Guglielmi[3], Kyle D. Fink[4], David J. Segal[5], Danielle Huk[6]

[1]Advanced Materials and Energetics, Battelle Memorial Institute, Columbus, OH,[2]Health Analytics, Battelle Memorial Institute, Columbus, OH,[3]Biological Chemistry and Molecular Medicine, University of California, Davis, Davis, CA,[4]UC Davis, Sacramento, CA,[5]UC Davis Genome Center, Davis, CA,[6]Drug Development and Precision Diagnostics, Battelle Memorial Institute, Columbus, OH

Introduction & Methods: Nervous system delivery remains a key challenge for gene therapy. To overcome this challenge, a proprietary design-build-test-learn (DBTL) polymer nanoparticle (PNP) discovery platform was used to make and test novel PNP designs *in vitro* and *in vivo*. PNPs were synthesized via reversible addition-fragmentation chain transfer polymerization, purified, and characterized for size, polydispersity, loading efficiency, cytotoxicity, and transfection efficiency. In parallel, proprietary nucleic acid barcoding technology was used to evaluate PNP biodistribution in C57BL/6 wild type (WT) mice. After two DBTL cycle iterations, new, non-intuitive PNP designs capable of distributing to the brain and sciatic nerve following intrathecal administration were identified. *Results*: Previous biodistribution screening of >1,000 PNPs revealed a few PNPs that distributed well within mice. In parallel, *in vitro* results revealed that 5 of 10 top PNPs out of the >100 PNPs that showed high cell viability and transfection efficiency in HEK cells were from the same polymer compositional family as the well distributed PNPs found in the biodistribution study. Altogether, these top PNPs from early *in vitro* and *in vivo* testing came from a single polymer compositional family "design box." These initial results provided key insights to non-intuitive polymer composition designs that warranted further investigation as part of a second DBTL cycle. Thus, a second polymer library focused on these design box parameters associated were synthesized, characterized, and tested *in vitro* and *in vivo*. The second DBTL cycle produced >500 PNPs, and 96 of them were focused on further interrogating the previously identified design box while the others included slight polymer composition modifications. These PNPs were evaluated *in vitro* for size, loading efficiency, immunogenicity, cytotoxicity, and transfection efficiency. In parallel, these PNPs were DNA barcoded, pooled, and injected intrathecally into WT mice to evaluate PNP biodistribution performance *in vivo*. Biodistribution test results revealed several PNPs with elevated accumulation levels in sciatic nerve and brain tissue. The PNP design parameters were compared back to the initial library design using both human intuition and machine learning (ML) to determine if any useful patterns emerged. Indeed, a clear trend was found where lead PNP candidates that distributed to the sciatic nerve and brain were clustered together within an even smaller design box. Moreover, the PNP molecular weight and size data within this design box fell outside of typical, intuitive rational PNP design criteria, so it is unlikely they would have been discovered using traditional non-viral delivery field dogma. *Conclusions*: These iterative DBTL studies have leveraged key elements of a proprietary DBTL platform, namely rapid PNP production and testing followed

by ML guidance, to dial in on a PNP design space with potential to produce lead candidate non-viral delivery vehicles capable of delivering to sciatic nerve and/or brain tissue. Future DBTL cycles will further refine the polymer design parameters within the design box of interest to identify if any new designs may enhance delivery to the brain and/or sciatic nerve tissues in mice. Ultimately, this work informs future non-viral delivery vehicle designs to deliver to the central and peripheral nervous systems.

AAV Vector Integration

345. The Genomic Architecture of 24 Carcinogenic rAAV Integrations in Mice: Implications for Human Gene Therapy

Randy J. Chandler[1], Eli J. Kaufman[2], NIH Intramural Sequencing Center (NISC)[3], Paul N. Valdmanis[2], Charles P. Venditti[1]

[1]Metabolic Medicine Branch, National Human Genome Institute, NIH, Bethesda, MD,[2]Division of Medical Genetics, School of Medicine, University of Washington, Seattle, WA,[3]Comparative Sequencing Program, NIH, Bethesda, MD

Background: Hepatocellular carcinoma (HCC) in mice exposed to recombinant adeno-associated viral (rAAV) vectors was first reported over 20 years ago. More recent reports of an association between human HCCs and wildtype AAV infections, rAAV integrations with clonal expansion in hemophilic dogs, and the formation of an HCC in patient following rAAV treatment for hemophilia continues to raise concerns about the possibility of rAAV integrations causing insertional mutagenesis in humans. Elucidating the mechanisms by which rAAV causes HCC in mice after integration will provide critical insights that might help mitigate potential toxicity after AAV gene therapy in humans. However, the complete genomic characterization of AAV associated HCCs has been limited to the capture of a single ITR junction, with PCR based genomic walking across integrations attempted by several groups in the past without success. Using a combination of sequencing approaches, we have performed the first, and most comprehensive, analysis of toxic rAAV integrations associated with HCC formation in a large cohort of mice. **Methods:** We used LAM PCR, locus specific amplification, Sanger sequencing, and 2 distinct linked-reads platforms and a long-read sequencing platform (10x Genomics, TELL-Seq and Oxford Nanopore) to study HCCs from a historic cohort of 33 MMA mice and unaffected control littermates treated with a variety of single stranded therapeutic and reporter AAV vectors (R. Chandler JCI 2015, PMCID:4319425). A subset of integrations obtained by linked- and long-read sequencing platforms were validated by Sanger sequencing. **Results:** We detected rAAV sequence in 24 murine HCCs: 17 captured the entire integration and 7 captured partial rAAV integrations. All 24 rAAV integrations mapped to the Rian locus. The 17 rAAV integration events completely sequenced ranged in size from 255 to 4,535 base pairs; 15 contained truncated AAV genomes less than 1.3 kilobases in size. Only 3 of the integrations contained the transgene coding sequence with 2 of these events having a majority of the packaged rAAV genome intact. ITRs were recovered in all but 2 integrations and the ITRs were substantially rearranged. One rAAV integration contained the origin of replication (ori) from the plasmid used to package the AAV in cis with a truncated vector genome. Common elements to all 24 AAV integrations included AAV enhancers and/or transgene promoters. **Conclusions:** The genomic architecture of pathogenic events reveals that all contain transcriptional transactivating elements with some as small as ~ 250 base pairs. Concatemers that underwent intramolecular recombination and subsequent deletion are predicted as the in vivo precursor(s) of some integrations, and the identification of an event that contains a reverse packaged vector plasmid *ori* suggest that future studies should focus on longitudinal assessment of integration events in animal models, with an expansion to query all elements of plasmids used to produce AAV as possible candidates for unwanted integration. Lastly, our data support a model where vector mediated enhancer insertion, with subsequent dysregulated transcription mediated by transgene encoded regulatory elements, appears to be the mechanism underlying tumorigenesis after AAV integration. In aggregate, our study helps define the approaches needed to assess AAV integration, supports a mechanism(s) by which rAAV can contribute to tumorigenesis, and should help inform the design of safe vectors for human gene therapy.

346. Comprehensive Preclinical Integration Site Analyses of SPK-3006, a Liver-Mediated AAV Gene Therapy for Pompe Disease Reveals a Low Theoretical Risk for Insertional Mutagenesis

Ali Nowrouzi[1], Daniela Cesana[2], Matthew Jennis[3], Valeriya Posternak-Ball[3], Francis Pankowicz[3], Carlo Cipriani[2], Laura Rudilosso[2], John Everett[4], Aoife Roche[4], Chao Di[1], Pichai Raman[1], Zhenming Yu[1], Ype P de Jong[5], Frederic D. Bushman[4], Federico Mingozzi[6], Eugenio Montini[2], Meena Jain[7], Michael Olaopa[3], Kathila Alatsis[8], Mehdi Sarmady[1], Christopher R. Riling[3]

[1]Genomic and Data Sciences, Spark Therapeutics Inc, Philadelphia, PA,[2]San Raffaele Telethon Institute for GT (Sr-Tiget), IRCCS San Raffaele Scientific Institute, Milan, I, Milan, Italy,[3]Systemic Gene Therapy, Spark Therapeutics Inc, Philadelphia, PA,[4]Department of Microbiology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA,[5]Division of Gastroenterology and Hepatology, Weill Cornell Medical College, New York, NY,[6]Former employee, Spark Therapeutics Inc, Paris, France,[7]Clinical Development, Spark Therapeutics Inc, Philadelphia, PA,[8]Toxicology and Pharmacology, Spark Therapeutics Inc, Philadelphia, PA

Adeno-associated virus (AAV) gene therapy for Pompe disease marks an innovative approach to treating this rare genetic disorder caused by a deficiency of acid alpha-glucosidase (GAA). In contrast to the current enzyme replacement therapy (ERT), with its patient burden for lifelong treatments and potential development of neutralizing antibodies, AAV presents a promising alternative. Recent preclinical studies with our clinical candidate SPK-3006 demonstrated the efficacy of AAV-mediated hepatic GAA expression in animal models. The therapy demonstrated enhanced pharmacokinetics and greater enzyme uptake in relevant tissues compared to ERT. To balance the therapeutic benefits of AAV-based gene therapy with long term safety, addressing concerns regarding AAV integration into hepatocyte genomes is essential.

Such integration poses potential risks, including the development of hepatocellular carcinomas (HCCs) through insertional mutagenesis. To assess the genotoxic safety of AAV-based gene therapy for Pompe disease, a comprehensive analysis of over 8000 integration sites was conducted in non-human primates (NHPs) and in primary human hepatocytes engrafted in a liver chimeric humanized mouse model treated with SPK-3006 at various doses. A low and dose-dependent integration frequency of SPK-3006, calculated as retrieved integration events per haploid genome, was observed ranging between 2.8 and 5.8 x 10^{-4} in NHPs and 3.3 x 10^{-4} and 1 x 10^{-3} in the liver chimeric humanized mouse model. These integrations exhibited typical intragenic preferences common for AAV vectors, with no observed enrichment in cancer genes or regulatory regions of cancer genes with increasing doses of hepatic AAV transduction. Long-term follow-up studies in NHPs showed no signs of integration-driven clonal expansions or pathological findings in the liver. Preliminary analyses of cell-free DNA from NHP plasma confirmed detectable SPK-3006 integration sites, warranting further development of this method as a potential minimally invasive approach for monitoring AAV integration sites *in vivo* AAV-mediated gene therapies. In conclusion, the SPK-3006 AAV candidate for Pompe disease treatment chromosomally integrates at a low frequency comparable to reported AAV vectors. Importantly, there are no abnormal preferences for integration near cancer genes, supporting a low theoretical risk of SPK-3006 for insertional mutagenesis mediated induction of HCCs in patients. This work further expands our understanding of AAV integration of a liver tropic capsid in primary NHPs and human hepatocytes, serving as a resource for future AAV integration related studies in preclinical models.

347. Predicting Truncation Events in AAV Vector Genome Designs Using Deep Learning

Sandhiya Ravi[1,2], Mitchell Yip[1], Ngoc Tam Tran[1,2], Suk Namkung[1], Guangping Gao[1,2], Phillip Wl Tai[1,2]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA

The presence of truncated genomes found in recombinant adeno-associated virus (rAAV) vector preparations can have significant impact on the effectiveness of gene therapy treatments that are based on these biodrugs. The packaging of truncated genomes is known to be caused by template-switching events centered on inverted repeats and sequences with high GC content. Importantly, they may result in the production of non-functional vectors with unknown consequences. The propensity of some vector design elements to drive the formation of truncated genomes is not completely known and can vary from batch-to-batch. Therefore, the ability to predict truncation hotspots in AAV vector designs is crucial for developing future vectors. In this study, we aimed to use deep learning (DL) to identify truncation events in AAV vector genome designs, *de novo*. We have accumulated multiple datasets related to the detection of truncated genomes from previous and ongoing studies that profile vector genome heterogeneity in packaged rAAVs using single molecule, real-time (SMRT) and nanopore sequencing technologies. Additionally, recognizing the intricate variability in AAV vector design, our study encompasses an extensive diversity of design features including different promoters, transgenes, single-stranded (ss) versus self-complementary (sc) configurations, diverse capsid types, and various preparation methods. We hypothesized that using a large dataset of detected truncation events found among a diversity of rAAV designs to train a DL model, we can develop an accurate truncation hotspot prediction algorithm. We have defined as features across the training data, the relative positions of the truncation events and truncation abundances centered at 200-nt sliding windows. Using this metadata, we trained a combination of convolutional neural network (CNN) and bi-directional long short-term memory (LSTM) models to predict truncation events. The model is designed to classify whether a sequence will house a truncation hotspot and predict the "strength" of the truncation. The model incorporates both regression and classification components and achieved remarkable classification accuracy (98%). Crucially, the algorithm can accurately predict truncations in vector designs tested thus far. This study illustrates the potential for DL models to predict genome truncations in rAAVs. These advancements will not only aid in designing more effective gene therapy vectors, but also deepen our understanding of AAV's biophysical properties. G.G. and P.T. are corresponding authors.

348. Characterization of AAV Vectors in Tissues Transduced with Conventional and Novel ITRs Show Differences in ITR Recombination, Episome Structures, and Transgene Expression

Suk Namkung[1], Elisabet Mandon[1,2], Jialing Yang Liang[1], Fang Zhang[1], Mitchell Yip[1], Tapan Sharma[1], Jackson McGowan[1], Sophia Liu[1], Thomas Leland[1], Mengtian Cui[3], Dan Wang[1,2], Phillip Tai[1], Guangping Gao[1]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]RNA Therapeutics Institute, UMass Chan Medical School, Worcester, MA,[3]Horae Gene Therapy Center, Umass Medical School, Worcester, MA

The inverted terminal repeat (ITR) sequences that were originally isolated from the genomic termini of adeno-associated virus serotype 2 (AAV2) have been obligatory for producing recombinant (r)AAVs. The ITRs are also essential for driving intramolecular recombination to form stable circular episomes in the host cell. It has been reported that ITRs contribute to transgene expression via their promoter-like activities. However, the consequences of vector genome circularization on transgene expression have not been extensively explored. We serendipitously found a vector design that when transduced into mouse livers, showed a complete lack of transgene expression. Through Hirt DNA extractions and nanopore sequencing of episomal vector species in transduced tissues, we revealed that the episomes completely lacked the ITRs. Some species even included deletions of transgene segments. AAV-GPseq of the vector preparation showed that the vectors were intact and homogenous, suggesting that the lack of ITRs in episomes extracted from transduced tissues were not due to truncations found in vector preparations. We hypothesize that recombination to form episomes is error prone and stochastic. This premise offers another facet to poor transduction seen in certain vector designs. To understand how transgene expression is impacted by ITRs post-circularization, we are developing a novel platform that permits the examination of different episomal forms (*e.g.,* monomers, dimers/concatemers), and the characterization of ITR structures post-transduction. Using third-

generation, single molecule, real-time (SMRT) sequencing, this study aims to reveal, 1) the fate of ITRs post-transduction and circularization, and 2) outcomes of recombination between ITRs of different AAV serotypes that yield distinct transgene expression profiles. This work also uncovers concepts that contrasts with common knowledge regarding the retention and conformation of ITRs post-transduction *in vivo*. These results shed light on an unexplored property of vector genome processing and has paradigm-shifting impact for AAV vector biology and the field of gene therapy. In addition, our findings open the door to new avenues of research aimed at improving the transgene expression and stability of rAAV episomes in order to increase the efficacy of rAAV-based gene therapies. G.G. and P.T. are corresponding authors

349. *In Silico* Reconstruction of Genotoxic AAV Integration Events from a Tiling Array Dataset: Proof of Concept and Validation

Tyra G. Wolfsberg[1], Stephanie N. Smith[1], Sumeeta Singh[1], Mark S. Sands[2], Charles P. Venditti[1]

[1]NIH/NHGRI, Bethesda, MD,[2]Washington University School of Medicine, St Louis, MO

Introduction: Prior studies have established that genotoxic vector integrations into the mouse *Rian* locus are associated with hepatocellular carcinoma (HCC) following systemic administration of AAV vectors. The Twitcher (Twi) mouse, a model of Krabbe disease, is deficient in galactosylceramidase (GALC) activity. While CNS-directed AAV2/9-GALC-mediated gene therapy combined with bone marrow transplantation and a substrate reduction drug results in an impressive increase in the lifespan from ~ 110 days to ~ 400 days, >90% of the Twi mice, as well as 100% of the wild type controls, succumb to HCC (PMC7854295). HCC has never been reported in untreated Twi mice. A targeted sequence capture analysis using a tiling array with probes specific for the AAV vector was used to map ITR-containing integrations in or near *Rian*, or other cancer- and cell-growth/death-associated genes, in the HCCs. However, the integrations were not further characterized. Indeed, although several groups have attempted to use targeted PCR and chromosomal walking to recover the full length nucleotide sequence of the genotoxic events in other AAV-associated HCCs, the structure of the integrated vectors has remained elusive. **Methods:** We developed a bioinformatics method designated as AVITA (Assembly of Viral Integrations from Tiling Arrays) to mine tiling array sequence data to reconstruct full length integrations. The first step in AVITA uses the VSeq-Toolkit (PMC7177155) to identify chimeric sequencing reads, i.e., those that span the vector-genome breakpoint at any position in the vector. A pileup of independent reads, aligning to the same vector and genomic coordinates, flags a potential integration junction. Two pileups at nearby genomic regions define the ends of candidate integration sites. The second step assembles overlapping reads from one genome-vector integration junction, through the vector, to the mate vector-genome integration at a nearby genomic position, allowing full length reconstruction of the inserted sequence. One of these complete integrations was selected for validation by locus-specific PCR and Sanger sequencing. **Results:** To test the method, captured sequencing reads from 4 of 10 tumors (PMC7854295) were examined in detail. Each tumor contained two high read count genome-vector breakpoints, which delineated the ends of an integration site. The four integration events, which range in length from 444 - 1326 nt, were fully reconstructed *in silico*. All lack the GALC cDNA, have a single truncated 5' ITR, and contain parts of the enhancer/promoter of the AAV vector transgene. One full-length integration event was confirmed by PCR amplification of genomic DNA, followed by Sanger sequencing. The integration disrupts 12 nt in the mouse *Rian* locus and contains a 444 nt insertion of AAV vector DNA that includes the final 76 nt of the 5' ITR followed by the CMV enhancer. **Conclusions:** Using AVITA, we demonstrate, for the first time, that genotoxic full-length AAV integrations can be fully recovered from a tiling array using an *in silico* approach. Because tiling arrays can capture partial and/or rearranged integrations, hybridization approaches present a less biased method to identify integration events than LM-PCR, which relies upon anchoring amplification in the ITR. Indeed, in 2 of the 4 samples analyzed here, the AAV vector is rearranged and/or the ITR is truncated, both of which would likely preclude capture by LM-PCR. We anticipate applying AVITA to sequence data from all publicly available tiling array datasets to assess global patterns of AAV integration. Enhancements to automate AVITA, coupled with improvements to tiling array performance, might enable AAV integration studies to be conducted in a multiplexed fashion, and amenable to broad implementation.

350. Characterization of AAV Integrations in Preclinical Models of Gene Therapy Using RAAVioli Pipeline with Long and Short Sequencing Reads

Carlo Cipriani[1,2], Laura Rudilosso[1], Giulio Spinozzi[1], Dhwanil Dalwadi[3], Markus Grompe[3], Giuseppe Ronzitti[4], Marco Masseroli[2], Eugenio Montini[1], Andrea Calabria[1], Daniela Cesana[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Department of Electronics, Information and Bioengineering, Politecnico Di Milano, Milan, Italy,[3]Papé Family Pediatric Research Institute, Department of Pediatrics, Oregon Health and Science University, Portland, OR,[4]Genethon, Inserm, Univ Evry, Université Paris-Saclay, Evry, France

Adeno Associated Viral vectors (AAVs) have been successfully exploited in gene therapy (GT) applications for the treatment of several genetic disorders. Even though they are considered episomal vectors, it has been shown that fragmented or full-length AAV DNA can integrate within the genome of host cells leading to hepatocellular carcinoma and clonal expansion events in some preclinical models. However, methods and bioinformatic tools that provide a reliable and efficient assessment of AAV integration sites (IS) are required. Here we propose a sonication-based PCR approach combined with short-read sequencing and a bioinformatics pipeline known as RAAVIoli (Recombinant Adeno-Associated Viral Integration analysis) as a new approach to identify and characterize AAV IS and vector rearrangements. The pipeline is based on Python and R scripts that parse the alignments to identify IS and reconstruct vector rearrangements using CIGAR strings. The robustness of our method was evaluated by characterizing AAV IS in a humanized liver mouse model where human primary hepatocytes were transduced with a tomato-expressing AAV. In this model vector insertions were previously characterized using an AAV-specific probe base selection method and

long-reads PacBio sequencing. Sequencing reads were analyzed in both cases using the same RAAVioli pipeline. A greater number of AAV IS were identified by PCR/short-read sequencing (N=811) as compared to the long-read sequence approach (N=370). AAV IS distributed similarly within the human genome showing the typical preference of targeting CpG islands and transcriptional start sites. Moreover, 32 IS were shared among the two datasets, demonstrating the consistency of the results obtained independently from the platform adopted. A comparable number of AAV IS (~25%) were composed of rearranged AAV genomes, although, a higher number of vector re-arrangements (up to 7 AAV fragments) were identified by long-read compared to short-read sequences. Therefore, our PCR-based method produced comparable findings to long-read sequencing approaches regarding the pattern and structure of AAV integration and rearrangements. Nevertheless, it demonstrates significantly greater efficiency in retrieving AAV insertion sites. Thanks to this platform, we observed that in functionally corrected T cells of immune-deficient ZAP70 knock-out mice intra-thymically injected with an AAV expressing the therapeutic transgene, AAV Inverted Terminal Repeats (ITRs) were enriched at DNA breaks created by the enzymatic activity of recombination activating genes (RAG) during V(D)J recombination. Furthermore, AAV IS were successfully identified in the liver and cell-free DNA of Non-Human Primates systemically injected with therapeutic AAVs, underlining the feasibility of the methods as a potential minimally invasive approach for monitoring AAV IS in in-vivo GT applications. In summary, our work indicates that the development of reliable methods for the characterization of AAV integrations is fundamental to providing insights on the safety and efficacy of these vectors in gene therapy applications.

351. A Highly Potent Engineered AAV Capsid, STAC-150, Enables High-Throughput AAV Production and Arrayed Epigenetic Regulator Screening Directly in Cultured Neurons

Patrick Dunn, Jisoo Lee, Ankitha Nanjaraj, Kimberly Marlen, Andrew Olin, Hung Tran, Amy Pooler, David Ojala, Bryan Zeitler

Sangamo Therapeutics, Inc., Richmond, CA

Epigenetic regulators have the potential to transform the treatment of neurodegenerative and neurodevelopmental disorders by specific and tunable modulation of disease-associated genes. Identifying potent and highly specific epigenetic regulators, however, can require extensive empirical testing with iterative rounds of on- and off-target screening. Candidate epigenetic regulators can be evaluated in immortalized cell lines that are well-suited for high-throughput screening, but these cells often fail to recapitulate the gene expression levels, disease biology or native epigenetic signature present in more biologically relevant cells. Alternatively, screening can take place directly in the target cell type of interest, such as primary neurons, but these tend to be less experimentally tractable and can require production of viral vectors for each transgene - a step that increases cost and significantly lowers throughput. We sought to circumvent these challenges by building a robust platform for screening epigenetic regulators directly in primary- or iPSC-derived neurons. We accomplished this by engineering a novel AAV capsid, STAC-150, with two important properties: (1) the ability to transduce neurons *in vitro* at substantially lower MOIs than conventional serotypes, and (2) significant partitioning of total production yield into the cell culture medium during HEK293 capsid production. We leveraged these capsid properties in a microliter-scale, automation-compatible AAV production and purification process capable of efficient and cost-effective generation of thousands of arrayed AAVs directly from crude AAV production media. In the first step of this workflow, we transiently transfected plasmids encoding the viral genome, helper genes and epigenetic regulator transgenes into 1000 µL HEK293 suspension cell cultures in 96-well deep well plates. After 48 hours, we centrifuged the culture plates and purified secreted virus from the production medium supernatants using AAVX magnetic beads. We assessed viral protein ratios and capsid purity by total protein stain and western blot. Purified virus showed the expected viral protein ratios and minimal contaminating proteins. DNAse-resistant viral particles were quantified by qPCR, where we observed yields exceeding 1e10 viral genome copies with corresponding titers up to 5e11 vg/ml. We evaluated the functionality of these purified STAC-150 AAVs by assessing the on-target activity of their epigenetic regulator payloads in primary mouse cortical neurons by RT-qPCR. We observed potent and dose-dependent target gene regulation with strong epigenetic regulators showing EC_{50} values as low as MOI 10. In summary, we identified a secreted, neurotropic AAV capsid, STAC-150, with high potency *in vitro* and built a high-throughput, scalable production workflow to enable epigenetic regulator arrayed library screening in primary- or iPSC-derived neurons. Together, these advancements establish a platform with the potential to accelerate the development of potent and highly specific epigenetic regulators for the treatment of neurological disorders.

AAV Vectors - Immune Modulation

352. C3 Complement Inhibitor Limits Transaminitis and Immune Response Following Liver Targeted Adeno-Associated Virus (AAV) Gene Transfer (GT) in Non-Human Primates (NHPs)

Elizabeth Kropf, Corinne J. Smith, Ali L. Kamal, David M. Markusic, Anna Majowicz, Klaudia Kuranda

Spark Therapeutics, Philadelphia, PA

AAV vector-based gene therapies are shifting the medical paradigm by offering a potentially curative treatment for genetic diseases that previously lacked effective treatments. Although systemic AAV gene therapies are very promising, durability may be limited by liver transaminitis that usually subsides with corticosteroid treatment. Additionally, in clinical trials that use high systemic AAV doses (≥ 1×10^{13} vector genome [vg/kg]), complement activation accompanied by a form of thrombotic microangiopathy (TMA) was reported. TMA is defined by the presence of hemolytic anemia, thrombocytopenia and subsequent organ damage. Excessive complement activation has been observed in multiple trials across a range of disease indications and capsid serotypes. The emergence of severe adverse events at high doses of AAV presents a clear need for more effective immune suppression protocols to address the impact of complement

activation. Further, there is growing evidence that complement mediates immune response to AAV also at lower doses and thus, may warrant complement inhibiting over a broad range of AAV doses. We have previously demonstrated that inhibition of complement C3 activation, *in vitro,* in human whole blood, significantly reduced AAV vector internalization and activation of certain dendritic cells subsets. Additionally, C3 inhibition significantly reduced the levels of proinflammatory cytokines and chemokines secreted in response to AAV stimulation. These findings suggested that complement may play a major role in driving phagocytosis and priming of the immune responses to AAV. To further test this hypothesis *in vivo*, we investigated the role of prophylactic C3 complement inhibition in NHPs administered with high dose of AAV vector. Eight male, *Macaca fascicularis* were injected intravenously on day 0 with 1×10^{14} vg/kg of AAV-Spark100-hFIX (human Factor IX). Four of eight NHPs were treated with 140 mg/kg of C3 inhibitor (Pegcetacoplan, APL-2) by daily subcutaneous injections from days -5 to 23. At week 14 all animals were sacrificed. Systemic administration of high-dose AAV in control NHPs resulted in complement activation and elevated liver enzymes 1.5-fold above baseline for both alanine transaminase and aspartate aminotransferase, as seen in clinical trials. The peak of alternative pathway complement activation (Bb) was observed one week post AAV infusion. C3a showed transient elevation at day 3 post AAV infusion followed by another more pronounced increase around week 4. Interestingly, C3a elevation at week 4 coincided with the onset and duration of liver enzyme elevation. APL-2 treatment significantly lowered AAV-induced complement activation, was well-tolerated and did not interfere with AAV-based GT as measured by plasma levels of hFIX protein, liver hFIX expression, and vector genome copies. As previously observed *in vitro*, C3 inhibition also limited immune response to AAV vector *in vivo*. Compared to control animals, the APL-2 treated group showed lower systemic levels of pro-inflammatory cytokines (*i.e.*, IL-6 and IL-8) and reduced capsid-specific hepatic T cells at week 14. Importantly, C3 inhibition with APL-2 significantly limited hepatotoxicity following AAV gene transfer as demonstrated by lower liver enzyme levels**.** Further studies are ongoing to elucidate the mechanism underlying this observation. These promising results may encourage clinical evaluation of C3 inhibitors' capacity to limit AAV immunogenicity and concomitant transaminitis in gene therapy trials using moderate AAV doses. In trials using high AAV doses, C3 inhibitors may additionally prevent adverse complement activation and TMA, to improve safety of patients.

353. Orthogonal B Cell and Plasma Cell Immunosuppression Strategies Prevent and Suppress High-Titer Antibody Immunity to Enable AAV Vector Re-Dosing

Nicholas Giovannone, Carley Tasker, Nyanza Rothman, Sofia Caruso, Julia Provino, Laura Johnsen, Johanna Napetschnig, Jamie Orengo, Christos Kyratsous, Andre Limnander, Katherine Cygnar, Andrew Baik

Regeneron Pharmaceuticals, Tarrytown, NY

Adeno-associated virus (AAV)-based vectors hold tremendous promise to transform treatment of genetic diseases. Yet, the potential of AAV gene therapies has so far been limited by development of host neutralizing antibodies (nAbs) that block transduction on subsequent exposures. Clinically, the inability to re-dose AAVs presents challenges because efficacy cannot be restored if transgene expression is subtherapeutic or lost (*e.g.* due to cell division, silencing, or a cytotoxic immune response). Moreover, due to natural AAV exposure, many patients develop nAbs prior to treatment that render them ineligible for even a single dose. Therefore, strategies that prevent or attenuate anti-AAV nAb responses could vastly expand the utility and accessibility of existing AAV gene therapies, while safeguarding eligibility for future AAV-based advances. Here, we report on two distinct B cell immunosuppression approaches that enable AAV vector re-transduction at levels equal to seronegative animals, either by preventing development of post-treatment nAbs (via CD40 blockade) or depleting pre-existing nAbs (via combined plasma cell and immunoglobulin depletion). We first tested whether antagonizing CD40, a receptor crucial to generation of long-lived antibody responses, could suppress development of post-treatment AAV nAbs. Using fully human anti-CD40 antibodies and CD40 humanized mice, we found CD40 blockade prior to systemic AAV8 treatment resulted in potent and durable suppression of post-treatment AAV IgG titers. When administered a second AAV8 vector, mice that received CD40 antibody, but not isotype control, achieved levels of re-transduction equal to seronegative control mice. Mechanistically, we observed CD40 blockade prevented B cell activation and germinal center differentiation, including of AAV-specific B cells. Together, these data suggest transient CD40 blockade preserves immunological naivety to AAV to enable repeated AAV dosing. Long-lived plasma cells (LLPC) mediate constitutive antibody production to most antigens and are the likely reservoir of persistent anti-AAV antibody immunity. While CD40 is required for generation of LLPC, it is not known to impact LLPC survival or antibody secretion. Therefore, we evaluated whether the source of pre-existing AAV nAbs could be directly eliminated *in vivo* by LLPC depletion with linvoseltamab, a fully-human T cell-bridging bispecific antibody targeting B cell maturation antigen and CD3 (BCMAxCD3), either alone or in combination with B cell depletion (to eliminate non-LLPC sources of anti-AAV nAbs), and/or FcRn blockade (to accelerate serum IgG clearance). To model a rigorous scenario of pre-existing AAV immunity, we treated dual CD3/BCMA-humanized mice with AAV8 to induce high-titer nAbs. Ten weeks later, we administered weekly doses of BCMAxCD3, anti-CD19/20, and/or FcRn blocker and monitored anti-AAV IgG titers over time. Whereas treatment with BCMAxCD3, anti-CD19/20, or FcRn blocker alone elicited minor titer reductions, mice treated with both BCMAxCD3 and FcRn blocker showed rapid titer declines to naïve or near-naive levels, and mice additionally treated with anti-CD19/20 universally exhibited undetectable titers after five weeks of treatment. Strikingly, upon administration of a second AAV8 vector, 3/6 and 6/6 mice receiving BCMAxCD3 + FcRn blocker or all three agents, respectively, achieved re-transduction equivalent to seronegative control mice. Collectively, these data suggest CD40 blockade and plasma cell depletion are orthogonal approaches that may enable repeated dosing of AAVs, or other immunogenic gene therapy vectors, regardless of serostatus.

354. Vectorized Antibody-Mediated Anti-Eosinophil Gene Therapy

Maria Gioulvanidou[1], Selenay Sarklioglu[1], Hailey C. Lephart[1], Xinlei Chen[2], Irina V. Lebedeva[2], Yeliz Inalman[2], MaryAnn Pohl[2], Lloyd Bourne[2], David Andrew[2], Ivo C. Lorenz[2], Katie M. Stiles[1], Odelya E. Pagovich[1], Neil R. Hackett[1], Miguel de Mulder Rougvie[1], Stephen M. Kaminsky[1], Ronald G. Crystal[1]

[1]Genetic Medicine, Weill Cornell Medical College, New York, NY,[2]Sanders Tri-Institutional Therapeutics Discovery Institute, New York, NY

Chronic hypereosinophilia, characterized by persistent elevated blood levels of eosinophils (≥1500/μl), is characteristic of chronic eosinophilic leukemia, hypereosinophilic syndrome, allergic disorders and helminth parasite infections. Hypereosinophilia is often associated with tissue infiltration of eosinophils and consequent organ damage by toxic mediators released by the eosinophils. The current therapy for chronic hypereosinophilia is systemic corticosteroids, and if that is not successful, patients are treated with imatinib, vincristine, hydroxyurea, mepolizumab, benralizumab or reslizumab all of which require repetitive administration. As a novel approach to treat hypereosinophilia, we hypothesized that adeno-associated virus (AAV)-mediated delivery of an anti-eosinophil antibody would provide one-time therapy that would mediate persistent suppression of blood eosinophil levels. To generate a humanized anti-human eosinophil monoclonal, the AlivaMab kappa/lambda, AlivaMab kappa and Balb/c mouse strains were immunized with the human Siglec8 protein or DNA (pTTS-huSiglec8). This generated 25 anti-human eosinophil IgG1 monoclonals directed against Siglec-8, a sialic-acid binding Immunoglobulin-like lectin, expressed on high levels on the cell surface of human eosinophils and mast cells and to a lesser extent on basophils. Based on ELISA and flow cytometry assays, the 08C07 monoclonal was chosen for further study. The human variable regions of 08C07 were joined to human Ig constant region, creating H08C07, a fully humanized anti-human eosinophil monoclonal. Using the gene sequence of H08C07, we created EN01, an AAVrh.10-based vector with the CAG promoter driving the heavy and light chains of H08C07 separated by the furin-2A cleavage site. Intravenous administration of EN01 (10^{11} gc) to C57Bl/6 mice resulted in elevated serum levels of H08C07 (3.0±1.5 mg/ml at 4 weeks). Serum from the treated mice was used for a series of *ex vivo* assessments of EN01 function. Gene therapy generated H08C07 bound to recombinant Siglec8 protein in a dose-dependent manner and specifically to human eosinophils. Importantly, overnight incubation of human purified interleukin-5-activated eosinophils with the EN01-generated antibody, mediated higher apoptosis of human eosinophils compared to the untreated group (control 17.3% *vs* 74.9% Annexin-V^+ cells, $p<0.001$). In the presence of human purified NK-cells, the EN01-generated antibody mediated higher antibody-dependent cellular cytotoxicity (ADCC) activity against human eosinophils (control 48.4% *vs* 97.7% Annexin-V^+ cells, $p<0.05$). This data supports the conclusion that EN01 is an effective anti-human eosinophil AAV-based vector that, with intravenous administration, generates high levels of H08C07, a fully humanized monoclonal that mediates potent apoptosis of human eosinophils. EN01 holds the potential to offer therapeutic benefit to patients with chronic hypereosinophilia.

355. Modulating the AAV-Specific Immune Response: A Comparison of T Cell Co-Stimulation Blockade with Rapamycin Plus B Cell Depletion

Audry Fernandez[1], Layth Awartani[1], Abbie Svancarek[1], Mason Zlotnik[1], Dylan Southers[1], Rachel Hedinger[1], Charles Krause[1], Rohini Sheth[1], Alan Curtis[1], Erik Wielechowski[1], D'Shawna Burwell[1], Joerg Votteler[1], Yash Shah[1], Rob Junkins[1], Angela Mote[1], Sherif Gabriel[1], Anna Tretiakova[1], Shari Gordon[1], R. Jude Samulski[1,2]

[1]Asklepios BioPharmaceutical, Inc., Durham, NC,[2]University of North Carolina, Chapel Hill, NC

Immune modulation in the context of Adeno-Associated Virus (AAV) gene therapy aims to suppress the adaptive response to prevent immune-related adverse events and ultimately facilitate AAV redosing. To this end, the combination of the T cell inhibitory drug Rapamycin and B cell depletion with anti-CD20 antibody (rapa/aCD20) has become the strategy of choice in several AAV clinical studies. However, few studies have performed a comparative analysis with other regimens and to our knowledge there is no definitive data indicating that this combination can facilitate intravenous (IV) AAV redosing. Our study compares the effects of CTLA4-Ig to rapa/aCD20 on AAV induced adaptive responses in multiple murine models. CTLA4-Ig is a fusion protein containing the extracellular domain of Cytotoxic T-Lymphocyte Antigen 4 (CTLA4-4) and the Fc portion of a human immunoglobulin. This well characterized immune suppressive agent in clinical use (Abatacept) inhibits CD28-mediated T cell co-stimulation through preferential binding to B7 molecules on mature antigen presenting cells. In this work we evaluated the ability of rapa/aCD20 to facilitate AAV8 redosing in acid alpha-glucosidase (GAA) knockout mice, a pre-clinical model of Pompe disease. The vector was administered IV with secreted nano luciferase and GAA as selected transgenes for the first and second dose, respectively. Inhibitory agents were temporarily administered to control the immune response associated with each vector dose in all the mouse redosing models evaluated. Despite causing a significant reduction of AAV8-specific IgG antibodies (Abs) after the first vector dose, the administration of rapa/aCD20 did not facilitate redosing in this model as demonstrated by the evaluation of vector genomes and GAA enzymatic activity. Interestingly, this immune modulation regimen did not prevent the development of AAV8-specific IgG Abs after a second vector dose. These results supported the idea of testing other immune modulatory agents like CTLA4-Ig. In fact, using the highly immunogenic AAVrh32.33 vector as a model, we observed that CTLA4-Ig as a single agent was superior to rapa/aCD20 at blunting the $CD8^+$ T cell response and maintained long-term inhibition of both T and B cell responses specific for this vector. Next, we evaluated the ability of CTLA4-Ig to facilitate AAVrh32.33 redosing as this vector model allows the comparison of intramuscular (IM) and IV administration. Ovalbumin and luciferase were used as model transgenes for the first and second vector dose, respectively. CTLA4-Ig regimen caused an almost complete reduction of AAVrh32.33-specific IgG Abs after the first vector dose irrespective of the vector administration route. In the IV administration setting, CTLA4-Ig was unable to facilitate redosing and combination with other strategies will be tested in future studies. However, when the vector was dosed IM, an increased luciferase transgene expression

was detected in the group treated with CTLA4-Ig suggesting it could facilitate IM redosing when used as a single agent. In conclusion, our results suggest that co-stimulation blockade with CTLA4-Ig could be superior to rapa/aCD20 to control AAV-specific immune response but would need combination with other immune modulatory agents to facilitate intravenous AAV redosing.

356. Vectorized Expression of a Pan-Filovirus Bispecific Monoclonal Antibody in a Murine Model for Protection Against Ebola and Marburg Virus Challenge

Elena S. B. Campbell[1], Wenguang Cao[2], Arielle N. Gillies[1], Yanlong Pei[1], Jordyn A. Lopes[1], Amira D. Rghei[1], Leonardo Susta[1], Logan Banadyga[2,3], Sarah K. Wootton[1]

[1]Pathobiology, University of Guelph, Guelph, ON, Canada,[2]National Microbiology Laboratory, Public Health Agency of Canada, Winnipeg, MB, Canada,[3]Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, MB, Canada

Filoviruses, such as Ebola virus (EBOV), Sudan virus (SUDV), and the more divergent Marburg virus (MARV), have known epidemic potential. Despite this knowledge, there is a lack of funding and resources being allocated to vaccine development for a number of these infectious diseases. Furthermore, elderly, and immunocompromised populations have a limited capacity to respond robustly to classical antigen-based vaccines, and the production of clinical-grade monoclonal antibodies (mAb) for passive immunization remains labor-intensive, technically challenging, and confers short-lived immunity. One novel strategy to circumvent these pitfalls involves the use of the host as a "bioreactor", whereby host cells are stimulated to generate pathogen-specific mAbs capable of accumulating to therapeutic levels, a platform known as vectored immunoprophylaxis (VIP). Vectored mAb expression mediated by adeno-associated virus (AAV) gene delivery can produce protective and sustained concentrations of therapeutic mAbs in animal models for several infectious diseases. Our rationally engineered AAV6 triple mutant capsid, AAV6.2FF, facilitates rapid and robust mAb expression following intramuscular (IM) administration. Using AAV6.2FF mediated expression of anti-EBOV mAbs 100 and 114 and pan-ebolavirus mAbs FVM04, ADI15878, and CA45 as human IgG1 antibodies, we previously demonstrated protection against mouse-adapted EBOV (MA-EBOV) at low serum concentrations. A follow-up study further showed rapid and robust AAV6.2FF mediated expression of two potent human antibodies, MR78 and MR191, protected against mouse-adapted MARV (MA-MARV) infection in a murine model. Notably, both high ($1x10^{11}$ vector genomes [vg]) and medium dose ($1x10^{10}$vg) groups were protected from lethal MA-MARV challenge. Here, we have re-engineered our AAV-VIP platform to create a broadly protective anti-filovirus immunotherapy. Our bispecific human IgG1 mAb design is comprised of the pan-ebolavirus mAb, FVM04, variable heavy and light chain domains and the self-complimentary variable fragment (scFv) of the MARV mAb, MR191. In a preliminary investigation, IM injection of $1x10^{11}$vg AAV6.2FF-FVM04-MR191 was administered to BALB/c mice (n=4). Antibody expression kinetics resulted in serum human IgG concentrations of 85-201 μg/ml for over 8 weeks post vector administration. Binding of FVM04 and MR191 to the EBOV and MARV glycoproteins, respectively, was also confirmed using a glycoprotein binding ELISA. In a subsequent study, IM injection of BALB/c mice (n=16) with $1x10^{10}$ (low dose), $5x10^{10}$(mid dose), and $1x10^{11}$ (high dose) vg of AAV6.2FF-FVM04-MR191-WPRE resulted in average serum human IgG concentrations of 0.62 μg/mL, 1.43 μg/mL, and 23.41 μg/mL respectively, immediately prior to MA-EBOV and MA-MARV challenge. Six of eight mice in the high-dose group and two of eight in the mid-dose group were protected against lethal MA-EBOV challenge, and none were protected against the MA-MARV challenge. In a follow-up experiment, we have re-designed the bispecific antibody orientation to allow for stronger protection against both viruses and are evaluating a higher dosing regimen. Evaluation of human IgG expression kinetics is currently being measured ahead of repeat challenge experiments. Overall, AAV-mediated antibody gene transfer is a viable method for prolonging the therapeutic effect of recombinant mAbs, representing an alternative "vaccine" strategy for those with compromised immune systems or in possible outbreak response scenarios.

357. Evaluating the Effects of IdeS-Generated Anti-AAV IgG Fragments on AAV Gene Delivery

Saghana Muraleetharan, Valeriya Posternak, Conor Dickson, Scott Ashley, Lindsey Peed, Robert Straub, Victor Howard, Pavani Ponnela, Pitchaimani Kandasamy, Heena Beck, Timothy Luongo, Jeffrey Alexander, Christopher Riling

Spark Therapeutics, Philadelphia, PA

Humoral immunity to wild-type adeno-associated virus (AAV) has the potential to present significant challenges for treating patients with AAV-based gene therapy. A considerable proportion of otherwise eligible patients become ineligible due to pre-existing anti-AAV capsid neutralizing (NAb) antibodies. IgG-degrading enzyme (IdeS), a cysteine endopeptidase derived from *S. pyogenes*, has demonstrated efficacy in degrading pre-existing anti-AAV IgG enabling AAV-mediated gene transfer through systemic delivery. IgG fragments are generated following IdeS cleavage (single-chain IgG (scIgG), Fc, $F(ab')_2$) with different levels of neutralizing properties. While the impact of intact IgG NAbs on AAV gene delivery is relatively well-characterized, the neutralizing effect of IdeS-generated $F(ab')_2$ is not well understood. To assess the impact of $F(ab')_2$ on AAV gene delivery, we utilized a human FcRn (hFcRn) transgenic mouse model passively immunized with intravenous immunoglobulin (IVIg) from pooled human plasma containing anti-AAV NAbs. Efficacy of gene delivery was evaluated after the passively immunized mice received pretreatment with IdeS and were administered a single intravenous dose of liver-directed AAV at several timepoints (2, 4, 6, 24, 48, 72 hours) following pre-treatment. In vehicle pretreated mice, anti-AAV IgG remained in circulation for up to 72 hours and neutralized AAV transduction. In contrast, mice pre-treated with IdeS demonstrated complete cleavage of anti-AAV IgG at 2 hours post-IdeS administration. Additionally, anti-AAV $F(ab')_2$ fragments remained in circulation for up to 6 hours in IdeS treated animals. Although anti-AAV IgG was completely cleaved, neutralization was still observed in plasma up to 6 hours following IdeS pretreatment. AAV-derived transgene expression correlated with increasing time post IdeS pretreatment and decreasing $F(ab')_2$ levels, reaching peak expression at 48-72 hours following IdeS pretreatment.

Although pretreatment using IdeS demonstrates improved AAV transduction in seropositive animals, our data suggest that circulating $F(ab')_2$ fragment has the potential to neutralize AAV and impact gene transfer at early timepoints post-IdeS treatment. Taken together, our findings indicate that $F(ab')_2$ fragment clearance and careful selection of the ideal AAV administration window may be important when considering IdeS pretreatment in patients with pre-existing immunity to AAV.

358. IgG Enzymatic Cleavage Prevents Uptake of AAV and Activation of Phagocytic Immune Cells in Seropositive Human Donors

Blake J. Rust[1], Anita Kumari[1], Nathalie Uzcátegui[2], Lena Windstedt[2], Todd Yoder[1], Minnal Gupta[1], Sushmita Paul[1], Gaelle A. Mardy[1], Gavin George[1], Mengyan Du[1], Eisha Clyde[1], Vyohm Neel[1], Daniel J. Hui[1], Sam Hopkins[1], Greg Gojanovich[1], R. Jude Samulski[1], Simon F. Lacey[1]

[1]Asklepios BioPharmaceutical, Inc., Morrisville, NC,[2]Hansa Biopharma AB, Lund, Sweden

Background Adeno-associated viral vector (AAV) gene therapy is a clinical reality, aiming to bring life-altering treatments to subjects afflicted by genetic diseases. However, natural exposure to wild-type AAV results in ~30-70% seropositivity for neutralizing antibodies (nAb), limiting the treatable patient population. IgG-cleaving enzymes, like that from Streptococcus pyogenes (imlifidase), have demonstrated the ability to provide a unique window for treatment with AAV therapy in the context of pre-existing humoral immunity, although the impact on broader cellular immune modulation has not been thoroughly explored. **Purpose** Previous reports have indicated that AAV nAb+ blood has an increased ability to uptake AAV vectors (*Smith et al.*) and that this effect can be mitigated with complement inhibition. Further, imlifidase has been shown to be successful in altering the AAV/antibody interactions that activate complement, but its effects on AAV uptake into blood immune cells are unknown. We hypothesized that the enhanced uptake effect in the context of pre-existing humoral immunity was driven by immunoglobulin/Fc receptor interactions, therefore we sought to leverage imlifidase to determine if capsid uptake could be ablated in seropositive healthy human donor whole blood. **Results** Following 24-hour incubation of AAV with healthy donor whole blood, leukocytes were isolated and assessed with a comprehensive spectral cytometry panel capable of immune cell phenotyping and intracellular capsid detection. Uptake of both AAV8 empty capsid and an AAV8 construct expressing nano-luciferase was observed at a significantly higher degree in both monocytes, granulocytes, and to a lesser degree in dendritic cells (DC), in seropositive donors as compared to seronegative donors. Uptake of capsid was associated with significant upregulation of costimulatory molecules CD80 and CD86 in the monocyte and DC antigen-presenting subsets, but not granulocytes. Treatment with imlifidase prior to coincubation of donor whole blood with AAV not only ablated capsid uptake in seropositive donors, but also reduced costimulatory molecule upregulation. This finding was corroborated by imaging cytometry which revealed visual reduction of capsid uptake in single cells. Decreased levels of proinflammatory cytokines were also observed following treatment of donor blood with imlifidase. **Conclusions** While the enhancement of AAV transduction by imlifidase has been well documented, less is understood about its effect on cellular immunogenicity, which is ultimately related to toxicity and long-term efficacy in the clinical gene therapy space. Here we demonstrate that cleavage of IgG *in vitro* with imlifidase not only prevents AAV capture by phagocytic and antigen-presenting immune subsets within 24 hours, but also limits the ability of these subsets to activate and express costimulatory molecules that allow for the further propagation of adaptive immune responses. Further experiments are needed to clarify the relative contributions of antibodies and complement components to enhance AAV uptake into immune cells, which ultimately reduces target cell transduction and increases immunogenicity. Altogether, this study indicates that *in vivo* imlifidase treatment to ablate pre-existing antibodies may have further utility in attenuating the cascade of cellular immune responses against AAV - particularly in the context of antigen presentation and T-cell activation. Smith CJ, et al. *Front. Immunol.* 2022 Sep;13:999021.

Oncolytic Viruses

359. Additional Expression of T Cell Engager in Clinically Tested Oncolytic Adeno-Immunotherapy Redirects Tumor-Infiltrated, Irrelevant T Cells Against Cancer Cells, Enhancing Anti-Tumor Immunity

Daisuke Morita[1,2,3,4], Amanda R. Shaw[1,2], Caroline E. Porter[1,2], Greyson G. W. Biegert[1,2], Mae L. Woods[2], Spyridoula X. Vasileiou[2,5], Bora Lim[6], Masataka Suzuki[1,2]

[1]Department of Medicine, Baylor College of Medicine, Houston, TX,[2]Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital, Houston Methodist Hospital, Houston, TX,[3]Department of Pediatrics, Shinshu University School of Medicine, Matsumoto, Japan,[4]Institute for Biomedical Sciences, Interdisciplinary Cluster for Cutting Edge Research, Shinshu University, Matsumoto, Japan,[5]Department of Pediatrics, Baylor College of Medicine, Houston, TX,[6]Duncan Cancer Center-Breast, Baylor College of Medicine, Houston, TX

We have developed a binary oncolytic/helper-dependent adeno-immunotherapy (CAdVEC) expressing IL-12p70, PD-L1 mini-antibody and HSV-tk safety switch (CAd*Trio*) currently under clinical investigation in patients with advanced solid tumors (NCT03740256). Results to date are promising; a single, local injection of CAd*Trio* at the tumor site has safely led to complete eradication of localized tumors in some patients, despite being given at doses 100-fold lower than previous clinical trials of oncolytic Ad. However, all patients primarily amplified Ad-specific T-cells (AdVSTs) after treatment, similar to other OAd trials. Here, we investigate the mechanisms by which CAd*Trio* produces superior anti-tumor effects in patients. We demonstrate that HDAd-infected, but not OAd-infected, cells escape recognition by AdVSTs, and HDAd-derived immunomodulatory transgenes enhance tumor-specific T-cell activity. Tumor biopsy samples collected from patients showed increased CD8+ T-cell infiltration after CAd*Trio* treatment. Given the amplification of adenovirus immunity in patients after CAd*Trio*, these tumor-infiltrating CD8+ T-cells are likely enriched for AdVSTs. Since CAd*Trio* is insufficient to cure distant tumors in

some patients, we thus hypothesize that re-directing these irrelevant T-cells against cancer cells will enhance direct anti-tumor effects and tumor-antigen spread, resulting in increased tumor-specific T-cell development and systemic tumor targeting. CD44 variant 6 (CD44v6) is widely expressed in solid tumors and is known as a cancer stem cell marker. Therefore, we incorporated a T-cell engager molecule targeting CD44v6 (CD44v6.BiTE) into CAd*Trio* to form CAd*Tetra*. We confirmed that CAd*Tetra*-derived CD44v6.BiTE enables AdVSTs to kill CD44v6+ cell lines with AdVST proliferation in vitro and in different xenograft mouse models. Since all patients had adaptive immunity against Ad prior to CAd*Trio* treatment, we developed Ad pre-immunized humanized mouse models to evaluate the efficacy of CAd*Tetra*. CAd*Tetra* significantly controlled tumor growth by repolarizing local and systemic immune responses against cancer cells in immunologically "hot" and "cold" tumor models. Increased tumor-specific T cells also controlled re-challenged tumor growth in humanized mice. These results indicate that CAd*Tetra* effectively induces adaptive T cell responses against cancer cells by redirecting tumor-infiltrating irrelevant T cells, creating a more potent CAdVEC immunotherapy applicable to many solid tumor types.

360. Repurposing the Selective Serotonin-Reuptake Inhibitor (SSRI) Paroxetine to Combat Systemic Immunosuppression in an Orthotopic Syngeneic Murine Glioblastoma Model Treated with Oncolytic MV-s-NAP-uPA Based Immunovirotherapy

Georgios M. Stergiopoulos[1], Susanna C. Concilio[1], Kimberly Viker[1], Susan Clark[1], Steven Robinson[1,2], Evanthia Galanis[1,2]

[1]Department of Molecular Medicine, Mayo Clinic, Rochester, MN,[2]Department of Medical Oncology, Mayo Clinic, Rochester, MN

Introduction: Glioblastoma multiforme (GBM) is the most common malignant brain tumor and carries a dismal prognosis. Notably, GBM has yet to benefit from immunotherapy strategies: a major challenge relates to the systemic immunosuppression, in part mediated via sequestration of T cell populations in the bone marrow. The latter occurs through internalization of sphingosine-1 phosphate receptor (S1P1/S1PR1), which acts as an "exit visa" for T cells from lymphoid organs. S1P1 is internalized by G protein-coupled receptor kinase 2 (GRK2), a protein inhibited by the SSRI paroxetine, an FDA-approved antidepressant. Our team has developed an oncolytic measles virus strain armed with the neutrophil activating protein (NAP) a secretory bacterial peptide derived from *Helicobacter pylori*, which is a TLR2 agonist and potent immunomodulator. Preclinical work combining the NAP-armed virus with immune checkpoint inhibitors (ICIs) targeting programmed cell death protein 1 (PD-1) and T cell immunoreceptor with Ig and ITIM domains (TIGIT) demonstrated significant survival benefit in syngeneic GBM mouse models. We hypothesized that relieving the tumor-imposed T cell sequestration would further enhance the therapeutic effect of our previously established triple pronged immunovirotherapy combination. **Methods:** 7-week-old C57/BL6 female mice were implanted with syngeneic CT-2A murine GBM cells orthotopically. Two intratumoral doses of MV-s-NAP-uPA, a mouse-retargeted MV-s-NAP strain, were administered, complemented by six doses of anti-PD-1 + anti-TIGIT, and daily oral paroxetine administration throughout the treatment. Treatment with granulocyte-colony stimulating factor (G-CSF), an agent shown to increase T-cell circulation in C57/BL6 mice, was used as a positive control. Other controls included untreated mice, mice treated with MV-s-NAP-uPA, MV-s-NAP-uPa + anti-PD-1 + anti-TIGIT, and MV-s-NAP-uPa + anti-PD-1 + anti-TIGIT + paroxetine + G-CSF. **Results:** Mice that received paroxetine in addition to the triple treatment demonstrated survival benefit (P=0.008 compared to the untreated) and a higher cure rate, compared to the triple immunovirotherapy group, reaching an approximately 90% long term survival. Survival was comparable in mice receiving triple treatment with paroxetine, G-CSF, or paroxetine + G-CSF. Flow cytometry data from mice that received paroxetine (and/or G-CSF) in addition to immunovirotherapy, demonstrated an increase in circulating lymphocytes with upregulated S1P1 expression (P=0.055), a higher proportion of activated lymphocytes, particularly CD8+ cells expressing α chain of the high-affinity IL-2 receptor [CD25+, (P=0.048)], denser staining of CD25 on CD4+ and CD8+ cells (P=0.022), more circulating NKT cells (P=0.041) and fewer neutrophilic myeloid-derived suppressor cells (P<0.001) compared to the ones receiving only the triple immunovirotherapy. **Conclusions:** Paroxetine, an SSRI antidepressant, further enhances the efficacy of measles-based immunovirotherapy with ICIs in preclinical GBM models by increasing lymphocyte trafficking and promoting their activation. The commercial availability of paroxetine, coupled with its favorable side effect profile, enhances its potential clinical applicability. Ongoing experiments include cytokine analysis, immunohistochemistry of tumors, brain and spleen, and flow cytometry analysis of the bone marrow, spleen and thymus at different timepoints during the treatment. Updated data will be presented at the meeting. This work was supported by R01 CA258239.

361. The Clinical Oncolytic Virotherapy, VET3-TGI, Displays Potent Therapeutic Activity in Multiple Mouse Tumor Models through Blocking TGF-Beta and Augmenting Type-1 Immune Response

Steve Thorne

Kalivir Immunotherapeutics, Pittsburgh, PA

TGF-beta mediated resistance is one of the major mechanisms of immune suppression utilized across multiple solid tumor types. Blockade of TGF-beta (TGFBi) will therefore likely improve response to immunotherapy. Combining TGF-beta blockade with IL-12 expression may maximize therapeutic benefits through simultaneously reducing immunosuppression and enhancing anti-tumor immune response. Current studies have developed a vaccinia-based immunotherapy, VET3-TGI, combining enhanced systemic virus delivery to CXCR3 ligand rich tumors and locally expressed IL-12 and TGFBi within the tumor microenvironment, for efficient control of multiple tumor models.VET3-TGI infected cells expressed CXCR3 and showed enhanced migration to CXCR3 ligands *in vitro* and improved systemic delivery to tumors expressing CXCR3 ligands *in vivo*, even in the face of pre-existing anti-viral immunity. *In vivo* mouse studies using multiple tumor models demonstrated potent therapeutic activity, including 100% CRs, even at doses several logs below equivalent clinical doses.

Mechanism of activity studies suggested that the therapeutic efficacy of VET3-TGI is associated with considerable modification of the tumor microenvironment. In addition, toxicity studies demonstrated the safety of VET3-TGI in mouse models. VET3-TGI demonstrated an ability to reduce immunosuppression and dramatically enhance antitumor immune response leading to safe and potent therapeutic activity in multiple mouse tumor models. This data led to the selection of VET3-TGI as a lead clinical candidate. A human version has been manufactured for clinical use.

362. Where's Waldo: Finding Tumor Antigens in the Circus of the Immune Response to Oncolytic Viruses

Benjamin Kendall, Rosa Diaz, Jason M. Tonne, Richard G. Vile

Mayo Clinic, Rochester, MN

Oncolytic viruses (OVs) are a class of immuno-oncology agents engineered specifically to replicate in and kill tumor cells. OVs have recently entered the ranks of other cytotoxic T-cell focused therapies like chimeric antigen receptor T cells and immune checkpoint blockade (ICB) due to their ability to redirect T cells targeting either viral or tumor antigens to the site of infection. The current paradigm proposes that OV tumor cell killing induces immunologic cell death which triggers the innate and adaptive immune cascade culminating in the presentation of tumor antigens to their cognate T cell receptors. However, this paradigm largely ignores the impact a highly immunodominant antiviral response against the OV has on the subdominant antitumor response, particularly regarding how the relatively low frequency, low avidity tumor antigens (Waldos) can potentially be masked by a circus of viral antigens. To address this issue experimentally, we first studied the interaction between the two responses in the immunologically cold, B16 melanoma expressing the artificial, and potentially immunogenic, antigen ovalbumin (B16-ova) - a situation in which we would expect Waldo to be relatively visible to the immune system. Contrary to the accepted paradigm, the inflammatory Vesicular Stomatitis Virus (VSV) was unable to stimulate an antitumor T-cell response in C57 mice as determined by MHCI tetramer staining, even when transgenic OT-1 T-cells recognizing ovalbumin were added. Multiparametric phenotypic analysis of these T cell populations via cytometry by time-of-flight confirmed that antiviral T cell expansion induced by VSV led to the ablation of active antitumor T cells, shifting the tumor-reactive T cell landscape toward exhaustion via the increase of markers of dysfunction such as LAG3, TIM3, and CD39, correlating with lower rates of survival. Interestingly, we observed the rescue of tumor specific, activated T cell populations when VSV was armed with ovalbumin (VSV-ova), suggesting the chimerization of the antiviral and antitumor responses allowing the latter to overcome the oppressive effect of the immunodominant antiviral response. Finally, we utilized ICB therapy to bolster the therapeutic capacity of the tumor-specific T cells generated when the OV expressed ovalbumin. We observed that combining VSV-ova with αPD1 therapy greatly improved the survival of B16-ova tumor-bearing mice over VSV + αPD1, thus highlighting the therapeutic insufficiency of tumor antigens released from oncolysis alone. Taken together, these data challenge the conception that immunogenic viruses function as *in situ* tumor vaccines. However, our findings offer insight into how we can improve OV therapy by helping tumor-reactive CD8 T cells find Waldo in the crowded antiviral circus.

363. Anti-Tumor Immune Responses of Oncolytic Vaccinia Virus are Synergistically Enhanced by Induction of Cell-Cell Fusion and Delivery of Multiple Immunomodulators

Motomu Nakatake, Emi Kaitsurumaru, Hajime Kurosaki, Takafumi Nakamura

Division of Genomic Medicine, Graduate School of Medical Sciences, Tottori University, Yonago, Japan

Oncolytic virotherapy is a novel cancer treatment which shows remarkable clinical benefits against refractory tumors. Oncolytic virus induces powerful anti-tumor effects by causing the anti-tumor immune response following the local oncolysis. One of the candidates, vaccinia virus, once widely used as a smallpox vaccine, is now utilized as an oncolytic virus. We have developed two distinct oncolytic platforms, MAPK-dependent recombinant vaccinia virus (MDRVV) and fusogenic oncolytic vaccinia virus (FUVAC). MDRVV achieved tumor-specific virus replication by deleting two viral growth factors, VGF and O1L. FUVAC has isolated from the MDRVV as a mutant clone which has the fusogenic phenotype due to the nonsense mutation of viral fusion inhibitor, K2L. FUVAC-mediated cell-cell fusion more efficiently induced oncolysis and immunogenic cell death in the treated tumors, resulting in efficient anti-tumor immune response through decreasing tumor-associated immune suppressive cells locally and increasing cytotoxic T lymphocytes systemically. Thus, FUVAC changed tumor immune microenvironment from "cold" to "hot", especially in the direct treated tumors. However, it was insufficient to cure the distant untreated tumors such as metastatic tumor region which depends only on the induction of anti-tumor immunity. To remodel the tumor immune microenvironment systemically, we have engineered FUVAC armed with two immunostimulatory cytokines, interleukin 12 (IL12) and C-C motif chemokine ligand 21 (CCL21). At first, IL12 or CCL21 single-armed FUVACs were evaluated by alone or in combination. Mice bearing bilateral CT26 tumors were treated with each virus unilaterally. As expected, the combination of IL12-armed and CCL21-armed FUVAC showed much higher therapeutic efficacy than each viral monotherapy. Therefore, the gene cassettes expressing IL12 and CCL21 were inserted into the VGF and O1 gene loci of FUVAC, respectively. This dual-armed FUVAC-IL12/CCL21 was compared with non-armed FUVAC or dual-armed MDRVV-IL12/CCL21, to identify whether the key therapeutic factor was cell-cell fusion and/or cytokine activity. In bilateral CT26-bearing mice, unilateral injection of MDRVV-IL12/CCL21 induced 29% of complete response (CR) in both tumors, whereas non-armed FUVAC couldn't eliminate the non-injected tumors. Notably, FUVAC-IL12/CCL21 more prolonged the survival of mice than MDRVV-IL12/CCL21 and reached 72% of CR. It suggests the therapeutic efficacy of FUVAC-IL12/CCL21 was synergistically enhanced by both cell-cell fusion and cytokine activity. Moreover, biochemical analysis demonstrated that the IL12 and CCL21 was mostly accumulated in the injected tumors, and the levels of those cytokines in blood were tolerable. On the contrary, anti-tumor IFN-γ was elevated systemically by FUVAC-IL12/CCL21 treatment. Furthermore, whole tumor immune microenvironment was analyzed

by single cell RNA-seq. FUVAC-IL12/CCL21 changed the tumor immune microenvironment systemically, by decreasing tumor cells and increasing tumor-infiltrating lymphocytes in the distant untreated tumors. FUVAC-IL12/CCL21 increased the effector memory T cells while decreasing the exhausted T cells. The detailed analyses including TCR repertoire are ongoing. Our results demonstrated that fusogenic oncolytic vaccinia virus armed with IL12 and CCL21 augmented the antitumor activity via inducing potent and durable antitumor immune responses following extensive viral oncolysis. Thus, FUVAC would be a next generation platform of oncolytic immunotherapy.

364. Oncolytic Adenovirus Armed with a Type I Interferon Exerts Systemic Antitumor Immunity in Immunocompetent Replication-Permissive Models

Kazuho Inoko[1], Shuhei Shinoda[1], Natalia Calixto Mancipe[2], Margarita Romanenko[1], Sacha Robert[1], Mizuho Sato-Dahlman[1], Kari Jacobsen[1], Takuro Noguchi[3], Masato Yamamoto[1], Julia Davydova[1]

[1]Department of Surgery, University of Minnesota, Minneapolis, MN,[2]Minnesota Supercomputing Institute, University of Minnesota, Minneapolis, MN,[3]Department of Medical Oncology, Hokkaido University, Sapporo, Japan

Background: Oncolytic adenoviruses (OAds) are emerging as a promising class of oncolytic virotherapy. The appeal of OAds relies on their intrinsic ability to overcome tumor immunosuppression by inducing immunogenic cell death (ICD). In addition, OAds are capable of highly efficient delivery of immune-stimulating genes to cancer cells with the intent of improving antitumor immune responses. Type I interferons (IFNs), the class of cytokines employed in this study, are known for their pivotal role in both antiviral and antitumor responses. Thus, understanding IFN interactions with replicating viruses, tumor cells, and immune cells is particularly important. However, such fundamental insight is still poorly understood in the field of OAds. Here, we assessed the immunological activities of a replication-competent OAd expressing a syngeneic IFN-α (OAd-IFN) in immunocompetent, replication-permissive Syrian hamster models. **Methods and Results:** We developed novel immunological tools, including spatial transcriptomics (ST), to assess the immunomodulatory role of OAds in Syrian hamsters. Unlike commonly used mouse models, Syrian hamsters are permissive to OAd replication, providing an advantage in simulating OAds behavior in humans. We first evaluated the cytotoxicity of OAd-IFN in hamster pancreatic adenocarcinoma (PDAC) cell lines. OAd-IFN showed significantly higher cytotoxicity compared to the identical luciferase-expressing control virus (OAd-Luc). Next, we examined inflammatory responses by RT-PCR, and found that a variety of proinflammatory chemokine genes, including CXCR3 ligands, and MHC I were upregulated in OAd-IFN infected cells compared to OAd-Luc infected or uninfected cells. The level of extracellular ATP measured by bioluminescent assay was also significantly increased in OAd-IFN infected cells, suggesting the potential of OAd-IFN to induce ICD. We then investigated *in vivo* immunomodulatory activities of OAd-IFN. Hamsters bearing PDAC subcutaneous tumors were treated intratumorally with OAd-Luc or OAd-IFN ($2.5x10^9$ pfu/injection on days 0, 3, 6, 9), and analyzed on day 12. ST analysis revealed that a cluster enriched for various immune-related processes was more prevalent in the OAd-IFN treated responder compared to the OAd-IFN treated non-responder, or the OAd-Luc treated tumors. Interestingly, the immune-rich cluster spatially overlapped with the regions enriched for IFN-α response. We further evaluated *in vivo* systemic efficacy ("abscopal effect") of OAd-IFN. Hamsters bearing PDAC tumors on both flanks were treated intratumorally with PBS, OAd-Luc, or OAd-IFN unilaterally as above. Compared to the control groups, OAd-IFN showed significant tumor growth inhibition in not only the injected tumors, but also in the non-injected distant sites. Infectious viral particles (as quantified by TCID50 assay) were restricted to only the injected sites and were not responsible for the abscopal effect. To reveal the underlying cellular immunity responsible for the abscopal effect, we investigated tumor infiltrating lymphocytes by flow cytometry and found that natural killer cell population was significantly increased in the OAd-IFN group in both injected and non-injected tumors. **Conclusions:** This study demonstrated the potential of OAd-IFN to induce systemic antitumor immunity in immunocompetent OAd-permissive models and will provide crucial knowledge for clinical translation of OAd-type I IFN-based therapies for immunosuppressive tumors, such as PDAC.

365. Novel Vesiculovirus Library Characterization for Repeat Dosing Strategies

Natalie M. Elliott[1], John Vant[2], Yumei Zhou[1], Alex Baker[3], Mitesh Borad[1]

[1]Molecular Medicine, Mayo Clinic, Scottsdale, AZ,[2]Biodesign Center for Applied Structural Discovery, Arizona State University, Tempe, AZ,[3]Division of Cancer and Genetics, Cardiff University, Cardiff, United Kingdom

Vesicular stomatitis virus (VSV) is a negative strand oncolytic RNA virus with a pro-inflammatory life cycle that can immunomodulate the tumor microenvironment. In early phase clinical trials, recombinant VSV vectors have yielded preliminary evidence of anti-tumor activity. Combinatorial pre-clinical studies have shown additive/synergistic effect with checkpoint inhibitors providing the impetus for development as cancer vaccines and in conjunction with cell therapies. A critical limitation in virotherapy is repeat dosing when given intravenously due to host generated neutralizing antibodies. As such, we pursued the development of a library of novel pseudotyped vesiculovirus vectors. Herein we describe host antiviral responses, vector kinetics, and cross-neutralization of these vectors with an ultimate goal of attaining repeat dosing. We obtained five novel vesiculoviruses: Morreton (MORV), Jurona (JURV), Malpais Springs (MSPV), Isfahan (ISFV), and Radi virus (RADV). Neutralization assays with commercially available αVSV-G antibodies, clones 1E9F9 and 8G5F11, showed evasion of cross neutralization with all tested novel vesiculovirus glycoproteins. Protein modeling identified key amino acid residues in the αVSV-G antibody epitopes, followed by sequence alignment that confirmed epitope variability amongst novel vesiculovirus glycoproteins. Patient sera collected from VSV-IFNβ trial NCT01628640 mediated neutralization assays that corroborated these findings. Kinetics assays showed high titer production of all novel vesiculovirus vectors within 24-hours of viral infection. Plaque assays yielded equivalent plaque sizes in wild type vesiculoviruses compared to the respective pseudotyped VSV vector, indicative of the role the glycoprotein serves in efficient viral budding from the host cell. To

assess the ability of the novel vesiculoviruses to antagonize interferon responses we compared IFNβ release from a panel of 32 cancer cell lines following viral infection. ISFV was able to inhibit IFNβ expression at a similar capacity as VSV, with more variable IFNβ inhibition in MORV and JURV. MSPV displayed a surprising lack of interferon response inhibition. Sequence alignments identified matrix protein mutations comparable to the VSV-ΔM51 variant, engendering interest for additional mechanistic studies. Overall, our *in vitro* characterization of these novel vesiculoviruses provides a framework for logical vector engineering strategies and a toolkit that can be advanced towards clinical translation.

Other Cellular and Regenerative Therapies

366. Abeta-Responsive Cells as a Therapy in Alzheimer's Disease

Madeline R. Spetz, Dylan J. Conger, Hyosung Kim, Matthew S. Schrag, Ethan S. Lippmann, Jonathan M. Brunger

Vanderbilt University, Nashville, TN

Introduction: Alzheimer's Disease (AD) afflicts over 6.5 million Americans and has an estimated national cost of $321 billion annually. Biochemically, AD is characterized by aggregation of amyloid beta (Aβ) into plaques and tau into neurofibrillary tangles. Despite significant effort, clearance of Aβ from the brain via monoclonal antibody (mAb) therapies has not demonstrated clear cognitive benefit in clinical trials. Thus, new treatment approaches that do not rely solely on the clearance of protein aggregates are needed. Here, we present a potential cell therapy for AD. Astrocytes are engineered to express a synthetic receptor that recognizes Aβ and allows them to respond in a therapeutic manner via expression of a programmed transgene. **Methods:** We are leveraging the synthetic Notch (synNotch) platform to program cells. We have exchanged Notch domains for synthetic components to construct receptors Aβ-specific receptors: the extracellular domain for a single-chain variable fragment (scFv) of an anti-Aβ mAb and the intracellular domain for a synthetic transcription factor. As in Notch signaling, recognition of the input (ie. Aβ) will result in cleavage of the transcription factor, driving expression of programmed transgenes (Fig 1A). We built a panel of receptors by repurposing mAbs that have been tested in clinical trials. The panels were expressed in fibroblasts with reporter transgenes (mCherry, luciferase) to characterize signaling dynamic range. We then expressed high-performing receptors in human iPSC-derived astrocytes with neuroprotective (BDNF) or anti-inflammatory (soluble TNF receptor, sTNFr) transgenes. Receptor expression in engineered cells was measured by flow cytometry; receptor activity was measured by microscopy, luminescence, qPCR, and ELISA. **Results:** As measured by luciferase expression, Aβ receptors constructed from bapineuzumab, gantenerumab, and donanemab recognize synthetic $A\beta_{42}$ and $A\beta_{40}$. Each receptor exhibits a wide dynamic range - up to an 80-fold increase in transgene expression in response to Aβ - with limited background activity (Fig 1B). Human astrocytes programmed with the bapineuzumab synNotch (Bap-Notch) recognize human brain-derived Aβ, as demonstrated by mCherry (Fig 1C). Bap-Notch astrocytes produce BDNF upon recognition of synthetic $A\beta_{42}$ (Fig 1D). When stimulated by inflammatory cytokines (IL-1α, TNFα), astrocytes take on a reactive state with increased *IL6*, complement *C3*, and *SERPIN3A* expression. This inflammatory gene signature is partially attenuated by synthetically regulated sTNFr in Bap-Notch astrocytes. In astrocytes expressing a reporter transgene (SEAP) via Bap-Notch, Aβ trends toward exacerbating the inflammatory phenotype; this is not seen when synNotch drives the production of the sTNFr transgene (Fig 1E). **Conclusion:** Aβ synNotch receptors represent a promising new therapeutic approach to treating AD. Capitalizing on their native surveillance functions in the brain, astrocytes expressing these receptors can specifically recognize Aβ as a marker of disease and respond via direct upregulation of a programmed therapeutic transgene. Due to the versatility of the synNotch platform, it is possible to produce many different transgenes under control of Aβ receptors (Fig. 1A). Additionally, the expression of synNotch receptors in other cell types, such as microglia or neurons, opens the possibility of alternative cell therapies. Future studies will investigate Aβ synNotch as a gene therapy in mouse models of AD.

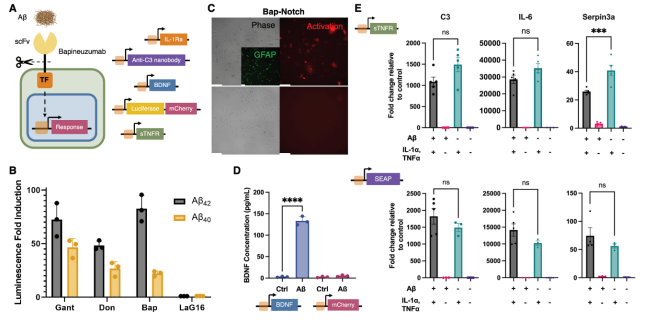


Fig. 1. Testing of Aβ synNotch receptors. **(A)** Replacement of the Notch extracellular domain by an Aβ scFv results in an Aβ-specific receptor. Recognition of Aβ cleaves the synthetic transcription factor from the transmembrane core and results in subsequent transgene expression. An mCherry-luciferase transgene was used for receptor panel screening; sTNFr and BDNF are candidate therapeutic transgenes tested. The modularity of synNotch allows for many possible transgenes. **(B)** Aβ-dependent expression of luciferase in L929 fibroblasts. LaG16 is an off-target synNotch receptor. **(C)** Activation (nCherry) in Bap-Notch astrocytes by patient-derived Aβ. GFAP confirms astrocyte phenotype. Scale bar = 200 μm. **(D)** SynNotch-driven production of BDNF transgene in Bap-Notch astrocytes cultured on synthetic $A\beta_{42}$. Magenta bars represent Bap-Notch astrocytes without BDNF transgene. ****p<0.00005. **(E)** Gene expression analysis of Bap-Notch astrocytes show partial attenuation of inflammation when Aβ results in sTNFr transgene expression; Aβ results in exacerbation of inflammatory gene signature in Bap-Notch astrocytes with control SEAP transgene. ***p<0.0005.

367. Improvement of Disease Phenotype in Beta-Thalassemic Mice with a Novel Radiation-Free Conditioning Regimen of Pro-Apoptotic mRNA

Laura Breda[1], Michael Triebwasser[2], Naoto Tanaka[1], Ariel Rivera[1], Tyler Papp[3], Awarama Akyanu[3], Jayalakshmi Ramani[3], Barbara Mui[4], Ying K Tam[4], Drew Weissman[3], Peter Kurre[5], Hamideh Parhiz[3], Stefano Rivella[1]

[1]Pediatrics, Hematology, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Pediatrics, University of Michigan, Ann Arbor, MI,[3]Infectious Diseases, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[4]Acuitas Therapeutics, Vancouver, BC, Canada,[5]Bone Marrow Failure, University of Pennsylvania, Philadelphia, PA

Hematopoietic stem cells (HSC) are the precursors of all blood cells. HSC with inherited genetic defects can be replaced with healthy HSC through allogeneic HSC transplantation (HSCT). However, current conditioning protocols carry significant short- and long-term side effects (i.e., sterility, secondary malignancies), and can significantly delay patient recovery after HSCT, putting them at risk of additional

complications. We previously developed CD117/LNP-mRNA, a lipid nanoparticle (LNP) targeted to stem cell factor receptor (CD117), which can significantly increase the delivery of mRNA to HSC *in vivo*. We sought to test the ability of CD117/LNP to deplete BM cells using a pro-apoptotic p53 upregulated modulator of apoptosis (PUMA) mRNA that acts on the anti-apoptotic gene Mcl-1, which is implicated in survival of human and mouse HSC. We had found that treatment of murine C57BL/6 BM cells with particles loaded with (PUMA) mRNA reduced short and long-term HSC (LT-HSC) viability in culture. Competitive transplants using a combination of *ex vivo* CD117/LNP-PUMA-treated bone marrow and untreated GFP$^+$ congenic donor bone marrow, confirmed selective survival and engraftment of untreated GFP$^+$ HSC over time, indicating effective *ex vivo* depletion of the LT-HSC by CD117/LNP-PUMA. To minimize PUMA off-target expression in the liver, we modified mRNA cargo to incorporate liver-specific miRNA binding sites for mir-122 into the 3'UTR of our PUMA mRNA. Non-genotoxic conditioning by direct I.V. administration of pro-apoptotic CD117/LNP-PUMA^{mir122} accomplished the engraftment of donor HSC at rates sufficient to improve or cure congenital immune disorders, like severe combined immunodeficiencies (SCID). To improve and test the ability of this technology to correct the phenotype of diseases that require higher levels of donor chimerism (i.e. hemoglobinopathies), we utilized our conditioning regimen in a murine model of beta-thalassemia (*Hbb$^{th3/+}$*). In *Hbb$^{th3/+}$* mice, we observed that 0.125mg/kg of CD117/LNP-PUMA^{mir122} injected I.V., followed by transplantation of GFP$^+$ bone marrow, led to up to 30% red blood cells (RBC) donor chimerism. Anemia was improved: hemoglobin raised from 7.5g/dL in control mice up to 11g/dL in treated mice. Other blood parameters that are major features of ineffective erythropoiesis (RBC morphology, hematocrit levels, RBC number, and reticulocytosis) were all improved. Additionally, secondary transplantation of the bone marrow obtained from the primary chimeras showed continued GFP expression in blood cells, suggesting self-renewing and multilineage capacity of the HSC engrafted after conditioning with CD117/LNP-PUMA^{mir122} particles. Together our data indicate that *in vivo* targeting with CD117/LNP-PUMA^{mir122} particles can effectively deplete endogenous HSC, allowing successful donor engraftment without toxic chemotherapy or irradiation. The improved administration regimen allowed us to correct the phenotype of thalassemic animals, expanding the range of application of this technology to diseases that require higher level of engraftment, such as thalassemia. The ability to target and selectively deplete HSC *in vivo* offers a novel non-genotoxic conditioning regimen for HSCT and could overcome some of the limitations with current genotoxic conditioning, offering a potential new and advantageous tool to the HSCT armamentarium.

368. A Gene Agnostic Stem Cell Therapy for Mid-Stage Retinitis Pigmentosa Treatment

Deepti SIngh[1], Pierre Dromel[2]

[1]Ophthalmology, Ingel Therapeutics, Allston, MA,[2]Ophthalmology, InGel Therapeutics, Allston, MA

Retinitis pigmentosa (RP) comprises a group of progressive inherited retinal dystrophies characterized by sequential loss of rod then cone photoreceptors, resulting in disabling vision impairment. Our investigational treatment, IGT001, aims to rescue diseased photoreceptors by utilizing encapsulated human rod precursor cells that act by secreting protective protein factors. We demonstrate robust anatomical and functional efficacy across three mutant mouse models of RP. Additionally, unbiased proteomic analysis identified a subset of key secreted proteins underlying these phenotypic improvements in models. **Methods:** Rho, rd10, and P23H mutant mouse lines were used and IGT001 was intravitreally injected at disease stage analogous to mid-stage RP. IGT001 comprises hRPs encapsulated within a biomimetic hydrogel scaffold for intravitreal injection using a translational delivery platform. Testing included histological, electrophysiological, and behavioural assessments longitudinally. In parallel experiments, conditioned media harvested from hRP cultures underwent quantitative mass spectrometry-based profiling followed by sequential in vitro and in silico screening steps to delineate key factors and mechanisms conferring neuroprotective and repair properties. **Results:** Across models, IGT001 elicited >40% increased cone photoreceptor survival versus sham controls based on central retinal histology. Rescued cells maintained outer segment integrity, suggesting preserved function. This anatomical preservation was accompanied by significant gains in electrical responses to light stimuli, with x2 fold greater photopic b-wave amplitudes and cognitive optomotor reflexes, together indicating recovery of visual capacity compared to rapid deterioration in control. Proteomics identified over 300 secreted proteins, of which subsequent target validation studies functionally confirmed 10 factors modulating pathogenic processes from glycolysis dysfunction to protein misfolding as central drivers of the multifaceted treatment effects observed. **Conclusions:** In three distinct genetic RP models, sustained delivery of trophic factors by intravitreally injected allogeneic hRPCs safely preserved cone photoreceptors and visual function. Elucidating responsible protein agents and mechanisms enables optimizing potency. This platform technology may enable mutation-agnostic intervention amidst disease heterogeneity by restoring cone functions.

369. REGEN-HEPS: Expandable and Functional Hepatocytes Derived from Primary Cells

Arnav Chhabra, Sunil Mallanna, Soujanya Karanth, Sophie Cazanave, Maxwell V. Buskirk, Joseph Marturano, Tim Norman, Esha Uddin, Abhijith Kudva, Andrew M. Hashkes, Mehar Cheema, Lindsay Tomczak, Aarohi Thakkar, David Broderick, Billy Scholl, Kevin Macera, Michael Ret, Julie Morse, Eric Vanderploeg, Tom Lowery, Alireza Rezania

Satellite Biosciences, Watertown, MA

Introduction The current landscape of liver disease in the United States is alarming, with 4.5 M patients affected and over 50,000 deaths annually[1]. Cellular therapy holds promise as a potential solution, yet its progress has been hampered by the lack of a sustainable source of highly functional hepatocytes. Although primary cells function well, their availability for widespread patient use is severely limited. On the other hand, progenitor-derived cells can be produced in large quantities but do not recapitulate mature hepatocyte functions[2]. To address this critical gap, we leveraged insights from developmental biology and liver regeneration to generate REGEN-HEPS (Regenerative Expansion, Growth, and ENgraftment of primary HEPatocyteS), a novel source of primary cell-derived hepatocytes that combines

expansive growth with retention of mature hepatocyte phenotype and functions. **Methods** We utilized primary human hepatocytes (PHHs) from multiple donors, subjected them to our expansion protocol, and verified growth through a cell count assessment. During the expansion process, we confirmed maintenance of hepatic identity through immunofluorescent co-staining of HNF4α and albumin. For hepatic functional assessment, we evaluated markers across axes of synthesis, metabolism, and detoxification. To engineer the expanded cells, we employed lentiviral vectors and overexpressed a protein of interest. For in vivo assessments, we conducted intrasplenic injections of REGEN-HEPS, PHHs, and iPSC-derived hepatocytes (iPSC-Heps) into *Fah*$^{-/-}$*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ (FRG) mice[3] and evaluated engraftment using secreted biomarkers like human albumin and explant assessment of Fumarylacetoacetate hydrolase (FAH) staining. **Results** Our process effectively expanded primary human hepatocytes from several different donors, with 36% (4 out of 11) of the donors showing more than 20-fold expansion in the first passage (Fig. 1Ai). We achieved > 1,000-fold expansion over two passages for one donor (Fig. 1Aii). Post-expansion, the cells retained key hepatocyte lineage markers, HNF4α and albumin, ensuring preservation of cellular identity (Fig. 1Bi). Moreover, we successfully engineered REGEN-HEPS with high transduction efficiency (94%), which is typically unattainable with standard, non-cycling PHHs (Fig. 1Bii). When placed in a three-dimensional culture, REGEN-HEPS demonstrated functional capacities on par with those of PHHs, across both synthetic and metabolic functions (Fig. 1C). Most notably, REGEN-HEPS exhibited robust repopulation abilities in vivo within the FRG mouse model, markedly outperforming iPSC-Heps, which highlights their potential for future clinical applications (Fig. 1D). **Conclusions** REGEN-HEPS offers a scalable, cGMP-compatible hepatocyte source that maintains hepatic identity during expansion and exhibits functionality on par with primary hepatocytes, both in vitro and in vivo in the FRG model. This breakthrough addresses a vital gap in the field of liver cell therapy and potentially represents a substantial step forward in the ability to treat chronic liver diseases.

References 1. Everhart+, *NIDDK*, 2008 2. Zhang+, *Cell Stem Cell*, 2018 3. Azuma+, *Nat Biotechnol*, 2007

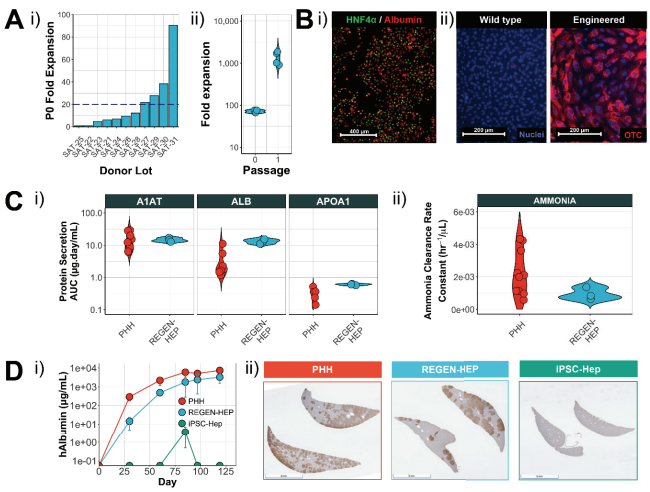


Figure 1: Comprehensive analysis of REGEN-HEP expansion, function, and therapeutic potential in vivo. **A)** Expansion of primary human hepatocytes (PHHs): i) Expansion across multiple donors with >20-fold expansion observed in 4 out of 11 donors during the initial passage (P0); ii) Over 1,000-fold expansion across two passages for one donor. **B)** i) Retention of key hepatocyte lineage markers (hepatocyte nuclear factor 4 alpha, HNF4α, and albumin) post-expansion; ii) Lentiviral-mediated overexpression of a specific protein (ornithine transcarbamylase, OTC, indicated in red) in REGEN-HEPS with 94% transduction efficiency. **C)** Functional comparisons: i) Secretion levels of liver-specific proteins (A1AT, ALB, APOA1) as measured by ELISA area under the curve (AUC) values, and ii) ammonia clearance capabilities as measured by in vitro rate constant, showing equivalence between REGEN-HEPS (n = 3 lots) and PHHs (n = 10 donors) in a 3D culture environment. **D)** In vivo validation: Successful repopulation of REGEN-HEPS in Fah$^{-/-}$/Rag2$^{-/-}$/IL2rg$^{-/-}$ (FRG) model, comparable to PHHs and superior to iPSC-derived hepatocytes (iPSC-Hep), as determined by i) circulating human albumin (hAlbumin) levels and ii) Fumarylacetoacetate hydrolase (FAH) staining.

370. A Synthetic Receptor Platform to Program Mesenchymal Stromal Cells for the Detection and Treatment of Osteoarthritis

Bonnie L. Walton[1], Rebecca Shattuck-Brandt[1], Catherine A. Hamann[1], Victoria W. Tung[1], Juan M. Colazo[1], David D. Brand[2], Karen A. Hasty[3], Craig L. Duvall[1], Jonathan M. Brunger[1]

[1]Biomedical Engineering, Vanderbilt University, Nashville, TN,[2]Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN,[3]Department of Orthopaedic Surgery and Biomedical Engineering, University of Tennessee Health Science Center, Memphis, TN

Introduction: Osteoarthritis (OA) is a joint disease that affects over 32 million Americans, leading to pain and limited mobility. No disease-modifying drugs exist. Cartilage catabolism, which exposes a collagen II (CII)-rich extracellular matrix, is a biophysical hallmark of OA brought about by several factors including inflammation from cytokines such as IL-1. Current cell engineering strategies to treat OA rely on unregulated expression of therapeutic transgenes and thus do not guide cell behaviors based on disease-specific pathology. Here, we present a synthetic receptor design strategy to activate therapeutic transgene expression by engineered cells that is spatially constrained to damaged cartilage. **Methods:** We leveraged the synthetic Notch (synNotch) receptor platform, which is based on native Notch signaling and requires an immobilized ligand for receptor activation. By engineering a scFv from a monoclonal antibody targeting CII (mAbCII) as an extracellular receptor domain, as well as a synthetic intracellular transcription factor to regulate inducible transgene expression, we engineered a CII-synNotch receptor that allows cells to recognize an OA-specific biomarker and perform regenerative functions that ameliorate the disease (**Fig. 1A**). Lentiviral vectors encoding the

receptor and inducible transgenes were used to co-transduce primary marrow-derived murine mesenchymal stromal cells (MSCs). For CII-decorated surface experiments, purified CII was adsorbed to culture vessels overnight for ligand presentation to CII-synNotch MSCs. In tissue-based activation experiments, porcine cartilage explants were cryosectioned and enzymatically degraded with 0.25% trypsin-EDTA to expose the CII network, mimicking arthropathy. Activation was assessed via bioluminescence and fluorescence microscopy. Gene expression was quantified with qRT-PCR. **Results:** CII-synNotch MSCs demonstrate robust (40-fold) activation on 25 µg/ml CII (**Fig. 1B**). Engineered cells expressing activation-dependent mCherry on porcine cartilage explants reveal spatially regulated expression, but do not activate when occupying regions out of contact with the cartilage substrate (**Fig. 1C**). SynNotch-induced expression of pro-anabolic TGFβ3 led to an increase in *Acan* and *Col2a1* gene expression, as well as a decrease in hypertrophic *Col1a1* (**Fig. 1D**). CII-synNotch mMSCs engineered to inducibly express anti-inflammatory IL-1 receptor antagonist (IL-1Ra) were plated in a co-culture with ATDC5 chondrocytes stimulated with IL-1 (**Fig. 1E**). IL-1Ra-producing synNotch MSCs antagonized chondrocyte inflammation in a CII-dependent manner (**Fig. 1F**). **Conclusions:** Using CII as an OA biomarker for synNotch transgene expression enables cells to be functional detectors of arthropathy and serve as living therapies. The regulation conferred by CII-synNotch MSCs may circumvent off-target effects of biologic drugs by restricting expression to niches displaying direct signatures of OA pathology. This work demonstrates the first use of the synNotch platform for therapeutic transgene expression in the context of musculoskeletal systems.

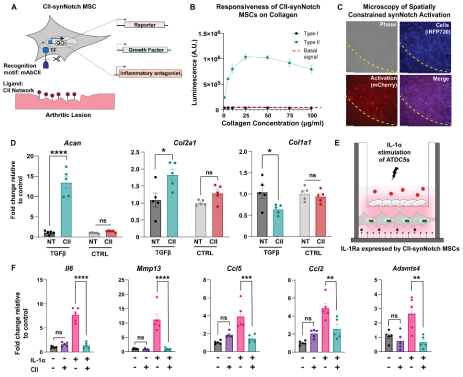


Figure 1: CII-synNotch MSCs detect CII in arthritic cartilage for programmable transgene expression. **(A)** The synNotch receptor works by 1) receptor recognition of exposed CII; 2) transmembrane cleavage; 3) release of a transcription factor that regulates gene expression. **(B)** Engineered cells activated potenty on 25µg/ml CII (t=72hr). **(C)** Cells (constitutive near-infrared fluorescent protein, or iRFP, false-colored blue for visualization) activate spatially restricted transgene expression (inducible mCherry) on physically damaged cartilage cryosections (t=48hr, scale=200µm). **(D)** Gene expression of engineered cells with either pro-anabolic TGFβ3 or control transgenes (t=72hr). **(E)** Trans-well schematic of co-culture with or without stimulated chondrocytes (top) and engineered MSCs with or without CII (bottom). **(F)** Gene expression of chondrocytes demonstates antagonism of inflammation when co-cultured with activated IL-1Ra-expressing CII-synNotch MSCs (t=5d). *p<0.05, **p<0.01, ***p<0.001,****p<0.0001.

371. Genetic Modification of Vascularized Composite Allografts via *Ex Vivo* Machine Perfusion

Raphaela Bento[1,2,3], Irina C. L. J. Filz von Reiterdank[1,2,4], Antonia T. Dinicu[1,2], A. B. Mink van der Molen[4], J. H. Coert[4], Curtis J. Cetrulo[2], Biju Parekkadan[1,3], Korkut Uygun[1,2]

[1]Center for Engineering in Medicine and Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA,[2]Organ Reengineering Laboratory, Shriners Children's Boston, Boston, MA,[3]Biomedical Engineering, Rutgers University, Piscataway, NJ,[4]UMC Utrecht, Utrecht University, Utrecht, Netherlands

Introduction: Lentiviral vectors have been extensively explored as gene delivery tools due to their ability to provide long-term and stable transgene expression, being promising candidates to promote genetic modification of organs. Ex vivo machine perfusion can be used in clinic to improve graft function, providing hours during which an organ can be preconditioned and modified as desired. This study aims to determine the feasibility of combining both approaches to enable targeted modifications of Vascularized Composite Allografts (VCA). VCA transplantation is one of the only options to restore motor function and reconstruct severe defects arising from trauma and burn injuries. However, its use in clinic remains limited due to the high incidence of acute rejection and side effects. Therefore, the field would significantly benefit from genetic engineering applications to locally resolve inflammation and rejection. This proof-of-concept study demonstrates the first successful genetic modification of VCAs post-transplantation. **Methods:** Lentiviral vectors expressing a secreted biomarker (*Gaussia Luciferase* - GLuc) under the constitutive promoter EF1α were generated using triple-transfection methods. Viral supernatant was collected, filtered, centrifuged, purified, and stored at -80oC. Vector titer was determined by qPCR. In vitro transduction efficiency studies were performed with primary rat myocytes and supernatant was analyzed for GLuc secretion. In vivo, hindlimbs of male Brown Norway rats were perfused at 37°C for 12h. $1.3x10^9$ viral particles were injected and recirculated for 11h. Perfusate was collected for gas and tissue injury marker analyses. Partial hindlimbs were transplanted to male Lewis rat recipients; animals were monitored for 8 days. Blood was drawn on post-operative days (PODs) and analyzed for biomarker secretion. Protein was extracted from tissue samples and analyzed for biomarker expression. **Results:** Viral transduction efficiency in primary cells exhibited a dose-dependent response (**Fig. 1 A-B**), guiding the selection of parameters for in vivo applications. Perfusion apparatus was used for ex vivo delivery of viral vectors before transplantation (**Fig. 1C**). Plasma at POD3 showed significantly increased GLuc levels compared to pre-operative levels, and signal strengthened over time. (**Fig. 1D**). In vivo bioluminescent imaging on POD8 showed a stark difference compared to control graft, with a 10-fold increase in radiance signal and comparable clinical evolution (**Fig. 1E**). Tissue assessment showed significant GLuc levels in transplanted tissues only, suggesting lack of off-target effects (**Fig. 1F**). **Conclusions:** This study demonstrates the feasibility of using ex vivo machine perfusion as a delivery system to achieve successful genetic modification of VCAs. Lentiviral vectors are effective in transducing primary cells and perfused grafts, however higher doses and longer exposure times are necessary to obtain such results. Post-

transplantation, transgene is increasingly expressed in target tissues. This approach opens many possibilities for graft modification and extended preservation.

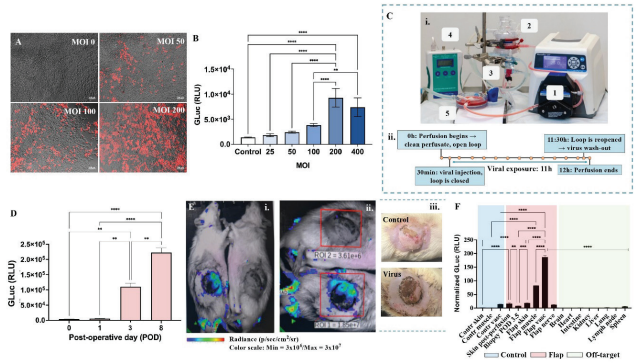


Figure 1. Genetically modified VCAs exhibit transgene expression post-transplantation. (A) Primary cells were treated with increasing MOIs (multiplicity of infection) of lentiviral vectors and exhibited dose-dependent transgene expression. **(B)** Similarly, biomarker secretion confirmed dose-dependent transduction efficiency (One-way ANOVA, **p≤.01; ***p≤0.001; **** p≤.0001). **(C) i.** *Ex vivo* machine perfusion apparatus, consisting of: 1) Roller pump; 2) Oxygenator; 3) Bubble trap; 4) Pressure monitor and 5) Basin with limb. **ii.** Schematic of viral perfusion protocol, with viral exposure of 11 hours. **(D)** GLuc expression in plasma increased significantly over time (POD = post-operative day). **(E) i.** Luminescent signal in transplanted partial hindlimb (left) compared to no signal in control transplant (right); **ii.** Enlarged regions of interest (ROI) show a 10-fold lower radiance signal in control hindlimb (top) compared to viral-perfused hindlimb (bottom). **iii.** Clinical state of VCA transplants at POD 7. **(F)** Each VCA tissue type and other organs exhibited significant local transduction without off-target effects (One-way ANOVA, **p≤.01; ***p≤0.001; **** p≤.0001).

372. Nongenotoxic CD45-ADC-Based Conditioning Maintains Potent Suppression of Plasma Viremia in Nonhuman Primate Model of HIV

Jason D. Murray[1], Teresa Einhaus[1], Carly Starke[1], Stefan Radtke[1], Christopher W. Peterson[1,2], Hans-Peter Kiem[1,2]

[1]Fred Hutchinson Cancer Center, Seattle, WA,[2]University of Washington, Seattle, WA

Introduction: Although hematopoietic stem and progenitor cell (HSPC) gene therapies provide lifelong benefit in numerous hematological diseases and disorders, safety and toxicity remain a critical barrier for routine application. In the setting of immunodeficiency syndromes and infectious diseases such as human immunodeficiency virus (HIV) infection, conditioning regimens may exacerbate immune dysfunction, undercutting measures of efficacy. We previously showed this in our nonhuman primate (NHP) model of HIV, where myeloablative conditioning with total body irradiation potentiated rather than reduced viral reservoirs due to a conditioning-induced loss of immune control. Our NHP model is ideally suited to identify regimens that replace or augment hematopoietic subsets with minimal impact on host immunity. In this study, we further sought to maximize engraftment of CRISPR-edited HSPCs and redirect cytoreductive approaches to i) reduce the number of infected cells *in vivo*, and ii) maintain robust virus-specific immunity. Methods: Enriched CD34$^+$ HSPCs were edited with NHP *CCR5*-specific CRISPR ribonucleoprotein (RNP) complexes, then cryopreserved. Three conditioning regimens were compared: antibody-drug conjugate (ADC) regimens targeting either CD117 or CD45 and myeloablative busulfan. We quantified extent of myeloablation/immunosuppression, time to hematopoietic recovery, long-term engraftment of CRISPR-edited autologous HSPC, and resistance to infection when challenged with increasing concentrations of an HIV-like virus. We further compared busulfan and CD45-ADC in our preclinical model of antiretroviral (ART)-suppressed HIV infection. Results: We observed myeloablation with similar rates of hematopoietic recovery, preserved lymphocyte counts, and long-term engraftment of *CCR5*-edited cells with all three conditioning regimens. Neither ADC conditioning nor busulfan augmented SHIV susceptibility in a prophylaxis model. Engraftment of edited cells was highest with busulfan conditioning, with frequencies as high as 40% in peripheral blood. While both CD117-ADC and CD45-ADC conditioning supported similar levels of editing, some CD45-targeted animals showed engraftment with reduced or no neutropenia. In our preclinical model of HIV latency, both busulfan and CD45-ADC elicited myeloablation and mild lymphopenia while maintaining potent suppression of plasma viremia and similar long-term *CCR5* editing efficiencies in peripheral blood. In animals assessed shortly after transplantation, we observed tissue-specific depletion of CD45$^+$ cells after conditioning with ADC. Conclusions: We have advanced our model of HIV-specific HSPC gene therapy in a clinically relevant model and observe long term persistence of CCR5-edited HSPC and progeny *in vivo* after targeted conditioning. Myeloablative busulfan, CD117-ADC and CD45-ADC conditioning all supported engraftment without impaired resistance to SHIV. In our model of antiretroviral therapy-suppressed HIV infection, hematopoietic recovery and engraftment of edited cells after CD45-ADC conditioning was comparable to busulfan and achieved tissue-specific depletion of CD45$^+$ cells. We are investigating the ability of this approach to clear latent viral reservoirs at these sites, and the ability of *CCR5*-edited HSPC to synergize with treatment strategies utilizing anti-HIV broadly neutralizing antibodies. These results are a promising step towards developing targeted strategies to engraft cells with therapeutic edits and antibody-based selection without the need for alkylating chemotherapies which are also relevant for malignancies and hemoglobinopathies.

Novel Immune Effector Cell Manufacturing

373. Single-Chain Variable Fragment Affinity Tuning Can Optimize Anti-AML CAR-NK Cell Functionality

Ruyan Rahnama[1], Huilin Yang[2], Monika Kizerwetter[2], Natalie Holl[1], Jun Choe[1], Stamatia Vorri[1], Arjun Modi[1], Jamie Spangler[2], Challice Bonifant[1]

[1]Johns Hopkins School of Medicine, Baltimore, MD,[2]Johns Hopkins University, Baltimore, MD

Background: CD123 is overexpressed on AML blasts relative to healthy myeloid progenitor cells. We hypothesize that CD123 targeting Chimeric Antigen Receptor (CAR)-modified NK cells with affinity tuned single-chain variable fragments (scFvs) can be engineered to discriminate target based on surface density for enhanced killing of leukemic cells. **Methods:** We designed CD123 targeting CARs using published scFv sequences of empirically validated affinity (7G3 WT K_d 8.75nM; variant K_d range 23.9-64.9nM) fused to intracellular portions of the NK-activating receptors 41BB and CD3ζ. CARs were retrovirally transduced into primary NK cells and surface expression measured by flow cytometry. CD123 negative Raji parental cells were engineered to generate pure model cell lines with low (Raji.CD123Low: 4.7x10^4

CD123/cell) and high (Raji.CD123High: 5.8x10^5 CD123/cell) CD123 expression correlative to healthy cells and AML blasts, respectively. We tested antigen-specific CAR NK cell activation and cytotoxicity using short- and long-term coculture assays. We used confocal microscopy to quantify membrane polarization at the CAR-NK and target cell immunological synapse. We studied CAR-NK activity in vivo with systemic inoculation of the AML cell line MOLM13. Firefly Luciferase (ffLuc) expressing CAR-NK cells were injected via tail vein and trafficking and persistence monitored with bioluminescence imaging. **Results:** CAR-NK cells showed antigen-specific cytotoxicity against CD123$^+$ target cells compared to non-transduced control NK cells. Low affinity (7G3L) CAR-NK cells demonstrated more powerful short-term cytotoxicity when compared to higher affinity (7G3M, 7G3 WT) CAR-NK cells against Raji.CD123High (7G3L vs 7G3 WT $p < 0.5$) and MOLM13 (7G3L vs 7G3 WT: $p<0.5$; 7G3L vs. 7G3M <0.001) at a 1:2 effector to target ratio. 7G3L CAR-NK discriminated on antigen density with significantly higher short-term cytotoxicity against Raji. CD123High compared to Raji.CD123Low in vitro (7G3L vs 7G3 WT: $p<0.01$) at 1:2 and 1:1 effector to target ratios. 7G3L CAR-NK demonstrated greater persistence in killing relative to 7G3 WT CAR-NK with serial stimulation of the AML cell lines MOLM13 and MV411, though overall persistence of activity was limited. 7G3 and 7G3L CAR-NK had significantly higher polarization of perforin granules at the immune synapse when compared to non-transduced control NK cells but no differences were observed related to binding affinity. 7G3L CAR-NK cells displayed more robust expansion in vivo relative to 7G3 WT CAR NK cells. **Conclusions:** Lower affinity 7G3L CAR-NK cells demonstrate greater short-term and long-term cytotoxicity against high CD123-expressing targets and can discriminate antigen density in vitro with preferential killing of high CD123-expressing targets. 7G3L CAR-NK demonstrate greater expansion in a systemic AML mouse model. These data support the role of affinity tuning to fully optimize cellular therapy designed to treat cancer.

Figure 1

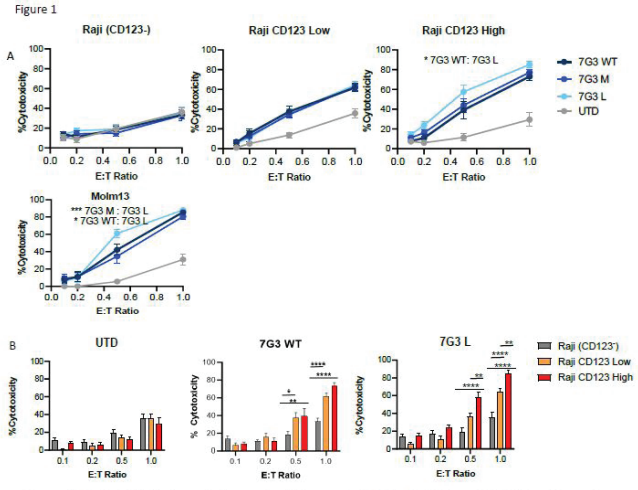


Figure 1. 7G3L CAR-NK demonstrate superior killing against Raji.CD123High and MOLM13 with greater discrimination between high and low antigen density targets. (A) 7G3 WT and variant CAR-NK cells were cultured with ffLuc expressing target cells. %Cytotoxicity was calculated based on residual bioluminescence; (B) Comparison of %Cytotoxicity by 7G3 WT and variant CAR NK cells on Raji.CD123High and Raji.CD123Low.

Figure 2

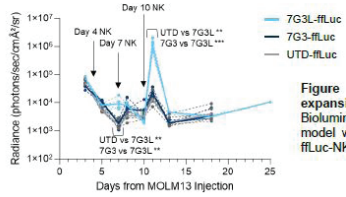


Figure 2. 7G3L CAR-NK demonstrate superior expansion in a systemic AML mouse model. Bioluminescent quantification of NK cells in systemic AML model with MOLM13 inoculation on Day 0 followed by ffLuc-NK cells administration on Day +3, +7, and +10.

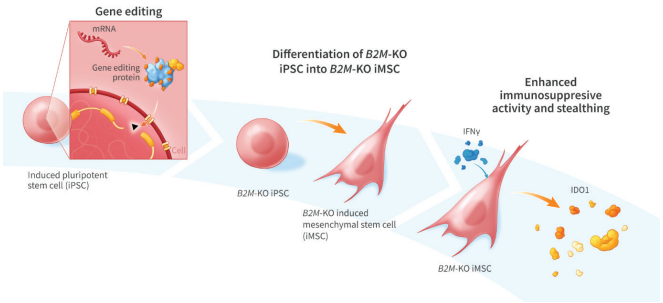
374. iMSCs Derived from mRNA-Engineered B2M-KO iPSCs Exhibit Enhanced Immunosuppressive Activity and Stealthing Features

Raven Dance Hinkel[1], Elizabeth Belcher[1], Christopher B. Rohde[2], Matthew Angel[2], Kyle M. Garland[1]

[1]Eterna Therapeutics Inc., Cambridge, MA,[2]Factor Bioscience Inc., Cambridge, MA

While mesenchymal stem cells (MSCs) have repeatedly demonstrated significant therapeutic potential in numerous preclinical models, their clinical translation has been greatly impeded by variability in therapeutic responses. This variability is often attributed to donor and source heterogeneity and limited expansion potential. Furthermore, MSCs can exhibit limited *in vivo* persistence due to clearance by host immune cells, which can also contribute to deficient therapeutic responses. Induced pluripotent stem cell (iPSC)-derived MSCs (iMSCs) promise to directly address many of the fundamental challenges facing MSC translation. We have previously shown that iMSCs generated using mRNA reprogramming exhibit ~4-fold greater population doublings *in vitro* relative to tissue-derived MSCs and can significantly attenuate the severity of sepsis-induced acute lung injury in an ovine model of acute respiratory distress syndrome (ARDS). In addition to improved consistency and therapeutic potency, the upstream iPSCs are an ideal platform for cell engineering applications, where a clonal population of edited cells is desired. Here, we report the development of a beta 2 microglobulin-knockout (*B2M*-KO) iMSC line with enhanced immunosuppressive activity and stealthing features that may further augment the therapeutic potential of MSCs in treating inflammatory diseases by precluding batch-to-batch inconsistencies, promoting *in vivo* persistence, and enhancing the effector function of the cells. *B2M* was inactivated in iPSCs by introducing a biallelic 14 bp deletion at exon 2 of the *B2M* gene using mRNA encoding NoveSlice™, a proprietary dimerizing chromatin-context-sensitive gene-editing endonuclease. Following colony selection and knockout verification via amplicon sequencing, *B2M*-KO iPSCs were differentiated to *B2M*-KO iMSCs using our previously published protocols. Successful MSC differentiation was confirmed by flow cytometry (CD90+, CD73+, CD105+, TRA-1-60-, TRA-1-80-, CD34-) and trilineage differentiation (adipogenesis, osteogenesis, and chondrogenesis). *B2M*-KO iMSCs displayed no evidence of HLA-I or HLA-II expression both before and after interferon gamma (IFNγ) stimulation, whereas wild-type iMSCs expressed HLA-I (59% HLA-ABC) and no HLA-II (0% HLA-DR) prior to IFNγ stimulation and both HLA-I (99%)

and HLA-II (94%) after IFNγ stimulation. Additionally, *B2M*-KO iMSCs significantly upregulated the immunoregulatory enzyme Indoleamine 2,3-dioxygenase 1 (IDO1) upon IFNγ stimulation relative to wild-type iMSCs as demonstrated by both western analysis and immunofluorescence staining. The immunomodulatory potential of the *B2M*-KO iMSCs was evaluated using a PBMC-proliferation assay. Cells were co-cultured with CFSE-labeled PBMCs in the presence of a CD3/CD28 T cell stimulator for 96 hours. Cells co-cultured with *B2M*-KO iMSCs exhibited significant reduction in PBMC proliferation (92% suppression), as compared to wild-type iMSCs (82% suppression; p=0.04) and tissue-derived MSCs (40% suppression; p<0.0001). *B2M*-KO iMSCs are more likely to evade immune clearance and exert their immunomodulatory effects, as demonstrated by their enhanced sensitivity of IDO1 expression and their improved ability to inhibit PBMC proliferation. Our data suggest that *B2M*-KO iMSCs may be a promising therapeutic agent for T-cell mediated autoimmune and inflammatory indications.



375. Massively Parallel CAR-T Cell Phenotyping Enables Identification of High Efficiency Candidates

Yanping Yang[1], JangKeun Kim[2], Richard Yau[3], Ronan Russell[3], Pier Federico Gherardini[3], Magnolia Bostick[3], Liz Wu[3], Tarun Khurana[3], Moonsoo M. Jin[1], Christopher E. Mason[2], Mostafa Ronaghi[3], Gary Schroth[3]

[1]Houston Methodist, Houston, TX,[2]Cornell University, Ithaca, New York, NY,[3]Cellanome, Palo Alto, CA

Chimeric Antigen Receptor - T cell (CAR-T) therapy is a rapidly progressing field within cancer treatment and beyond, aiming to harness the immune system to fight disease. Despite early successes, there are remaining hurdles to making this treatment strategy more efficacious, safe, and applicable to larger patient populations. One hurdle is the heterogeneity of cell products typically generated through lentiviral CAR delivery to patient T cells. Considerable interest exists in identifying and deeply characterizing which cell populations are most amenable to target cell killing to enable the next generation of CAR-T's. However, current efforts to characterize cell products are inefficient or yield limited results because they utilize multiple independent assays and reconstruct a patchwork overview of cellular states, leading to experimental noise. Here, we describe a novel platform integrating multiple assays on live single cells or heterogenous groups of cells, with the aim to accelerate and streamline cell product characterization. By employing photo-guided polymerization of hydrogels, we can create tens of thousands to hundreds of thousands of individual semi-permeable compartments per run, configurable in size from microns to millimeters. This enables continuous reagent delivery to integrate cellular phenotypes, including cell proliferation, morphology, cell-cell interactions (e.g. cell killing), cytokine secretion, and intracellular or cell surface immunostaining coupled with real-time live cell imaging. Our image analysis tools are trained to accurately differentiate heterogeneous cell populations, providing automated quantification of cell phenotypes and numbers, thereby simplifying data analysis. Following the desired period of longitudinal functional analysis, cells can then be lysed directly on the flow cell to generate barcoded libraries for downstream sequencing (e.g. single-cell RNA). We applied this technology to investigate and characterize an affinity-tuned CAR T (AIC100) targeting intercellular adhesion Molecule 1 (ICAM1). ICAM1 is a cell surface glycoprotein overexpressed in many types of tumors and the associated stroma. AIC100 is generated via lentiviral transduction ICAM1 with myc-tag to human primary T cells and is currently being assessed in clinical trials in patients with anaplastic thyroid cancer. Using this platform, we co-cultured individual CAR-T effector cells with HeLa target cells that overexpress ICAM1 in hydrogel compartments and tracked target cell killing with Sytox live/dead dye. We could simultaneously differentiate between myc-tagged CAR+ and CAR- cells and further characterize them as CD4+ or CD8+ T cells. We further coupled this with cytokine analysis (e.g. IFNγ and Perforin) to identify subsets of CAR-T cells that have higher target cell-killing efficiency. Single-cell RNA seq data of the heterogenous CAR-T populations, linked to phenotype and function, is being mined for detailed cell annotation, differential gene expression, and pathway analysis to interrogate whether there are unique gene signatures that correlate highly potent cells. Taken together, these data demonstrate the power of the Cellanome platform to incorporate multiple functional readouts from heterogeneous cell therapy products and elucidate complex phenotypes via longitudinal proteomics, imaging, and transcriptomics in a single workflow. This technology is well-suited to characterize the functionality of cells longitudinally and multi-modally correlate their properties to patient outcomes (e.g. durable response, cytokine release syndrome) and improve cell engineering and cell product criteria.

376. Chimeric Antigen Receptor Engineered Tregs with Donor Specificity and Enhanced Functional Stability for the Induction of Liver Transplantation Tolerance

Madhav Kishore, Jenny Louise McGovern, Georgios Eleftheriadis, Eva Crespo-Rodriguez, Nathalie Belmonte, Julieta Karegli, Anthony Sowerbutts, Marc Martinez-Llordella

Quell Therapeutics, London, United Kingdom

Regulatory T cells (Tregs) are essential for the maintenance of immune tolerance and to regulate inflammatory responses. It has been reported that Tregs can mediate donor-specific responses and induce allograft tolerance in multiple transplantation models. Therefore, multiple strategies have been proposed to modulate Treg function in the clinic to induce transplantation tolerance. However, several challenges are limiting their therapeutic effectiveness, such as the lack of antigen specificity to allow the activation and accumulation of Tregs in

the allograft, and the loss of phenotypic stability in inflammatory microenvironments hampering their suppressive function. QEL-001 is an autologous engineered Treg therapy designed to overcome the limitations encountered in previous clinical approaches to induce donor-specific tolerance in liver transplanted patients. Natural Tregs from liver recipients are selected and transduced with a multi-modular lentiviral construct that mediates constitutive expression of a chimeric antigen receptor (CAR) specific for the donor graft, a phenotype lock™, and a safety switch. The use of an anti-HLA-A2 CAR mediates the specific targeting of liver allograft in those HLA-A2 negative recipients that received an organ from HLA-A2 positive donors. Furthermore, the transduction of the exogenous FOXP3 gene safeguards the constitutive expression of the Treg master transcription factor independently of microenvironmental cues. The CAR-Tregs from QEL-001 products show consistent expression of the 3 transgenes, while preserving the transcriptional and protein expression patterns of non-transduced Tregs. Optimal expression levels of hallmark Tregs-associated markers such as FOXP3, HELIOS and CTLA4, together with persistent demethylation status of the TSDR region of FOXP3 gene and minimal expression of pro-inflammatory cytokines characterise the phenotype of the QEL-001 product. The in vitro culture of QEL-001 with HLA-A2 expressing cells promotes antigen-specific activation of the CAR-Tregs driving the expression of CD69 and CD137 markers and triggering cellular proliferation. In addition, the CAR-Tregs show efficient suppression of effector T cell proliferation and modulation of B cell costimulatory receptors in an antigen-specific matter. The addition of the phenotype lock™ maintains the suppressive capacity of CAR Tregs in the presence of pro-inflammatory cytokines. Finally, QEL-001 displays durable immunoregulation of inflammatory responses in a humanized model of xenoGvHD supporting its therapeutic efficacy and product safety. Overall, the QEL-001 multi-modular approach overcomes the limitations of previous Treg-directed therapies and pioneers the clinical implementation of autologous cell therapies for the induction of immune tolerance in liver transplanted patients.

377. NK510, a Small Genetic Edit, a Giant Leap for NK Cells

Han Ying[1], Chao Yao[2], Cheng Fang[2], Guanglei Li[3], Lixin Wang[2], Yufu Zhou[2], Chenyuan Gong[2], Juewan Li[3], Yiming Wang[3], Wenwen Fan[3], Fenghua Lv[3], Xiaohu Shao[3], Haiyang Zhao[3], Liling Wang[3], Jun Yan[3], Tianhong Xu[3], Shiguo Zhu[2]

[1]Base Therapeutics (US) Co., Ltd, San Mateo, CA,[2]Shanghai University of Traditional Chinese Medicine, Shanghai, China,[3]Base Therapeutics (Shanghai) Co., Ltd, Shanghai, China

Natural Killer (NK) cells hold great promise for cancer therapy owing to their innate ability to detect cells expressing signs of stress or infection. However, NK cell-based therapeutics face significant challenges: 1. Limited Expansion: It has been challenging to grow a sufficiently large quantity of NK cells for costeffective therapeutic infusions. 2. Genetic Modification Resistance: NK cells have exhibited notable resistance to genetic modifications that could enhance their ability to target specific cancer cell types. In response, we present our pioneering work in the development of Base Editing-Directed NK cell products, known as BED-NK, tailored for cancer treatment. We used the second-generation High-Fidelity Base Editor AccuBase™ developed within our company to knockout inhibitory proteins that bind to the receptors that are overexpressed by many cancer types. AccuBase™ (ceBE) has the highest editing efficiency in the industry, with zero off target events. This was achieved by strategically inserting deaminases into Cas9 at specific, tolerance-verified sites, a process guided by an extensive transposon-based genetic screening, protein engineering, and direct evolution approach. AccuBaseTM protein forms a complex with the sgRNA, but the inserted deaminase has not yet engaged with the target DNA and is not in contact with any non-targeting dsDNA because it is hidden inside the protein. This mechanism avoids guide RNA independent indiscriminate offtarget mutations that was a common problem of the first-generation base editing tool. Upon guided by sgRNA, the AccuBaseTM protein associates with the target double-stranded DNA, triggering structural alterations within the AccuBaseTM protein. This results in the outward exposure of the deaminase domain, facilitating the editing of the target base. AccuBaseTM (ceBE) substantially reduces off-target effects while maintaining on-target editing when compared to BE4max. This improvement has been confirmed in multiple human cell lines, mouse cell lines, human primary T and NK cells, human embryonic stem cells, and in animal embryos and liver. Through a meticulously planned optimization process, we have achieved over 90% editing efficiency in Peripheral Blood Mononuclear Cell (PBMC) NK cells using our high-fidelity Base Editor, AccuBase™. Additionally, we established NKBoost+ expansion system that is capable of generating a millionfold expansion of BED-NKs, referred to as NK510, within a 21-day expansion protocol. Notably, NK510 cells exhibit exceptional levels of purity, cytotoxicity, and viability. By knocking out inhibitory receptors in NK cells, we have prompted tumor cell-derived inhibitory ligands to interact with activating receptors (AR), initiating activating signals within the NK cells. This transformation effectively converted an inhibitory ligand into an activating one, resulting in tumor regression in multiple mouse models, including sarcoma. These results underscore the feasibility of combining base editing with ex vivo expansion of NK cells, significantly enhancing their therapeutic potential in clinical settings.

378. G Protein-Coupled Receptor Class C Group 5 Member D (Gprc5d) and B-Cell Maturation Antigen (BCMA) Bi-Specific Dual Chimeric Antigen Receptors (CARS) Effectively Address Antigen Escape and Tumor Heterogeneity Challenge in Multiple Myeloma (MM)

Chia-Wei Chang, Hui Ding, Lukasz Wlodarek, Kevin Nguyen, Yonghua Luo, Mandana Karimi, Shailaja P. Rao, Arpita S. Pal, Yi-shin Lai, Sihui Guan, Weimin Li, Weijie Lan

Overland Pharmaceuticals (CY) Inc, South San Francisco, CA

BCMA-directed CAR-T therapy shows remarkable efficacy in multiple myeloma (MM) patients, however, challenges like heterogeneous tumor BCMA expression, antigen escape, and limited treatment options upon resistance still post significant hurdles in relapsed patients. Dual targeting of MM-associated antigens could address these challenges and offer durable efficacy in BCMA-resistant MM patients. In this study, we present the development of a bi-specific CAR targeting GPRC5D

and BCMA simultaneously, demonstrating its efficacy, both *in vitro* and *in vivo*. Altogether, 41 GPRC5D and 36 BCMA VHH binders were identified from synthetic library screening and alpaca immunization. These binders were incorporated into lentiviral CAR constructs and tested for NFAT mediated signals in a Jurkat reporter platform. CARs with high antigen specific response and low non-specific and tonic signaling (Table 1) against antigen positive and negative target cell lines were prioritized for downstream functional assessments. Multiple GPRC5D and BCMA CARs were shown with better *in vitro* efficacy than benchmark (BM) GPRC5D and BCMA CARs (Table 1). Long-term and repetitive tumor challenge assays were performed using MM tumor lines OPM-2, NCI-H929 and RPMI-8226 to assess *in vitro* anti-tumor activities of the selected CARs. Several CARs were proved to have superior durable tumor growth inhibition comparable to BMs (Table 1). Next, bi-specific CARs (bsCAR) targeting both GPRC5D and BCMA were created using validated monovalent binders. During *in vitro* tumor restimulation assay against NCI-H929 tumor line, multiple bsCAR designs demonstrated superior long-term tumor growth control versus BM (Table 1). In this setting, our lead bsCAR T cells showed comparable antigen specific proliferation to BCMA BM and GPRC5D BM after 2 rounds of NCI-H929 challenging *in vitro* (Table 1). For cytokine production, the lead bsCAR T cells also generated high levels of GM-CSF, Granzyme-B, IFN-γ and TNF-α upon antigen stimulation (Table 1). Furthermore, in antigen escaping models, GPRC5D and BCMA bsCAR T cells efficiently suppressed growth of cells expressing only GPRC5D or BCMA antigens but spared double negative cells. Finally, in a disseminated NSG mouse model bearing MM cell line OPM-2, our top bsCAR T cells demonstrated highly efficient tumor growth inhibition, exhibiting competitive profiles to BCMA and GPRC5D BMs (Table 1) with an improved survival rate observed at Day 34 post-infusion. In conclusion, we have developed a novel VHH-based GPRC5D and BCMA bi-specific CAR product that efficiently suppresses tumor growth *in vitro* and *in vivo*. The dual-antigen targeting bi-specific CAR-T cells maintained robust anti-tumor efficacy even in models of antigen-escape. Together, these findings suggest the promising potential for treating MM patients resistant to BCMA-directed therapies due to antigen-escape or heterogenous BCMA expression.

Table 1. Characterization of tonic and antigen-specific signaling and anti-tumor activities of GPRC5D CAR, BCMA CAR and bi-specific CARs

Tonic and antigen specific NFAT signaling and anti-tumor activities of monovalent CAR leads						
	Tonic signal (%GFP)	Antigen-specific signal against NCI-H929 (%GFP)	Tumor growth suppression against OPM-2 (AUC normalized to BM)			
GPRC5D CAR#1	9.2%	89.1%	0.69			
GPRC5D CAR#2	5.0%	85.6%	0.84			
GPRC5D CAR#3	5.5%	87.6%	0.69			
GPRC5D BM	5.3%	82.2%	1.00			
BCMA CAR#1	12.0%	81.0%	0.61			
BCMA CAR#2	13.2%	79.9%	1.00			
BCMA CAR#3	13.3%	79.7%	0.83			
BCMA BM	15.1%	78.1%	1.00			
***In vitro* expansion and anti-tumor activities of bi-specific CAR leads**						
	Fold of expansion (2 rounds with NCI-H929)	*In vitro* tumor growth suppression against NCI-H929 (AUC normalized to BCMA BM)	Antigen-specific cytokine production against OPM-2 (concentration normalized to GPRC5D BM)			
			GM-CSF	Granzyme B	IFN-γ	TNF-α
bsCAR#1	289x	1.06	11.38	2.58	2.15	25.16
bsCAR#2	227x	0.88	3.43	1.66	1.84	5.41
bsCAR#3	313x	1.07	4.26	1.61	2.51	12.52
GPRC5D BM	384x	-	1.00	1.00	1.00	1.00
BCMA BM	282x	1.00	4.68	1.85	2.53	17.22
***In vivo* anti-tumor activities of bi-specific CAR: tumor growth suppression against OPM-2**						
	Total flux (D20 relative AUC)*		% Survival at D34			
bsCAR#1	1.74		0%			
bsCAR#2	2.38		20%			
bsCAR#3	0.44 (p=0.05, vs.BCMA BM)		80% (p=0.0719, vs.BCMA BM)			
GPRC5D BM	8.97		0%			
BCMA BM	1.0		10%			

Abbreviation: AUC, Area under the curve; BM, Benchmark
*Total flux at day 20 relative to AUC for tumor alone = 8.69

379. A Same-Day Manufacturing Platform Leveraging an RNA-Based Lipid Nanoparticle (LNP) Gene Writer System to Generate Chimeric Antigen Receptor (CAR) T Cells

Kassi T. Stein, Jason Rodriguez, Kevin Johnson, Elise Lambalot, Sayli Chavan, Michael S. Magee, Alberto De Iaco, James B. Rottman, Yu Cao, Rebecca Levy, Mohit Gupta, Jane Wang, Bill Querbes, Cecilia Cotta-Ramusino, Michael C. Holmes, Madhusudan V. Peshwa
Tessera Therapeutics, Somerville, MA

Autologous *ex vivo* chimeric antigen receptor (CAR) T-cell therapies have demonstrated significant clinical benefit across B-cell driven hematological malignancies. Despite these successes, there remain several challenges with respect to patient access, eligibility, cost, scalability and manufacturing. These barriers to patient therapy are beginning to be addressed through shortening CAR-T manufacturing times and reducing dose through improvements in potency. However, a true same-day bedside manufacturing paradigm or *in vivo*-based CAR-T has not been reached. We are combining our Gene Writing and delivery platforms to target primary human T cells in an effort to reduce the challenges associated with conventional autologous CAR-T approaches. RNA Gene Writers are designed to introduce a broad range of alterations to the genome, from writing in a transgene, inserting an exon, or introducing single nucleotide changes, providing an attractive toolkit for sophisticated cell engineering. RNA Gene Writers are inspired by the biology of non-LTR retrotransposons, a class of mobile genetic elements that can edit the genome through target-primed reverse transcription, or TPRT. RNA Gene Writers are designed to be delivered as all-RNA composition, which is intended to facilitate *in vivo* delivery by lipid nanoparticles, or LNP. In preclinical studies, we have observed that our RNA Gene Writers, delivered by our LNP platform as all-RNA compositions, drove stable integration of a green fluorescent protein (GFP) transgene in >70% of human T cells *in vitro*. Further, we demonstrated one of our RNA Gene Writers achieved the integration and CAR expression in an average of ~21% of T cells *in vitro*. These CAR-T cells also exhibited tumor cell killing *in vitro* and *in vivo* in an established human tumor xenograft model. Next, we demonstrated proof-of-principle of same-day manufacturing of CAR-T cells *in vitro* using RNA Gene Writers by packaging mRNA for RNA Gene Writer and an RNA template for CAR transgene into proprietary LNP formulations. The LNP-RNA composition was delivered to primary human T cells in fresh platelet-washed leukapheresis from healthy donors to generate CAR-T cells through this same-day manufacturing process. These CAR-T cells were subsequently expanded and evaluated for functionality both *in vitro,* where tumor cell killing and cytokine secretion was tested, and *in vivo* in immunodeficient NSG mice, where robust tumor clearance was observed post infusion compared to untransduced and vehicle controls. Lastly, as we begin to test our LNP-RNA compositions directly *in vivo* in murine models, we observed an average of ~6% GFP reporter gene expression in T cells in a humanized mouse model. In summary, we believe Tessera's Gene Writing and delivery platforms are uniquely positioned for same-day delivery of all-RNA components to enable more efficient manufacturing of CAR-T therapies, potentially removing the need for long expansion times and freeze-thaw cycles associated with conventional *ex vivo* CAR-T therapies.

Base Editing and Prime Editing II

380. Addressing the dNTP Bottleneck Restricting Prime Editing Activity

Pengpeng Liu[1], Karthikeyan Ponnienselvan[1], Thomas Nyalile[2], Sarah Oikemus[1], Anya T. Joynt[1], Karen Kelly[3], Dongsheng Guo[4], Zexiang Chen[3], Jeong Min Lee[5], Celia A. Schiffer[5], Charles P. Emerson[4], Nathan D. Lawson[1], Jonathan K. Watts[3], Erik J. Sontheimer[3], Luban Jeremy[2], Scot A. Wolfe[1]

[1]Molecular, Cell and Cancer Biology, UMass Chan Medical School, Worcester, MA,[2]Molecular Medicine, UMass Chan Medical School, Worcester, MA,[3]RNA Therapeutics Institute, UMass Chan Medical School, Worcester, MA,[4]Wellstone Program, Department of Neurology, UMass Chan Medical School, Worcester, MA,[5]Biochemistry and Molecular Biotechnology, UMass Chan Medical School, Worcester, MA

The goal of this project is to increase the efficiency of prime editing in post-mitotic cells and tissues. Prime editing has the potential for broad therapeutic application in the treatment of a variety of human diseases due to its versatility in performing targeted base conversions, deletions and insertions. Since the initial description of prime editing, many system components have been further optimized including: Cas9 nickase, M-MLV reverse transcriptase (MMLV-RT), nuclear localization sequences, pegRNA designs and DNA repair pathway activity. The original PE2 system utilizes a pentamutant M-MLV reverse transcriptase (MMLV-RT) that increases thermostability, affinity for the RNA-DNA template and processivity. To improve prime editing outcomes researchers have introduced additional point mutations into MMLV-RT to improve its functionality, substituted alternate RTs in the context of fused or split prime editor systems, or utilized molecular evolution to enhance the properties of multiple RTs for prime editing. Despite these improvements, precise editing rates remain modest in some primary cell types or in some tissue types *in vivo*. We believe that prime editing efficiency in challenging cellular scenarios can be enhanced by harnessing the wealth of optimization performed on MMLV-RT for *in vitro* applications. For example, protein aggregation observed during purification of the PEmax prime editor protein appears to be associated with the MMLV-RT domain, which suggests that its solubility may be one limiting factor. Additionally, most characterization and optimization of prime editing systems has been carried out in rapidly dividing mammalian cells, where high intracellular dNTP levels facilitate prime editing efficiency. Cellular dNTP levels can be more than 100-fold lower in post-mitotic or quiescent cells than cycling cells, which would restrict MMLV-RT activity due to its modest affinity for dNTPs. Consequently, we assessed the impact of two MMLV-RT mutations on prime editing activity: V223M, which increases dNTP affinity, and L435K, which increases MMLV-RT solubility. The presence of the L435K mutation translated into improved prime editing for every tested delivery modality (protein, mRNA and plasmid), as well as increasing prime editor protein yields when purifying from bacterial expression systems. Interestingly, the presence of the V223M mutation provided no appreciable benefit to precise editing rates in rapidly dividing transformed cell lines. However, the effect of the V223M mutation was pronounced (~2-fold improvement) in a variety of primary cell types (fibroblasts, induced myoblasts and primary human T cells). Combining the L435K and V223M mutations on the PEmax prime editing framework (PEmax**) yielded 2- to 3-fold improvement in precise editing rates in primary cells and *in vivo*. To further facilitate MMLV-RT processivity, we knocked down SAMHD1, which is involved in maintaining low intracellular levels of dNTPs, using VPX, a viral accessory protein that targets SAMHD1 for proteasome-mediated degradation. Using VPX with PEmax** increased prime editing rates up to 4-fold over PEmax alone in patient-derived fibroblasts and primary human T cells. We believe that the PEmax** variant in conjunction with SAMHD1 knockdown via VPX co-delivery will have broad utility for precise editing in quiescent and post-mitotic cells.

381. Characterization of Guide RNA Site Consistency Across Ancestries and the Potential for Off-Target Editing with the Clinical-Stage Base Editing Medicine, VERVE-101

Alexandra C. Chadwick, Jamie DeNizio, Sara Garcia, Anthony Federico, Manashree Damle, Hui-Ting Hsu, Estela Shabani, Daniel Weiner, Amit V. Khera, Joseph Biedenkapp, Sekar Kathiresan, Troy Lister, Andrew M. Bellinger

Verve Therapeutics, Boston, MA

Introduction: VERVE-101 is an investigational in vivo base editing medicine composed of an mRNA encoding an adenine base editor (ABE) and guide RNA (gRNA) packaged in a lipid nanoparticle (LNP). It is designed to permanently inactivate the *PCSK9* gene in the liver with a single base pair change and lead to durable reductions in low-density lipoprotein cholesterol (LDL-C). In an ongoing first-in-human clinical trial, a 55% reduction in LDL-C from baseline was observed after a single infusion of VERVE-101 in a patient treated with the highest dose studied to date. VERVE-101 is designed to enable robust editing at the intended *PCSK9* gene target site in hepatocytes while minimizing the risk for off-target edits 1) at other locations in the genome in any tissues and 2) at the *PCSK9* gene in tissues outside the liver. Here we describe the consistency of the gRNA target site at a population level to provide insight on the expected therapeutic utility of VERVE-101 across diverse ancestries. Rigorous off-target evaluations incorporating twenty different genomic backgrounds were performed to assess for potential off-target edits, as well as chromosomal structural variant formation. **Methods:** Consistency of the VERVE-101 *PCSK9* gRNA site was assessed using the Genome Aggregation Database (gnomAD v4.0) that aggregates gene sequencing data from approximately 800,000 individuals of diverse ancestral background (Chen, et al. *Nature*, 2024). Off-target analysis was performed in multiple lots of primary human hepatocytes and other liver, splenic, adrenal, and bone marrow cells. Cells were treated in vitro at doses $\geq EC_{90}$ for on-target editing and compared to untreated controls. To evaluate gRNA-dependent off-target editing, multiple orthogonal methods were used (ONE-Seq, ABE-Digenome-seq, and in silico analysis) to nominate approximately 6000 candidate sites. Hybrid capture was used to screen candidate sites for evidence of editing. gRNA-independent off-target assessments were performed using whole genome sequencing (WGS) to screen for excess deamination. Structural variant detection methods included optical

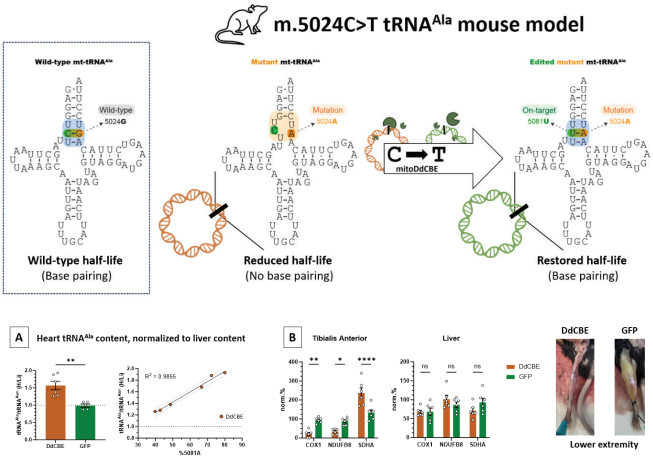
genome mapping and anchored Tn5-mediated unidirectional PCR (ATUP). **Results:** The VERVE-101 PCSK9 gRNA site was consistent in >99.9% of individuals in the total population captured in gnomAD (n=784,318) and in each separate ancestry group. None of the approximately 6000 candidate sites in the gRNA-dependent off-target screen showed evidence for editing in primary human hepatocytes, adrenal cells, or bone marrow. One candidate site in a liver cell line and one in primary human splenic cells showed evidence of editing. Targeted amplicon sequencing across a range of doses confirmed that editing at these two sites was a small fraction of on-target editing. Bioinformatic risk assessment of the off-target sites showed they were not in coding regions, were not near genes associated with cancer, and were not likely to impact gene expression. Editing of these two sites is also unlikely to occur at pharmacological doses in vivo. The WGS analysis showed no increase in global deamination, and there was no evidence for structural variant formation in VERVE-101 treated cells with either the optical genome mapping or ATUP method. **Conclusions**: VERVE-101 is the first in vivo base editing medicine to demonstrate proof-of-concept in humans. The consistency of the gRNA target site across populations suggests the potential therapeutic benefits of VERVE-101 should apply broadly across individuals with diverse ancestries. Comprehensive off-target assessment in different cellular contexts demonstrated VERVE-101 to be highly specific with a low risk for clinically relevant off-target edits.

382. The mitoDdCBE System as a Mitochondrial Gene Therapy Approach

Jose Domingo Barrera-Paez[1], Sandra Bacman[2], Till Balla[3], Beverly Mok[4], David R. Liu[4], Danny Nedialkova[3], Carlos T. Moraes[2]

[1]Genetics, University of Miami, Miami, FL,[2]University of Miami, Miami, FL,[3]Max Planck Institute of Biochemistry, Martinsried, Germany,[4]Broad Institute, Harvard University, and HHMI, Cambridge, MA

Introduction and Methods The mitoDdCBE system can induce cytosine-to-thymine transitions (C>T) in double-stranded, mitochondrial DNA (mtDNA). To test the therapeutic potential of this gene editing tool, we decided to make use of the m.5024T mutant mouse, one of the few existing animal models of heteroplasmic mtDNA mutations. This pathological mtDNA mutation disrupts a Watson & Crick base pair in the mitochondrial tRNA alanine (tRNA^{Ala}) secondary structure, reducing its half-life. Because the mutation (C>T) cannot be directly corrected with this approach, we created a compensatory edit (at position m.5081C>T) to restore the secondary structure and stability of the tRNA^{Ala} (Fig.1). **Results** AAV9-DdCBE viral preparations were retro-orbitally injected into young m.5024C>T mice. No expression nor editing was observed on the liver, whereas different muscle tissues (heart, Tibialis Anterior) were successfully transduced and edited. Using several AAV9 dosages, we achieved different levels of on-target editing, and noticed an exponential correlation with the generation of concomitant off-target editing. Furthermore, we also validated that DdCBE rescued the levels of tRNA^{Ala} exclusively on edited tissues in a dose-dependent manner (Fig. 2A). The highest AAV9 dosage generated numerous mtDNA off-target edits, causing a myopathic phenotype (myotonia, muscular hypoplasia, complex IV deficiency) with a corresponding lower steady-state levels of COX1 and NDUFB8 proteins (Fig. 2B). Next Generation Sequencing data generated using a specialized tRNAseq pipeline confirmed an increase in stability in the edited tRNA^{Ala} and showed that aminoacylation was not affected by the original or edited alterations. **Conclusions** We have successfully edited a cytosine in the mouse mtDNA that acts as a second site suppressor for the pathological m.5024C>T. We are thus showing, for the first time, that although the mitoDdCBE system has therapeutic potential *in vivo*, it is crucial to assess off-target editing performance to avoid a negative outcome.**Figure 1. Base editing in the mouse mitochondrial tRNA alanine gene.** The m.5024C>T mutation (G>A in the tRNA^{Ala}), present in the mouse mitochondrial tRNA^{Ala}, disrupts the base pairing at the acceptor stem, reducing the half-life of the molecule. Using the mitoDdCBE system, we have edited the m.5081C to a U, thereby restoring the base pairing. **Figure 2. Characterization of the phenotypical consecuences of the m.5081U edit in vivo.** (A) The steady state levels of mt-tRNA^{Ala} increased in mice injected with different doses of AAV9-DdCBE in a linear correlation with the amount of on-target editing achieved on edited tissues (heart) when compared to unedited tissues (liver). (B) The highest AAV9 dosage achieved 92% on-target editing but also high off-target editing, and therefore showed muscular hypoplasia and lower steady-state levels of COX1 and NDUFB8 proteins on edited tissues (tibialis anterior) but not on unedited tissues (liver).



383. Engineered IscB-ωRNA System with Expanded Target Range for Base Editing

Qingquan Xiao[1,2], Dingyi Han[2], Haoqiang Wang[1], Guoling Li[1], Yuan Yuan[1], Yingsi Zhou[1], Tong Li[1], Shaoran Wang[1], Xuan Yao[1], Alvin Luk[1], Xiangfeng Kong[1], Hainan Zhang[1], Hui Yang[1,2]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: As a miniature RNA-guided endonuclease, IscB is likely the ancestor of Cas9 and shares similar functions. Cas9 has proved challenging to deliver the protein into target cells due to the size, a feature reflecting the packaging limit associated with viral vector delivery such as adeno-associated virus (AAV). IscB is less than half the size of Cas9 and thus more suitable for *in vivo* delivery by a single AAV vector. We previously engineered the OgeuIscB protein and its corresponding ωRNA to generate a highly efficient OgeuIscB system.

Further work was conducted to generate miniature IscB-derived base editors (miBEs) with robust editing efficiency to induce DNA base conversions. Here, we identified several new variants of IscB with different transposon-associated motif (TAM) sequences, broadening the target range of miBEs. **Methods:** We employed a computational pipeline to annotate IscB orthologs and their corresponding ωRNAs by mining bulk metagenomic data (>200 GB). An EGFP-activation reporter system was designed and used to screen functional IscB systems in HEK293T cells. Protein engineering combined with sgRNA optimization was used to generate enhanced IscB variants with high editing efficiency and broad TAMs. *In-vivo* editing efficiency of engineered IscB variants was further evaluated in a mouse model of Duchenne muscular dystrophy (DMD). **Results:** By screening for IscB homologs in the metagenomic database, we identified 10 out of 19 uncharacterized IscB proteins from uncultured microbes showing activity in mammalian cells (Figure 1). Through protein and ωRNA engineering, we further enhanced the activity of IscB ortholog IscB.m16 and expanded its TAM scope from MRNRAA to NNNGNA, resulting in a variant named as IscB.m16* (Figure 2a-e). By fusing the deaminase domains with IscB.m16* nickase, we generated IscB.m16*-derived base editors that exhibited robust base editing efficiency in mammalian cells and effectively restored DMD proteins in disease mice via single AAV vector delivery (Figure 2f-h). **Conclusions:** Our research has successfully developed a suite of compact base editing tools originating from the IscB protein, which hold significant promise for both fundamental scientific inquiries and therapeutic interventions.

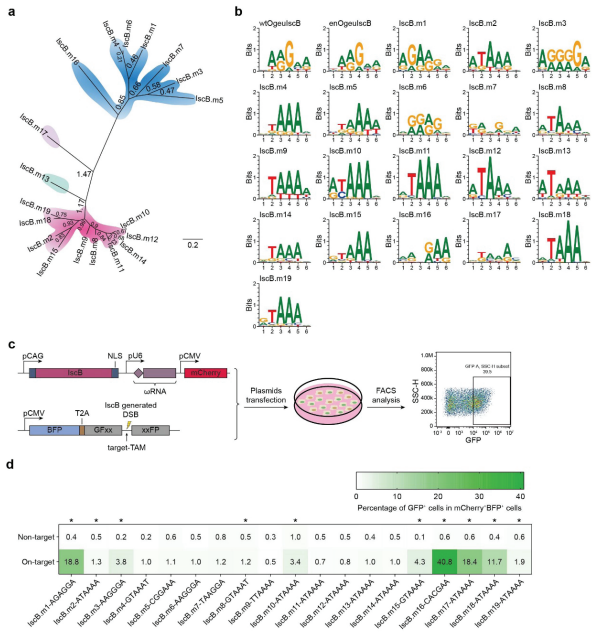


Figure 1. Identification and characterization of functional IscB orthologs. a. Phylogenetic tree of 19 uncharacterized IscB orthologs. b. TAMs of 19 active IscB proteins and two OgeuIscB variants determined by bacterial depletion assay. c. Schematics describing the detection of editing activity based on the fluorescence signal of GFxxFP reporter activation in HEK293T cells. d. Fluorescence signal of EGFP activated by IscB-mediated double-strand breaks (DSB) quantified by flow cytometry. Non-target means spacer with a random sequence. Asterisks mean >2-fold ratio of on-target and non-target, with on-target >1.0%, representing variants with activity in HEK293T cells.

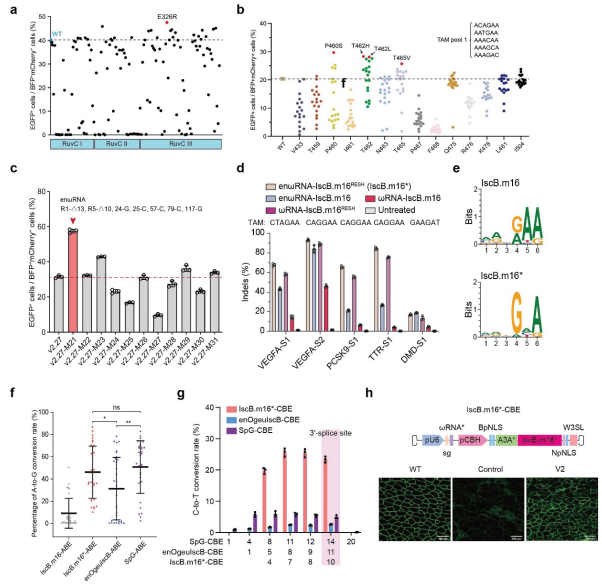


Figure 2. Protein engineering of IscB.m16 to improve editing efficiency and expand TAM range and base editing efficiency in mammalian cells. a. Screening for highly efficient variants by substitutions of amino acid residues in the RuvC domain of IscB.m16 protein with arginine. b. Screen for highly efficient variants with saturation mutagenesis at selected sites using GFxxFP reporters containing a pooled TAM target. c. ωRNA engineering by substituting C-G base pair on ωRNA-v2.27 based on IscB.m16RESH. d. Comparison of indel frequency of WT IscB.m16 and its variants at five endogenous sites in HEK293T cells. e. TAM logs of IscB.m16 and IscB.m16* system. f. Comparison of the A-to-G conversion efficiency of IscB.m16-ABE, IscB.m16*-ABE, enOgueIscB-ABE, and SpG-ABE at 33 endogenous loci. g. The C-to-T conversion efficiency of IscB.m16*-CBE, enOgeuIscB-CBE, and SpG-CBE at the splice acceptor site of the DMD intron between exon 49 and exon 50 in HEK293T cells. h. Dystrophin immunohistochemistry showed the restoration of dystrophin expression 3 weeks after TA injection of IscB.m16*-CBE. Dystrophin is shown in green. Scale bar, 100 μm.

384. Development of Deaminase-Free Base Editors by Engineering DNA Glycosylases

Huawei Tong[1], Haoqiang Wang[1], Nana Liu[1], Guoling Li[1], Yingsi Zhou[1], Danni Wu[1], Yun Li[1], Ming Jin[2], Xuchen Wang[3], Hengbin Li[1], Yuan Yuan[1], Tong Li[1], Shaoran Wang[1], Alvin Luk[1], Xuan Yao[1], Linyu Shi[1], Hui Yang[1,3]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Department of Neurology and Institute of Neurology of First Affiliated Hospital, Fujian Medical University, Shanghai, China,[3]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: Two categories of DNA base editors, deaminase-based base editor (dBE) and deaminase-free glycosylase-based base editor (gBE), enable direct editing of adenine (A), cytosine (C), or guanine (G), but there is currently no base editor for direct thymine (T) editing. Certain dBEs (e.g., AYBE, CGBE) enable transversion editing following a second-step generation of apurinic/apyrimidinic (AP) sites by engineered glycosylases after the deamination of A or C. **Methods**: To facilitate direct T and C base editing, we generated two prototype gBE versions, a deaminase-free glycosylase-based thymine base editor (gTBE) and a deaminase-free glycosylase-based cytosine base editor (gCBE), by fusing the two corresponding variants of nuclear UNG2 (Y156A and N213D, respectively) at the N-terminus of Cas9 D10A nickase (nCas9), respectively. To enhance the editing activity of gTBE or gCBE, we performed rational mutagenesis for engineering the UNG moiety based on structural and functional analyses. We characterized the editing profile of gTBE and gCBE by targeting dozens of endogenous

genomic loci in cultured mammalian cells as well as mouse embryos. Recently, we further improved the efficiency of gGBE and investigated its therapeutic potential by disrupting the *PCSK9* gene in mice. **Results**: We constructed UNG-NTD-truncated gTBE and gCBE versions to eliminate undesired protein-protein interactions. The gTBEv0.3 with UNG2Δ88-Y156A and gCBEv0.3 with UNG2Δ88-N213D fused at the N-terminus showed much higher editing activity than those at the C-terminus (10.2% vs. 1.0% and 51.4% vs. 13.3%, Fig. 1c-e), a 10- and 3.9-fold enhancement in the editing efficiency, respectively. After multiple rational mutagenesis screening, we obtained a variant gTBEv3 with elevated editing efficiency by 3.09-fold as compared with gTBEv0.3 and a variant gCBEv2 with enhancement of C-to-G editing activity by 1.3-fold compared to gCBEv0.3. We further characterized the editing profiles of gTBEv3 and gCBEv2 by targeting dozens of endogenous genomic loci and found that gTBEv3 achieved efficient T base editing activity (range of 24.3% to 81.5%) and obtained efficient C base editing activity (range of 31.8% to 77.7%) for gCBEv2. We then co-injected the gTBEv3 mRNA and sgRNA targeting the SD site of *DMD* exon 45 into zygotes of humanized DMD mice. 100% (20/20) mouse embryos harbored efficient base conversion (range of 35.0% to 97.0%) at the desired position T3, resulting in exon skipping which would be applicable to restore dystrophin expression in DMD. Finally, when targeting *PCSK9* with further engineered gGBE for exon skipping and the introduction of premature termination codons (PTCs), we obtained an editing efficiency superior to that of targeting ABE8e in cultured human cells. Additionally, we also developed a deaminase-free glycosylase-based adenine base editor (gABE) with direct A editing ability following one-step generation of AP sites. **Conclusions**: Our findings indicate that certain DNA glycosylases could be engineered into enzymes that selectively excise specific nucleotide bases and be applied to develop novel base editors with various features. The gBE novel base editors provide additional options for the sites that dBEs could not target, largely expanding the targeting scope of base editors.

385. Systemic Delivery of LNP mRNA In Utero Permits Editing in a Mouse Model of Spinal Muscular Atrophy (SMA)

Nicole Kus[1], Marco Carpenter[1], Rosa Choi[1], Ana Maria Dumitru[1], William Johnston[1], Hooda Said[1], Mohamad Gabriel Alameh[2], William Peranteau[3]

[1]Children's Hospital of Philadelphia, Philadelphia, PA,[2]Ecole Polytechnique de Montreal, Montréal, QC, Canada,[3]The Children's Hospital of Philadelphia, Philadelphia, PA

Spinal muscular atrophy (SMA) is a leading heritable cause of infant mortality and is caused by a mutation of the SMN1 gene. Pathogenesis of the disease begins prenatally and is often irreversible. The fetus has unique properties that make it favorable for gene editing: cells are highly proliferative and amenable to mRNA delivery, the fetus is immunological immature and tolerant, and diseases with a high morbidity and mortality in the prenatal and perinatal period can be targeted. In this novel study, we used *in utero* delivery of CRISPR-Cas9 via lipid nanoparticles to target the SMN2 gene in severe SMA mice. We hypothesized that in utero LNP-assisted mRNA delivery permits efficient base editing at the SMN2 locus, thereby improving survival in a SMA mouse model. To test this hypothesis, severely diseased SMA mice were injected intravenously (IV) prenatally (E14) and postnatally (P0) via either the vitelline vein or facial vein, respectively, and compared to direct ICV injections. Survival and weight data were collected. Brains, spinal cords, and peripheral organs were harvested from mice and analyzed for editing efficiency, SMN protein levels, and mRNA splicing. Editing levels in intravenously injected mice at E14 were significantly higher than P0 injected mice. An increase in editing was correlated with enhanced survival, with improvement in mouse lifespan from P7 - P8 days to P15-19. Mice with the longest lifespan demonstrated the largest improvement in SMN protein levels. This study demonstrates that adenine base editing technology can be used to increase SMN2 exon splicing and produce a functional protein through IV *in utero* LNP assisted mRNA delivery in a severe mouse model of SMA. These results highlight the potential for further studies to improve brain and spinal cord targeting.

386. Traceless Delivery of Genome Editors

Jakob Hansen Haldrup[1], Deborah R. Gill[2], Stephen Hyde[1]

[1]Nuffield Division of Clinical Laboratory Science, University of Oxford, Oxford, United Kingdom,[2]University of Oxford, Oxford, United Kingdom

Genome editing has enormous potential to transform the future of genetic medicines. Current delivery strategies suffer from several shortcomings including insufficient packaging capacity, long-term expression, and risk of unwanted genomic integration. Thus, there is an urgent need for developing new and clinically relevant vehicles to fulfil the vision of correcting genetic defects directly in patients. Not only should such vehicles accommodate large base and prime editors, but they should also deliver their cargo in a transient fashion to minimize issues surrounding genotoxicity. By incorporating ribonucleoprotein (RNP) complexes instead of vector genomes into lentivirus-derived nanoparticles (LVNPs), we address key limitations of viral and non-viral delivery modalities, including: (i) overcoming limited packaging capacity, (ii) preventing unwanted genomic integration, and (iii) reducing exposure to the therapeutic payload. LVNPs provide a short burst of gene editing RNPs that rapidly engages the target loci (within 12 hours) and undergo quick degradation (within 72 hours), resulting in reduced genotoxicity without compromising efficacy. Importantly, the practical utility of this LVNP platform has been demonstrated, directing robust levels of genome editing in both primary human T cells *ex vivo* and the murine retina *in vivo*. In addition to simple Cas9-based gene editors, we have also demonstrated LVNP-mediated delivery of larger base- and prime editors; however, the substitution of Cas9 with larger RNP complexes does cause a decline in both potency and viral yield (Haldrup et al., PMID 37678882). With a strategic emphasis on clinical applications, successful vehicles should converge in terms of potency, safety, and manufacturing. To improve potency of LVNPs loaded with prime editors, we attempted to reduce the number of "empty" particles by omitting co-transfection of Gag/GagPol-D64V during manufacturing. Despite achieving comparable prime editing levels with an equal (p24 normalized) amount of LVNP, we observed a ~2.5-fold decrease in manufacturing yield without any further advantages. To enhance our LVNP structure, we added (additional) effector domains to favour membrane anchoring during manufacturing and removed redundant viral proteins to improve LVNP packaging capacity. In addition, we incorporated protein/RNA aptamers to increase (e)pegRNA loading. Collectively, these

substantially improved prime editing efficacy (~3.9fold; ~8.7% versus ~34% across targeted loci (p-value ≤0.031). We also tested prime editor configurations that lack the RNAseH domain but observed a trend for slightly reduced efficacy. Finally, we established protocols for large-scale manufacturing by transitioning from (i) adherent cells to suspensions cells, (ii) ultracentrifugation to anion exchange chromatography, and (iii) tangential flow filtration for buffer exchange and final concentration. Encouragingly, early evidence supports that LVNPs are sufficiently robust to withstand the rigors of manufacturing processes at large-scale, when pseudotyped with the widely used VSV-G or novel Sendai F/HN glycoproteins. Collectively, these improvements in our LVNP platform will enable preclinical testing in patient-derived cells and *in vivo* settings to assess potency and safety.

AAV Vector Biology and Development III

387. Analyzing and Improving AAV Vector Design with Long Read Sequencing and Analysis

Amicia Elliott[1], Eric Talevich[1], Alpha Diallo[1], Brandi Cantarel[1], Nam Tonthat[2], Pranam Chatterjee[3], Douglas McCarty[4], Phillip WL Tai[5], Claire Aldridge[1], Elizabeth Tseng[6], Nadia Sellami[7]

[1]Form Bio, Austin, TX,[2]AskBio, Research Triangle Park, NC,[3]Department of Biomedical Engineering, Duke University, Durham, NC,[4]Nationwide Children's Hospital, Columbus, OH,[5]UMass Chan Medical School, Worcester, MA,[6]Pacific Biosciences, Menlo Park, CA,[7]PacBio, San Diego, CA

Recombinant adeno-associated viruses (rAAVs) are currently the dominant gene therapy vectors used in preclinical and clinical settings, with more than 200 vector platforms being tested in human trials to date. To ensure the safety and efficacy of the drug product, it is essential to have a comprehensive understanding of the purity and integrity of the product, as well as identify impurities of the vector genome. Long-read sequencing is a powerful analytical tool for characterizing rAAV vector genomes, with the ability to cover the entire 4.7-kb vector as a single intact read. However, the lack of standardized nomenclature on impurities and report metrics for analyzing long-read sequencing data have hindered comparability between production runs and have reduced the potential for improving vector designs. To address this need, we convened a working group of academic and industry experts in gene therapy, long-read sequencing, and bioinformatics analysis to draft the first standardized nomenclature and report metrics. Consensus emerged around reporting on analyses that addressed questions relevant to both vector design and critical quality attributes (CQAs) corresponding to the product's purity (such as full-length vs fragmented genomes) and identity of contaminations (such as host DNA or helper or rep/cap plasmids), which can have potential impacts on product quality and efficacy. Here, we will present our standardized report with non-proprietary data and sample output. The report includes measures that have not regularly been included in the past, such as normalizing and quantifying sequencing reads using lambda phage DNA, as well as higher resolution of partial and truncated species that PacBio long read sequencing enables. We will also present results from the re-analyses of published datasets and *de novo* manufacturing runs with reporter constructs that reveal specificity into the contents of filled and partially filled capsids that has higher resolution of full therapeutic genomes than today's commonly used assays such as analytical ultracentrifugation (AUC). While different payloads will vary between high fidelity sequencing as compared to orthogonal approaches such a AUC and Droplet Digital PCR (ddPCR) based on vector design and manufacturability, we will share data from multiple studies to show the improved accuracy with long read NGS. This work enables a deeper understanding of therapeutic vs non-therapeutic capsid content for AAV based drug products. This insight is crucial to inform dosing and safety risks due to chimeric or truncated vector genomes and the presence of viral or host residuals. To enable transparency and community adoption, we have implemented these standardized computational characterization and reporting methods as open-source software. We invite the community to freely use this code and potentially participate in guiding its future development. This software is publicly available under the permissive MIT license at: https://github.com/formbio/AAV

388. Unwanted Concatemeric Knock-Ins Occur Frequently with Cas9/AAV-Mediated Gene-Editing: Detection and Prevention

Fabian P. Suchy[1], Daiki Karigane[1], Yusuke Nakauchi[1], Maimi Higuchi[1], Jinyu Zhang[1], Katja Pekrun[2], Ian Hsu[1], Amy C. Fan[1], Toshinobu Nishimura[1], Carsten T. Charlesworth[2], Joydeep Bhadury[1], Toshiya Nishimura[1], Adam C. Wilkinson[1], Mark A. Kay[3], Ravindra Majeti[1], Hiromitsu Nakauchi[1]

[1]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Stanford, CA,[2]Stanford University, Stanford, CA,[3]Stanford University School of Medicine, Stanford, CA

Background: The combination of CRISPR/Cas9 protein and adeno-associated virus (AAV) has proven to be a highly efficient pair for generating targeted gene knock-ins. Indeed, the use of AAV6 to deliver homology-directed repair templates may boost gene knock-in rates ~100 fold compared to plasmid transfection for various cell types in vitro. However, when isolating dozens of human induced pluripotent stem cell (iPSC) colonies after knock-in and clonal expansion, we found inconsistent results across various PCR genotyping methodologies. In addition, subclones with seemingly identical genotypes often had highly variable levels of transgene expression. ***Problem:*** Since PCR failed to reliably genotype the iPSC subclones, Southern blots were used for clarification. Remarkably, over half of the edited cells contained concatemeric knock-ins of the AAV genome at the target editing site. These concatemers occurred at regular intervals, contained the full vector genome, were linked by the viral inverted terminal repeats (ITRs) in a head-to-tail fashion, and sometimes reached sizes greater than 25 kb (10 repeats). Although AAV is known to form episomal concatemers, they have not been shown to frequently integrate in the context of precision gene editing. Frustratingly, the chimeric ITRs found in the concatemeric knock-ins were significantly more resistant to PCR amplification/sequencing than the ITRs found in reported end-joining mediated knock-ins. This PCR resistance led to allelic dropout during target site amplification. Genotyping just the left and/or right side of the knock-in site with in-out PCR revealed no abnormalities,

further masking the editing error. Unfortunately, these frequent concatemeric knock-ins can cause adverse and unreliable phenotypes that are antithetical to the goal of precision genome engineering. ***Solution:*** Before seeking methods to disable concatemerization, we first sought to readily detect the concatemers by developing novel ddPCR-based assays for complex genotyping. We then characterized their frequency in clinically relevant cell-types (human iPSCs and hematopoietic stem and progenitor cells—HSPCs). Strikingly, ~60% of biallelically edited cells contained at least one concatemeric knock-in, independent of cell line, locus, or vector concentration. Finally, we sought to decrease the concatemer rate through post-transduction cleavage of the viral ITR, which decreased the concatemer rate ~10 fold and had minimal change on editing efficiency. Since this approach is compatible with established gene-editing pipelines, we hope to enable more robust genetic knock-ins that are safer, more reliable, and more reproducible. ***Summary:*** **(1)** AAV6-delivered homology-directed repair templates form on-target concatemeric knock-ins in ~60% of biallelically edited cells. **(2)** These concatemeric knock-ins differ from end-joining mediated knock-ins and are not detectable by common PCR/sequencing genotyping strategies. **(3)** Although Cas9/AAV6-mediated gene editing is used extensively in preclinical and clinical pipelines, these concatemeric knock-ins have not yet been well-characterized and could lead to unexpected phenotypes or possibly dangerous clinical outcomes. **(4)** We developed strategies to prevent these concatemeric knock-ins in clinically relevant cell types (human iPSCs and HSPCs) with minimal change to already established gene editing pipelines.

389. Molecular Origami: AAV Genome Recombination Patterns Mediated by ITRs in the Liver

Liujiang Song[1,2], Bethany Kuszlewicz[1], Lauren Nufer[1], Sinclair Cooper[1], Ross Fraser[1], Sherif Gabriel[1], R. Jude Samulski[1,2]

[1]AskBio, Durham, NC,[2]University of North Carolina at Chapel Hill, Chapel Hill, NC

In the livers of experimental animals, Adeno-Associated Viral (AAV) vectors transduce cells, leading to an initial increase and ultimately establishing steady-state levels of transgene expression in most cases. Earlier studies have shown that uncoated AAV genomes can rapidly undergo circularization and intermolecular recombination within the Inverted Terminal Repeat(ITR) region, forming extrachromosomal AAV episomes. However, it remains unclear how these episomes could persist long-term and in what genome configuration and molecular state. In the present study, a practical strategy and a bioinformatics pipeline were developed to profile AAV episomes' genome configuration, with a focus on genome intermolecular and/or intramolecular junctions, over time in both wild-type mice and a Pompe disease Acid alpha-Glucosidase (GAA) Knock Out mouse model. Additionally, we evaluated pattern similarities and differences between self-complementary (sc) and single-stranded (ss) AAV in the liver of wild-type mice. The long-read sequencing data from our current study demonstrates that irrespective of dose, capsid, promoter cassettes, ITR sequences, and mouse models, a prevalent head-to-tail configuration was observed in ITR junctions, while the head-to-head configuration appeared to be the least common. Notably, a nucleotide-level examination revealed that double D structures are common in ITR arrays (for ssAAV), accompanied by a remarkable, previously undiscovered deletion pattern at ITR junctional regions. Deletions with varying lengths at the junctions and across the ITR regions were observed, suggesting relatively sloppy viral genome recombination. Analysis of ITR Flip and Flop orientation at the junctions suggested that, while Flip and Flop configurations were not exactly equally distributed in either pre- nor post-transduction genomes, no conclusive data supported a preference for one configuration over the other. Notably, a substantial number of reads containing a tail-to-tail configuration were also observed at 8 weeks post-injection in Pompe disease mice injected with ssAAV8 carrying a liver-specific promoter and a GAA cassette. In addition, AAV genome configuration at 8 weeks compared to 16 weeks showed a similar pattern, suggesting that the genomes remain relatively stable during the time frame examined. Furthermore, scAAV exhibited a predominant head-to-tail junction pattern with lengths less than 200 nucleotides at 14-days post-injection. In contrast to ssAAV, scAAV carries the initial deletions of the D element at the mutated ITR ends pre-transduction. It displayed unique further truncations at the mutated ITR ends post transduction in most of the Head-to-Tail reads. This distinct pattern underscores the impact of specific ITR sequences on biological recombination processes. Epigenetic regulation of AAV genomes, including CpG/5mC modification across the genome cassette and analysis of histone modifications (H3K27me3, H3K4me3, and H3K27Ac) is under investigation. Collectively, this comprehensive investigation into AAV episomes and ITR-mediated recombination patterns may represent a significant advancement in understanding AAV virology, shedding light on the roles of ITRs in AAV genome conversion and episome durability, providing crucial optimization targets for enhancing the durability of future AAV-based gene therapy drugs and non-viral gene transfer.

390. Orthogonal Characterization of rAAV Genomes Reveals Vector Attributes That Drive Dimer Encapsidation and ITR Repair

Carla Llinares[1], Mitchell Yip[2], Ngoc Tam L. Tran[2], África Vales[1], Cristina Olagüe[1], Sergio Isola[1], Gloria González-Aseguinolaza[1], Phillip WL Tai[2,3], Carmen Unzu[1]

[1]DNA & RNA Medicine Division, Centro de Investigación Médica Aplicada, CIMA, Navarra, Spain,[2]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[3]Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA

Recombinant adeno-associated viral vectors (rAAVs) are the flagship vehicles for delivering DNA payloads for human gene therapy. However, in the past decade, only a few outstanding therapies have reached the market. One reason for the slow development of rAAV-based gene therapies has been our limited knowledge of basic AAV biology. Therefore, the goal of this work is to perform orthogonal evaluation of design elements within the packaged rAAV genomes and assess their impact on potency. Specifically, we investigated the influence of inverted terminal repeat (ITR) design, promoter use, and serotype on vector heterogeneity and transduction. We first analyzed the integrity of AAV-*Egfp* vectors containing either an alpha-1 antitrypsin (AAT) promoter (2 kb in vector genome size) or a chicken beta-actin (*CBA*) promoter (3 kb in size), packaged with six different

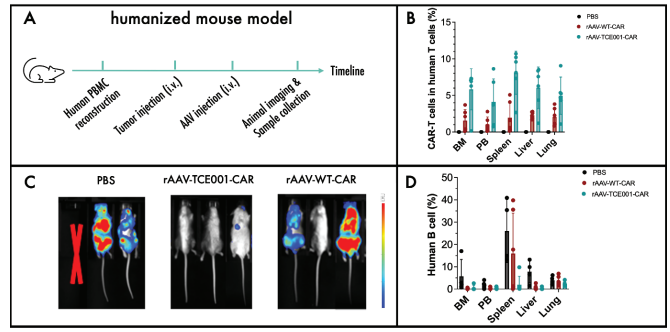
serotypes (AAV2, AAV3B, AAVLK03, AAV5, AAV8, and AAV9) by native agarose and automated electrophoresis. Whereas most of the packaged genomes migrated at their expected size, additional larger and smaller bands were also identified. Most of the bands were consistent among serotypes and were eliminated when the vector genomes were digested with SmaI, suggesting these DNA species were unresolved (*i.e.*, un-nicked and bound at the ITRs). Banding patterns were more heterogeneous with vectors harboring the CBA promoter, especially for those packaged with AAV8. We next produced constructs flanked by different ITR designs: two wildtype (wt)ITRs, a commercially available 11-nt deletion in the C arm of the 5'-ITR (dITR), or a 22-nt deletion in the B arm of the 3'-ITR (tITR). Alkaline gel analysis revealed monomer and dimer genomes for AAV3B, AAV5, AAV8, and AAV9 packaged vectors that carried the wtITR and dITR. However, only dimers were observed for vectors packaged with dITR-AAVLK03 and tITR-AAV8. Again, genome heterogeneity was observed for the dITR.AAV8.CBA.*Egfp* vector, but not for the dITR.AAVLK03 or dITR.AAV5.CBA.*Egfp* vectors; although, the three vectors showed an intriguing faint band larger than 5 kb. To validate these findings, long-read single molecule, real-time (SMRT) sequencing was performed on the vector genomes. Our analyses confirmed the presence of genome dimers with self-complementary configurations, including vectors over 3 kb in size. To determine whether the detection of oversized genomes was due to artifacts from target ligation/recombination during SMRT library preparation steps, 2-kb and 3-kb vector genomes were extracted and mixed before library preparation. Clear individual peaks and their respective self-complementary forms were detected, with no sign of recombination between the two genomes. Mutated ITRs have been shown to be repaired during rAAV genome replication and packaging, using the more functional of the two ITRs as a template. Interestingly, our bioinformatic analyses revealed that the promoter sequence has an impact on ITR repair. Finally, *in vitro* transduction studies confirmed superior expression from tITR genomes (dimers only) versus wtITRs, similar to classic self-complementary vectors. In conclusion, self-complementary dimer genome encapsidation is favored under specific ITR designs and can be influenced by other elements such as the promoter or the AAV capsid. C.L. and M.Y. are co-first authors.

391. An Evolved AAV Variant Enables *In Vivo* CAR-T Cell Generation, Tumor Cytotoxicity, and B Cell Elimination

Ke Ni[1], Ming Wei[1], Yanyi Cong[1], Lina Wei[1], Jing Wang[2], Qingkai Song[2], Hui Chen[1], Zhike Lu[1], Lijia Ma[2]

[1]Westlake Genetech, Hangzhou, China,[2]School of Life Sciences, Westlake University, Hangzhou, China

Background: While chimeric antigen receptor T (CAR-T) cell therapy has shown significant therapeutic benefits, its complex and expensive manufacturing process hinders its clinical use. A more viable approach is the in vivo generation of CAR-T cells, which could make CAR-T cells a universally applicable treatment. To achieve this, we must develop a delivery tool that primarily transduces payload to T cells while minimizing toxicities. **Results:** Here, we showed that T cells could be engineered into CAR-T cells in humanized mice by intravenous injection of rAAV-TCE001, an AI-generated novel AAV variant. We conducted a capsid screening to identify rAAV variants with high transduction to primary human T cells, from which the data was used to train an AI model to generate novel capsids. We validated that the rAAV-TCE001 exhibited a higher transduction efficiency to primary human T cells by more than two orders of magnitude in vitro. From the humanized mouse model **(Fig. A)**, data demonstrated that about 4.1-8.1% of human T cells could be successfully engineered into CAR-T cells across different organs **(Fig. B)**, in addition to a lowered liver transduction. When rAAV-TCE001 was loaded with an anti-CD19 payload, the in vivo generated CAR-T cells demonstrated potent tumor cytotoxicity compared to the control groups **(Fig. C)**. Further, the in vivo generated CAR-T cells also effectively eliminated human PBMC-derived B cells in humanized mice **(Fig. D)**. Finally, we applied whole-genome CRISPR screening on primary human T cells and identified receptors that promote the transduction of rAAV-TCE001 but not the parental wild-type AAV. **Conclusion:** Together, we proved the concept of in vivo CAR-T therapy by systematically injecting an rAAV variant evolved in silico from the parental serotype. The application of in vivo CAR-T in a humanized mouse model demonstrated its potential to treat tumors and autoimmune diseases, which promised great therapeutic potential in the clinic.



392. Deep Learning and RNN-Enhanced Models for Tissue-Dependent Codon Optimization

Tapan Sharma[1,2,3], Sandhiya Ravi[1,2,3], Mitchell Yip[1], Qin Su[1], Jialing Yang Liang[1], Huiya Yang[1], Joae Wu[4], Dan Wang[1,5], Jun Xie[1,2], Guangping Gao[1,2], Phillip Tai[1,2]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA,[3]Co-first authors, Worcester, MA,[4]Department of Pediatrics, UMass Chan Medical School, Worcester, MA,[5]RNA Therapeutics Institute, UMass Chan Medical School, Worcester, MA

A critical aspect of gene therapy design is enhancing the expression of protein-coding transgenes through codon optimization. Traditional approaches that are guided by codon-usage tables and/or species-specific expression of aminotransferases and transfer RNAs, typically rely on broad eukaryotic versus prokaryotic usage rules. This approach often neglects transcript expression levels, protein size, function, and tissue- or cell-type differences. These omissions highlight a significant gap in gene therapy vector engineering, and present avenues for innovation. In addition, publicly available tools typically yield various versions of codon-optimized sequences for the same transgene, which can show differences in expression - antithetical to being optimized. In response to tissue-related synonymous codon biases, our team

developed an advanced recurrent neural network (RNN)-based codon optimization tool, tailored for cell type-dependent expression. We trained a deep learning model with comprehensive datasets of genes highly expressed in mouse myocytes, neurons, and liver. This approach enabled the prediction of effective codon usage patterns for different cell types. To test the models' performances, the enhanced green fluorescent protein (EGFP) and firefly luciferase (FLuc) reporter genes were codon-optimized using the muscle-, liver-, or brain-trained models. *In vitro* testing showed enhanced expression of these transgenes as compared to wildtype and optimized sequences using a publicly available tool. *In vivo* testing through systemically-delivered adeno-associated virus (AAV) vectors in mice, showed that EGFP versions optimized for liver and muscle expression were detected at significantly higher levels than the wildtype version in their cognate target tissues. Moreover, we also discovered that the first version of our algorithm reduced expression of secretory proteins, which make up a significant portion of the gene replacement and addition platforms being tested in the clinic. We therefore retrained our algorithms with additional data to design new models tailored to secretory proteins of human and mouse. Our initial tests on benchmark secretory genes showed marked improvements in key metrics, such as codon adaptation index (CAI), GC content, codon frequency distribution (CFD), and CpG dinucleotide content. Our *in vitro* results showed that our DL-trained secreted gene (*Gaussia* luciferase) outperformed commercial optimization tools and demonstrate differential codon biases between secreted and non-secreted proteins. We are now validating the optimized transgenes *in vivo* to test our new DL-based models. Our findings so far represent a novel DL-based approach for codon optimization with a potential to improve gene therapy drugs. **TS and SR are equal contributors.** G.G. and P.T. are corresponding authors**.**

393. A Strong and Compact Bidirectional Neuronal Promoter for AAV-Mediated and CNS-Directed Delivery of Dual Transgenes

Xiupeng Chen, Hong Ma, Phillip WL Tai, Guangping Gao*, Jun Xie*

Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA

The recombinant adeno-associated viruses (rAAVs) vectors hold promise for delivering transgenes for human gene therapy. However, the packaging capacity of AAV is limited to <5 kb, which excludes larger and especially multiple therapeutic genes from conventional vector designs, necessitating the development of compact and efficient bidirectional promoters for transgene expression. Recently we identified a 155-bp promoter derived from the human survival motor neuron (SMN1) gene and demonstrated its robust and specific neuronal activity in rAAV-mediated *in vivo* gene delivery. In the present study, we aimed to test if this compact and CNS-specific promoter can direct the expression of two transgenes bidirectionally. To this end, we constructed an AAV plasmid harboring the 155-bp SMN1 promoter in the middle flanking with the mCherry gene in the upstream and EGFP gene in the downstream (i.e.mCherry-155-SMN-EGFP). By plasmid transfection into HEK293 cells, the 155-bp promoter is capable of efficiently driving both mCherry and EGFP expression at comparable levels. In contrast, a longer 900-bp SMN1 promoter can only drive its downstream transgene expression. We then packaged this dual reporter construct into rAAV9 for facial vein administration to neonatal C57BL/6 mice at P1 with a dose of 4E11 vgs/mouse. At the same time, we produced a rAAV9 with a flipped 155-bp SMN1 promoter in the dual reporter construct as control(mCherry-155-SMN_{flip}-EGFP). Four weeks after injection, we found that the 155 bp promoter directs neuron-specific expression in the brain and spinal cord with the GFP signals largely overlapped with mCherry. Interestingly, the flipped 155-bp SMN1 is also bidirectional but favors its upstream transgene expression over the downstream one. Taken together, our findings indicate that the 155-bp promoter is strong and bidirectional both in the cultured cells and mice.* **G.G., and J.X are co-corresponding authors**

Musculo-Skeletal Diseases

394. Ten-Year Durability of AAV8-MTM1 Gene Transfer in a Canine Model of X-Linked Myotubular Myopathy

SiWei Luo[1], Jessica M. Snyder[2], Julie Crudele[3], Robert W. Grange[4], Louise Mangin[5], Edith Renaud-Gabardos[6], Michael Lawlor[7], Alan H. Beggs[8], Martin K. Childers[9], Ana Buj Bello[6], David L. Mack[1]

[1]Rehabilitation Medicine, Bioengineering, Physiology & Biophysics, University of Washington, Seattle, WA,[2]University of Washington, Seattle, WA,[3]Department of Neurology, University of Washington, Seattle, WA,[4]Virginia Tech, Blacksburg, VA,[5]Gene Therapy Branch, Genethon, Evry, France,[6]Genethon, Evry, France,[7]Diverge Translational Science Laboratory, Milwaukee, WI,[8]Boston Children's Hospital / Harvard Medical School, Boston, MA,[9]Kinea Bio, Inc., Edmonds, WA

Mutations in the myotubularin gene (*MTM1*) result in X-linked myotubular myopathy (XL-MTM), a fatal congenital skeletal muscle disease characterized by small immature centrally nucleated myofibers, internal disorganization of mitochondria (ring fibers) and an absence of the T-tubule network. Patients typically present with severe hypotonia and respiratory insufficiency, and if patients survive infancy, about half die by 2 years of age from respiratory failure. The MTM1-mutant N155K canine model recapitulates all hallmarks of the disease observed in patients except the pre-cholestatic liver dysfunction identified in the ASPIRO clinical trial. Previous work from our group demonstrated that a single systemic infusion of AAV8-MTM1 driven by the muscle-specific desmin promoter prolongs survival, restores muscle strength and improves respiratory, gait and neurological function to near normal levels in the canine model[1]. Currently, several AAV-mediated gene transfer clinical trials for neuromuscular diseases are in progress, with initial cohorts receiving treatment approximately 5 years ago. However, critical questions remain about the longevity of transduced myonuclei over the span of years while undergoing normal turnover and how long transgene expression from AAV episomes will persist above therapeutic levels. Herein, we report that two MTM1 mutant dogs treated at 10 weeks of age with 2e14 vg/kg via regional hindlimb perfusion thrived to 11 years of age with no loss of effectiveness. Yearly efficacy readouts included neurological assessments, gait analysis, respiratory function (flow volume loops and peak inspiratory flow) and bilateral hindlimb force testing. Annual biopsies of the vastus lateralis, cranial tibialis, biceps brachii, and/or triceps brachii were analyzed for vector copy

number, MTM1 mRNA by qRT-PCR, and protein expression using western blots. Functional readouts between years 5-10 post dosing were similar to those from years 1-4[2,3] for neurological assessment scores, gait performance and hindlimb force output and were indistinguishable from one littermate carrier female control. Myotubularin protein expression remained stable, at or above normal endogenous levels in all muscles tested. Muscle histology at 11 years displayed nonspecific myopathic features (focal fiber size variation, internal nucleation, and endomysial fibrosis) but no findings that were characteristic of XL-MTM. Together, these data demonstrate that AAV-mediated gene therapy for a congenital myopathy is durable for at least a decade in a large animal model. [1]Systemic AAV8-mediated gene therapy drives whole-body correction of myotubular myopathy in dogs. Mack DL, et.al. Mol Ther. 2017 Apr 5;25(4):839-854. PMID: 28237839[2]Gene therapy prolongs survival and restores function in murine and canine models of myotubular myopathy.Childers MK, et.al. Sci Transl Med. 2014 Jan 22;6(220). PMID: 24452262[3]Long-term effects of systemic gene therapy in a canine model of myotubular myopathy. Elverman M, et.al. Muscle Nerve. 2017 Nov;56(5):943-953. PMID: 28370029

395. RGX-202, an Investigational Gene Therapy for the Treatment of Duchenne Muscular Dystrophy: Interim Clinical Data

Jahannaz Dastgir[1], Aravindhan Veerapandiyan[2], Vamshi Rao[3], Paulo Falabella[1], Stephen J. Pakola[1], Sarang Rastogi[1], Dawn Phillips[1], Catherine Wilson[1], Nidal Boulos[1], Michelle Gilmor[1], Lin Yang[1], Hiren Patel[1], Michele Fiscella[1], Olivier F. Danos[1]

[1]REGENXBIO Inc., Rockville, MD,[2]Arkansas Children's Hospital, Little Rock, AR,[3]Ann and Robert H. Lurie Children's Hospital of Chicago, Chicago, IL

Objective: To evaluate safety and efficacy of RGX-202 in patients with Duchenne muscular dystrophy (DMD) aged 4 to 11 years. **Background:** Duchenne is a rare, X-linked, progressive muscle disease due to pathogenic variants in the *DMD* gene which encodes dystrophin. The absence of functional dystrophin results in muscle cell damage and ultimately death with progressive weakness of skeletal muscle, loss of ambulation, and cardiac/diaphragm weakness. **Design/Methods:** RGX-202 is an investigational, one-time AAV therapeutic for Duchenne, using the NAV® AAV8 vector to deliver a transgene for a novel microdystrophin that includes the functional elements of the C-Terminal domain as well as a muscle-specific promoter to support a targeted therapy for improved resistance to muscle damage associated with Duchenne. **Results:** As of September 28, 2023, RGX-202 was reported to be well tolerated with no drug-related serious adverse events in three patients at dose level 1, $1x10^{14}$ genome copies (GC)/kg, with 3 weeks to over 5 months follow-up. The prophylactic immune suppression for RGX-202 gene therapy has been well tolerated. Initial biomarker data from two patients indicate increased expression of RGX-202 microdystrophin from bicep biopsies taken three months following one-time RGX-202 administration. RGX-202 microdystrophin expression measured via western blot (Jess) was 38.8% compared to control in the 4.4 year old and 11.1% compared to control in the 10.6 year old. Additionally, RGX-202 microdystrophin was detectable by immunofluorescence staining in muscle at three months, with RGX-202 microdystrophin protein localized to the sarcolemma. Updated interim results will be presented. **Conclusions:** RGX-202 has been well tolerated in 3 participants up to 25 weeks post-administration of RGX-202, with readily detectable RGX-202 microdystrophin levels at 12 weeks.

396. Sustained Clinical Effects of Single-Dose Intraarticular PCRX201 Injection in Moderate-to-Severe Osteoarthritis of the Knee

Cecilia Barese[1], Emily Walsh[2], Stanley Cohen[3], Alan Kivitz[4], Masato Nakazawa[1], Mary DiGiorgi[1], Jonathan Slonin[1]

[1]Pacira Biosciences, Inc., Tampa, FL,[2]Tremont Therapeutics Consulting, Boston, MA,[3]Metroplex Clinical Research Center, Dallas, TX,[4]Altoona Center for Clinical Research, Duncansville, PA

Osteoarthritis of the knee (OAK) is a severe disease associated with high disability, and current therapies are limited to providing temporary symptomatic relief. Targeting interleukin-1β (IL-1β) in OAK holds therapeutic potential for reducing pain, increasing function, and slowing structural progression. PCRX201 is a helper-dependent adenovirus serotype 5 vector for intraarticular (IA) injection in patients with OAK. This nonintegrating, nonreplicating vector is engineered to express the IL-1 receptor antagonist (IL-1Ra), an inhibitor of IL-1β, under the control of a nuclear factor-κB-inducible promotor in response to inflammation in the target knee, thereby reversing OAK pathology. This 52-week, open-label Phase 1 trial (NCT04119687) investigated the safety and efficacy of a single dose of PCRX201 in 72 participants aged 30-80 years with moderate-to-severe OAK. The first cohort (n=36) received an ultrasound-guided IA PCRX201 injection in the study knee at 3 ascending doses. The subsequent cohort (n=36) received pretreatment with IA 40 mg methylprednisolone immediately before PCRX201 administration at the same 3 ascending doses. PCRX201 was well tolerated in all participants at all dose levels and cohorts. The most common adverse event (AE) was dose-related index knee effusion reaction (23 participants [63.9%] in the noncorticosteroid cohort, most occurring with mid and high doses, versus 12 participants (33%) in the corticosteroid cohort). There were no serious AEs related to the study drug or treatment procedure. The effectiveness of PCRX201 was measured as percent change from baseline in pain (Western Ontario and McMaster Universities Osteoarthritis Index [WOMAC]-A). A substantial reduction in WOMAC-A pain was reported at all dose levels and cohorts by week 8 and through week 52 after injection (≥25%-50% decrease in noncorticosteroid cohort, ≥50%-75% decrease in corticosteroid cohort). A similar percent change improvement was observed in WOMAC-A stiffness. Favorable improvements were also observed in knee function as assessed by an independent patient-reported outcome (Knee injury and Osteoarthritis Outcome Score). Radiographic changes such as joint space narrowing and joint space width remained unchanged after 52 weeks for all dose levels, demonstrating PCRX201 was safe in the study knee. Additionally, bone shape changes in femur and tibia, candidates as imaging biomarkers of disease progression, revealed no demonstrable increases in B-score during the first 52 weeks of treatment. Last, the pharmacologic activity of PCRX201 measured by concentrations of IL-1Ra in synovial fluid of the study knee indicated that in participants with paired samples available from baseline and week 4 (n=4), increases in IL-1Ra levels ranging from 1.4- to 132.9-fold were detected. Collectively, these data

suggest the IA PCRX201 has an acceptable safety profile and provides sustained clinical effects at all dose levels up to 52 weeks postinjection as measured by validated patient-reported outcome assessments. These promising preliminary results warrant further investigation of PCRX201 in an ongoing randomized controlled Phase 1b trial.

397. Correction of Myotonia and Reduction of Toxic DMPK Foci After Treatment of the HSALR Mouse Model of Myotonic Dystrophy Type 1 Using an Adeno-Associated Virus Delivering U7snRNA Interfering with CTG Repeat Expansion

Camila Almeida[1], Alex Brinkman[2], Andrea Sierra Delgado[2], Liubov V. Gushchina[1], Dave Arnold[3], Robert Weiss[4], Nicolas Wein[2]

[1]Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH,[3]Department of Physical Medicine and Rehabilitation, University of Missouri School of Medicine, Columbia, MO,[4]Department of Human Genetics, The University of Utah School of Medicine, Salt Lake City, UT

Myotonic dystrophy type 1 is the most common form of muscular dystrophy in adults affecting the skeletal muscle, heart, and brain. It is caused by a CTG repeat expansion in the 3'UTR of the *DMPK* gene. Antisense oligonucleotides aiming to knock down *DMPK* expression or bind to the CTGs have been explored but require repeated injections to sustain therapeutic effects. To overcome this limitation, we designed modified U7 small nuclear RNAs containing a promoter and antisense sequences targeting the 3'UTR region to promote steric hindrance of the CUG repeats. We derived and characterized FibroMyoD cell lines from DM1 skin fibroblasts that were transdifferentiated into myotubes. We used several patient cell lines to test our approach, and the effect of the treatment was evaluated using RNA FISH combined with MBNL1 immunostaining for *foci* quantification and MBNL1 localization. Gene expression and splicing profiles were assessed by RNAseq and selected candidate genes were confirmed by RT-PCR. The AAV treatment reduced the *foci* number, changed MBNL1 localization, and shifted the splicing profile. Based on the *in vitro* results, we selected one vector candidate to test *in vivo*. We injected AAV1.U7snRNAs in the tibialis anterior (TA) and gastrocnemius (Gas) of HSA^{lr} mice, which express CTG repeats in skeletal muscles only. Eight weeks post-injection, we submitted the animals to electromyography (EMG) test and collected the muscles for further analysis. EMG revealed reduced mechanical myotonia (a major phenotype in DM1 muscles) and even absence of electrical myotonia in individual muscles. RNA FISH quantification showed reduced number and intensity of foci in TA and Gas. The reduction in foci led to improved splicing of *Serca1* and *Clcn1*. In conclusion, U7snRNAs interfering with the CUG-expanded mRNA can reverse DM1 pathology with the restoration of molecular features *in vitro* and *in vivo*, accompanied by significant amelioration of muscle physiological properties.

398. CSF Delivery of INS1201 AAV9-Micro-Dystrophin Demonstrates Efficacy in a Mouse Model of DMD

Gretchen Thomsen[1], Allan Kaspar[1], Laura Ferraiuolo[1], Binh Chu[1], Veronica Garcia[1], Theresa Slaiwa[1], Ryan Weiss[1], Robert Cano[1], Ashley Helmuth[1], Hana Julazadeh[1], Haunani Bautista[1], Melissa McAlonis Downes[2], W. David Arnold[3], Brian Kaspar[1]

[1]Insmed Gene Therapy, San Diego, CA,[2]UCSD, San Diego, CA,[3]University of Missouri, Columbia, MO

Insmed has developed INS1201, an AAV9-micro-dystrophin construct for thetreatment of Duchenne muscular dystrophy (DMD). INS1201 expresses a segment ofthe dystrophin protein that demonstrates efficacy in the *mdx* mouse and is delivered via a single intrathecal (IT) administration. IGT has shown that cerebrospinal fluid (CSF) delivery of AAV9 efficiently targets muscle and cardiac tissue in mice and non-human primate (NHP) models at lower dose levels than systemic administration. Biodistribution and efficacy of INS1201 was assessed in the *mdx* mouse model of DMD when delivered via a single-dose intracerebroventricular (ICV) injection at postnatal day (p) 31±4 with either 8.0E+09, 2.0E+11, 4.0E+11, or 8.0E+11 vector genomes (vg) peranimal. Tissue samples from INS1201-treated mice were analyzed by ddPCR using a probe specific to the INS1201 micro-dystrophin gene construct that is not present in untreated mice.INS1201 biodistribution studies were also performed in NHP following a one-time intrathecal delivery. These studies demonstrated that CSF delivery of 8.0E+11 vg per mouseresulted in .410 and 1.357 vg per nuclei in the gastrocnemius and heart, respectively, and a dose of 2.0E+14 vg per NHP resulted in .240 and 1.057 vgper nuclei in the gastrocnemius and heart, respectively. Comparable biodistribution among skeletal and cardiac muscle between mouse and NHP supports the equivalency of the intrathecal CSF route of administration by means of ICV in the mouse relative to intra-lumbar in larger animal models. Immunostaining of muscle tissues in *mdx* mice showed that membrane micro-dystrophin levels were significantly increased in each muscle examined in nearly every INS1201-treated *mdx* dose group, with 83% and 87% of all fibers positive for dystrophin in the gastrocnemius and TA, respectively at the 8.0E+11 vg dose. Relative to *mdx* vehicle controls, H&E-stained muscle from INS1201-treated *mdx* mice exhibited larger myofibers, fewer inflammatory cells, and reduced endomysial connective tissue. Picrosirius red staining for collagen provided quantitative evidence suggesting that fibrosis was significantly reduced in each muscle examined following INS1201 treatment in *mdx* mice. Quantitative analysis of fiber size measurements using laminin staining revealed significant increases in average fiber diameter in the gastrocnemius, TA and diaphragm across INS1201 treatment doses, relative to *mdx* vehicle controls, demonstrating an improvement of dystrophic pathology in the mouse model. INS1201 functional efficacy was demonstrated in *mdx* mice dosed with 2.0E+11, 4.0E+11 and 8.0E+11 vg. Significant improvement in grip strength was observed at all doses at postnatal day (p) ~115, and this was maintained at ~275 days when tested in the higher dosed animals. Using the Aurora Scientific Whole Animal System for Mice to assess plantarflexion muscle contractility, significant increases were observed in peak twitch torque following a single supramaximal stimulation and peak tetanic torque following a 200 ms train of stimuli delivered at 125 Hz. Significant torque improvement was

also noted across stimulation rates 20-160 Hz. These studies show that CSF delivery of INS1201 broadly transduced muscle tissues, leading to reduced DMD disease pathology and improved muscle function in a mouse model of DMD. This preclinical data provides support for further evaluation of therapeutic benefit in DMD patients.

399. Evaluation of Novel Muscle-Specific Promoters in Mice and Non-Human Primates

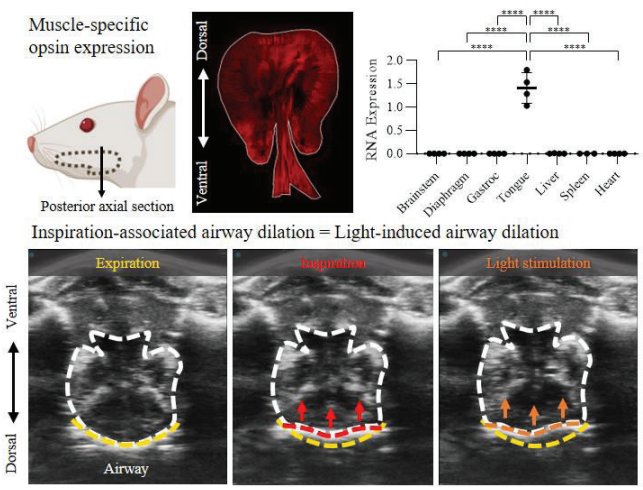
Randolph Qian, Steven J. Foltz, Kirk W. Elliott, Subha Karumuthil-Melethil, Olivier F. Danos, Ye Liu
REGENXBIO Inc., Rockville, MD

Many gene therapies for the treatment of muscle diseases require high systemic doses of AAV, which could pose risks to patients. Engineering stronger and more specific muscle promoters could enable lower therapeutic doses and decrease off-target activity, thereby enhancing the safety profile of these therapies. However, promoter size is an important consideration, as AAV vectors have a limited packaging capacity of 4.7 kb. Small promoters may enable the design of larger therapeutic transgenes and the inclusion of additional regulatory elements. Here, we describe a bioinformatics-guided design of several small but potent muscle enhancers and core promoters that were experimentally validated in mice and non-human primates (NHP) through a barcoded vector library or individual vectors. To create compact muscle-specific promoters, we first generated several small core promoters derived from the human and mouse alpha-actinin 2 (ACTN2) promoter region using a bioinformatics-guided approach. Each candidate core promoter was paired with a strong muscle enhancer to drive the expression of a barcoded Td-Tomato transgene and enable high-throughput screening via next-generation sequencing. Promoters were first screened for activity in differentiated C2C12 mouse myoblasts via plasmid transfection. Top candidates were packaged into AAVHu32 capsid and pooled at equal genome copies to produce an input AAV vector library. Evaluation of this AAV library in *mdx* mice identified a compact novel muscle promoter, EMP1, that drove robust expression in mouse skeletal and cardiac muscle compared to a commonly utilized muscle-specific promoter, CK7. EMP1, with a size of 280 bp, exhibited 5-, 7-, and 9-fold more activity in skeletal muscle, heart, and diaphragm, respectively, compared to CK7, despite being around half its length. The liver activity of EMP1 was similar to CK7, indicating high muscle specificity. Next, EMP1 was subjected to a second round of engineering by modifying the enhancer and core promoter sequences. Two additional muscle promoters were identified from a library study in *mdx* mice. EMP3 (500bp) demonstrated 8-, 14-, and 10-fold more activity in skeletal muscle, heart, and diaphragm, respectively, compared to CK7. EMP4 (400bp) exhibited 6-, 15-, and 8-fold increased activity in skeletal muscle, heart, and diaphragm, respectively, compared to CK7. Liver activity was similar between CK7, EMP3, and EMP4, indicating high muscle specificity. The top hits from the mouse studies were then cloned into a barcoded expression cassette encoding a microdystrophin to allow for their evaluation in association with a therapeutically relevant transgene. Twelve individually produced AAVhu32 vectors were pooled and intravenously administered into 2 cynomolgus monkeys at 1E14 GC/kg total dose. Necropsy was performed 3 months post-dosing, and barcoded microdystrophin mRNA expression levels were measured in multiple tissues, including muscle and liver. This NHP study confirmed the strong cardiac and skeletal muscle-specific promoter activities of EMP1, EMP3, and EMP4. Utilizing these novel muscle-specific promoters has the potential to improve the efficacy and safety of AAV gene therapies for muscle indications. Additionally, with their compact size, these promoters could enable the design of larger therapeutic payloads with additional functional domains or encode full-length human proteins that otherwise could not fit into the AAV genome.

400. An Optogenetics-Based Gene Therapy for Obstructive Sleep Apnea

Fiona L. Knapman[1,2], E. Myfanwy Cohen[2], Tom Kulaga[2], Nigel Lovell[2], Leszek Lisowski[3,4], Simon McMullan[5], Peter G. R. Burke[5], Lynne E. Bilston[1,2]
[1]NeuRA, Sydney, Australia,[2]UNSW, Sydney, Australia,[3]Children's Medical Research Institute, Sydney, Australia,[4]Military Institute of Medicine, Warsaw, Poland,[5]Macquarie University, Sydney, Australia

INTRODUCTION**:** Obstructive sleep apnea (OSA), where the upper airway collapses repeatedly during sleep, is challenging to treat as current therapies are poorly tolerated or have variable and unpredictable efficacy. We propose a novel, optogenetics-based gene therapy where direct light stimulation to the upper airway dilator muscles able to restore contraction, and prevent airway collapse. For proof-of-concept, a rodent model of human sleep-associated upper airway muscle atonia was developed. Using this model, we evaluated candidate viral vector constructs that generate the expression of light-sensitive proteins (opsins), including a muscle-targeted approach that is expected to provide a favorable safety profile. METHODS: Adeno-associated viral vectors (serotype 9, AAV9) expressing a standard opsin (channelrhodopsin-2, ChR2) and driven by either a muscle-specific (tMCK) or non-specific (CAG) promoter were injected into rat tongues to compare the strength and specificity of ChR2 expression. Light-evoked electromyographic (EMG) responses were recorded in an acute, isoflurane-based, rodent model of upper airway hypotonia and atonia. The promoter that generated the greatest expression in the tongue was then used with a highly myotropic AAV serotype (AAVMYO). ChR2 expression and light-evoked EMG responses were compared to that achieved with AAV9. Finally, ultrasound imaging measured light-evoked contractions of the upper airway muscles and the resultant airway dilation. RESULTS: The muscle-specific promoter tMCK produced 4.7-fold greater opsin expression across the tongue (RM-ANOVA, $F(1,8)=10.0$, $p=0.013$) despite a 3.6-fold lower viral load than the non-specific promoter(CAG). Moreover, the tMCK promoter abolished opsin expression in the brainstem, and viral DNA copy numbers and RNA expression were low or absent in all non-tongue tissues. Light stimulation on the tongue surface increased muscle activity by ~66% compared to unstimulated breaths (linear mixed model, $F=140.0$, $p<0.001$). AAVMYO produced greater response to light than AAV9, with light stimulation increasing EMG activity by ~194% (linear mixed model, $F=38.2$, $p<0.001$). Ultrasound imaging confirmed that light evoked muscle activity was sufficient to dilate the upper airway. CONCLUSIONS: Light stimulation of optogenetically modified tongue muscle cells was able to restore normal muscle activity and dilate the upper airway in a rodent model. This provides proof-of-concept for a non-invasive, optogenetic gene therapy for patients with OSA.



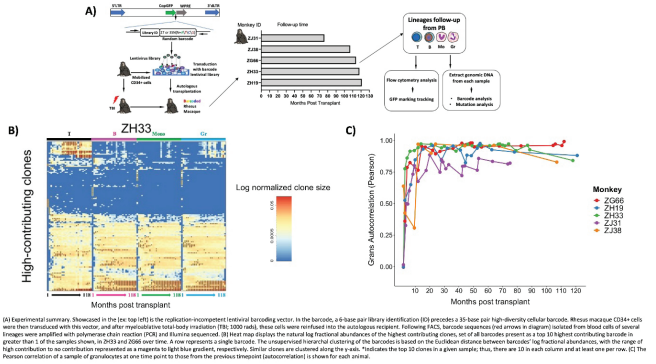
Molecular and Cellular Methods and Technologies

401. Long-Term Tracking of Hematopoietic Clonal Dynamics and Mutations in Nonhuman Primate Post-Autologous Lentiviral-Barcoded Hematopoietic Stem and Progenitor Cell Transplantation

Rohan V. Hosuru[1], Jack Yang[1], Yifan Zhou[1,2], Taha B. Hayal[1], Sogun Hong[1], Chuanfeng Wu[1], Cynthia Dunbar[1]

[1]Translational Stem Cell Biology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD,[2]Wellcome-MRC, Cambridge Stem Cell Institute, Cambridge, United Kingdom

Hematopoietic stem and progenitor cells (HSPCs) autologous transplantation-based gene therapies are promising for treatment of genetic blood disorders. Lentiviral vectors serve as clinically important vehicles for delivering therapeutic transgenes to HSPCs. Investigating the long-term clonal dynamics of HSPC output and disease-related mutations following lentiviral-mediated gene therapy is important to gain insights regarding efficacy and safety. We established an autologous lentivirally-barcoded HSPC transplantation rhesus macaque (RM) model allowing quantitative interrogation of many aspects of primate hematopoiesis, with relevance to human HSPC biology. Healthy RMs underwent total body irradiation, followed by transplantation of autologous HSPCs transduced with a lentivirus library containing high diversity genetic barcodes, uniquely labeling HSPCs and their progeny. Quantitative genetic barcode recovery enabled accurate assessment of HSPCs with greater accuracy than insertion site retrieval. The macaque model allowed repeated sampling of peripheral blood, which underwent a clonal tracking workflow (Figure A), in the absence of an underlying disease state that could impact on hematopoiesis. We characterized a broad range of clonal behaviors, including contribution levels, clonality, diversity, stability, and lineage biases in 5 barcoded RMs for up to 121 months post-transplantation. We revealed stable multipotent long-term hematopoietic clonal output of T, B cells, monocytes (Mono), and granulocytes (Gr) produced by a highly polyclonal repertoire of HSPCs in all 5 macaques for up to 121 months post-transplantation. Distinct clonal differences existed between early short-term (prior to 2 months post-transplantation) and long-term clones (persisting up to 121 months), excluding the emergence of some large T cell clones due to adaptive peripheral expansions likely in response to environmental cues. Clonal succession after stable hematopoietic reconstitution was minimal (Figure B). Granulopoiesis, reflecting active HSPC production, remained stable over time in terms of clone number and clonal diversity across all animals (Figure C). We also examined clonal bias, observing unbiased progenitor cells contributing to both Mono and Gr. For both B cell- and Gr-producing cells, the larger clones seem biased toward B cells but others biased to Gr, stabilizing over time. Interestingly, progenitors contributing to both T and B cells display high bias, with certain clones exclusively contributing to T cells at times. Our data suggests steady-state hematopoiesis is driven by multipotent progenitor cells. Recent research (Chapman et al., 2023) highlights an increased frequency of mutations associated with myeloid neoplasms and clonal hematopoiesis (CH) in both modified and nonmodified cells following gene therapy for sickle cell disease. Tracking of a panel of CH-related gene mutations comparing pre-/early timepoint samples with the longest follow-up timepoint post-transplantation is ongoing.



402. A Genotoxicity Mouse Model of HSPC Gene Therapy to Identify Early Biomarkers in the Interaction between Senescent Cells and the Immune System

Cristina Colleoni, Daniela Cesana, Pierangela Gallina, Laura Rudilosso, Leonardo Ormoli, Fabrizio Benedicenti, Ivan Merelli, Francesca Sanvito, Eugenio Montini

San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy

Gene therapy (GT) product approval relies on costly in vivo safety studies that characterize the genotoxic potential, or lack thereof, of an integrating vector. Past pre-clinical and clinical studies in hematopoietic stem and progenitor cell (HSPCs) GT have extensively described that semi-random integrations of retroviral vectors can occur near oncogenes, activating them and leading to tumorigenesis. However, adverse events after oncogene activation may occur before or beyond tumorigenesis. We previously showed that oncogene BRAFV600E expression in human HSPCs transplanted into immune-deficient (NSG) mice triggered senescence, causing lymphoid impairment and

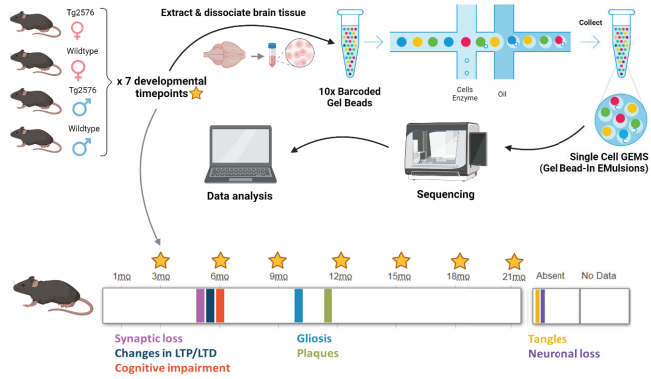
altered secretion of pro-inflammatory cytokines, leading to lethal bone marrow (BM) failure. Thus, senescence after oncogene activation may be detrimental before tumorigenesis, by inducing early aging in HSPCs. Our study aimed to characterize whether the interaction between senescent cells (SCs) and a functional immune system in an immune-competent (WT) mouse model can ameliorate the detrimental outcome observed in NSG mice. Secondly, we wanted to identify SCs signatures to ideally use them as genotoxicity biomarkers in future studies. To achieve these goals, we transplanted NSG or WT mice with mouse HSPCs transduced with lentiviral vectors expressing either the mouse oncogene BrafV600E or GFP as a control. Our results showed that oncogene expression in NSG mice (n=12) caused dose-dependent lethality (median survival = 36 days), while 40% of WT mice (n=7) survived up to 498 days post-transplant. The same mice that survived long-term exhibited elimination of SCs 12 weeks post-transplant. Both models showed histiocyte infiltration in the organs, reduction in peripheral blood cellularity, and altered levels of pro-inflammatory cytokines, but NSG mice had IL-9 and IL-10 levels, which exert immune suppression, correlating with the percentage of SCs. WT mice, instead, showed levels of IL-17a and IL-1α inversely correlating with engraftment levels, while senescence markers such as CCL-2 and IL-6 directly correlated with the engraftment of donor cells and inversely correlated with the number of recipient B and T cells. RNA-seq data revealed upregulation of inflammatory signaling (IFN-γ, IL-6, and TNF-α) in both mice backgrounds, as well as the upregulation of MHC Class II molecule genes and biological processes, suggesting an activation of the antigen presentation process in myeloid SCs. Intriguingly, gene set enrichment analysis revealed that donor myeloid and B cells of WT mice upregulated previously published SCs signatures (SenMayo, Purcell, Sencan) while NSG mice did not, despite showing other signs of senescence (i.e. upregulation of cell-cycle inhibitor gene *Cdkn2a*). Moreover, genes linked to immune suppression (*Il10, Pdcd1*) were upregulated in NSG myeloid SCs, while WT SCs upregulated immune response genes (*Cd80, Cd86, Icosl*), especially in B cells. This data hinted that functional recipient B and T cells, absent in NSG but present in WT mice, were able to recognize donor SCs, eliminate them, and partially dampen the inflammation. We tested our hypothesis by performing the same experiment on Rag1KO mice that lack B and T cells. Indeed, all Rag1KO mice transplanted with oncogene expressing HSPCs died just as NSG, showing B and T cell impairment. Altogether, these results identified SCs signatures in HSPCs and emphasized how their elimination is influenced by the immunological background of recipient mice. In the future, we will investigate SCs signatures, such as the cited cytokines and the identified genes, as genotoxicity biomarkers for cost-effective in vitro assays.

403. Exploring Novel Mechanisms of Alzheimer's Disease Pathophysiology through High-Dimension Transcriptomic Technology

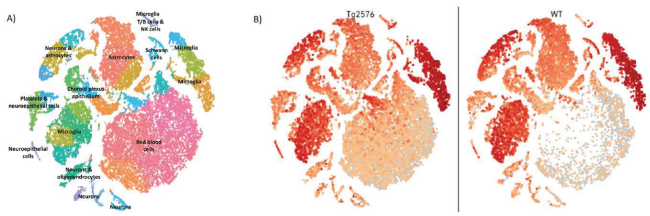
Marie Johns, Giorgia Caspani, Chaahat S. B. Singh, Maria-Elizabeth Baeva, Wing Yan Leung, Cheryl Pfeifer, Wilfred Jefferies

Medical Genetics, University of British Columbia, Vancouver, BC, Canada

BACKGROUND: Alzheimer's disease (AD) is a debilitating neurodegenerative condition characterized by cognitive decline, progressing to eventual dementia and death. Recent discoveries have challenged AD's traditional "amyloid hypothesis", warranting new approaches and prospective treatment agents in the AD therapeutic pipeline. Angiogenesis, the formation of new blood vessels, is characteristic of early AD. Growing evidence suggests that modulating angiogenesis may repair damage in the AD brain, though transcriptomic data is currently lacking. **OBJECTIVE:** To create the first single-cell view of AD progression in mice in order to identify alternative mechanisms of AD pathophysiology, and reveal putative molecular targets for AD drug therapy. **METHODS:** Cohorts of four mice representing both AD (using the Tg2745 APP overexpression mouse model) and control genotypes and both sexes (male and female) were humanely euthanized at six different developmental timepoints (N=24). Brains were dissociated and profiled at single-cell resolution using 10X Genomics software. *Figure 1:* Cohorts of four mice representing both AD and control genotypes (Tg2576 and wildtype) and both sexes (male and female) were humanely euthanized at six different disease progression timepoints (N=24). Brains were dissected and dissociated into a single-cell suspension. Individual cells were captured, and cellular mRNA was sequenced and profiled at a single cell resolution. Single-cell expression data was analyzed using 10X Genomics software: data was pre-processed with Cell Ranger then imported into Loupe Browser for differential expression analysis.



RESULTS: Pathway analysis of the 6- and 9-month cohorts demonstrated statistically significant enrichment in pathways related to: increased angiogenesis (p = 3.37E-02), vasculature development, (p = 3.71E-02), and amyloid-beta formation (p = 1.49E-02). *Figure 2:* (a) Cluster diagram for merged AD and wt samples. During the data analysis process, clusters are formed by grouping cells with similar transcriptomic profiles. (N=8) (b) Representative gene comparison. This representative gene, a negative regulator of angiogenesis, is upregulated in AD vs. wildtype mouse brains (N=8) (P=2.82E-08).



CONCLUSION: This study offers a unique cellular-level view of transcriptional alterations associated with AD pathology in mice. Preliminary findings support the 'vascular angiogenesis model' for AD, supporting growing evidence that neo-angiogensis-induced

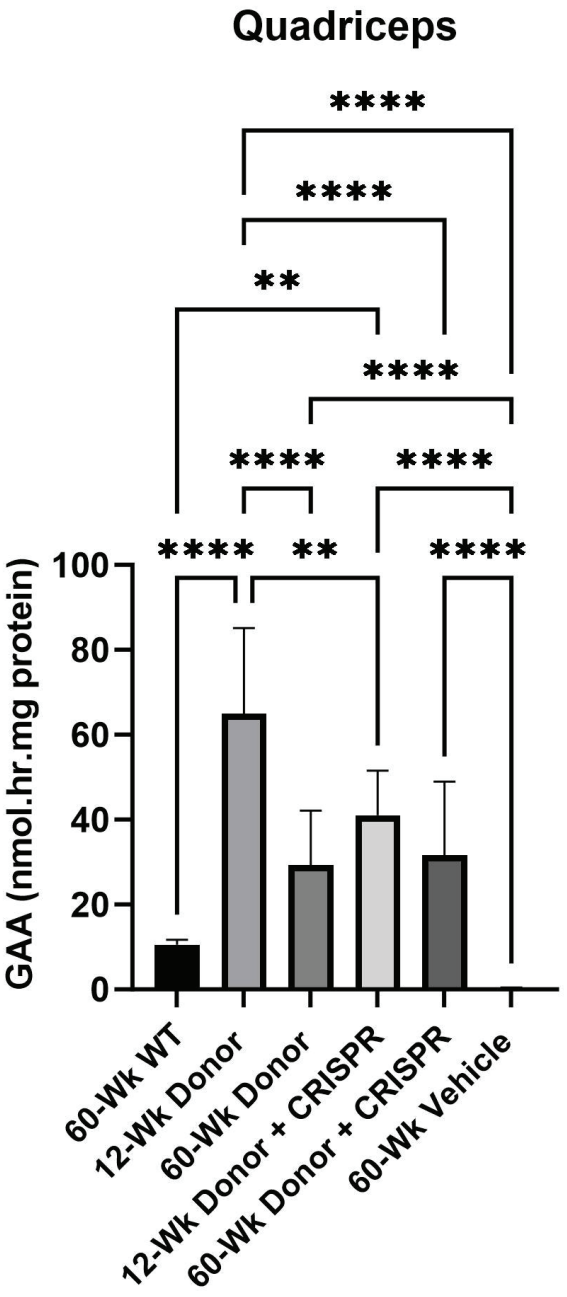
disruptions to BBB integrity lead to the increase in amyloid-beta accumulation and subsequent AD pathology seen in Tg2576 mice and AD patients.

404. Stable Treatment of Infant Mice with Pompe Disease by Direct Transduction of Muscle

Kate Ekaterina Ilich[1], Benjamin D. Arnson[1], Elizabeth Brooks[1], Songtao Li[1], Matthew Weinrub[1], Troy von Beck[1], Jasson Hoggard[1], Dwight Koeberl[2]

[1]Medical Genetics-Pediatrics, Duke University, Durham, NC,[2]Medical Genetics-Pediatrics, Duke University Medical Center, Durham, NC

Early treatment is needed to reverse the symptoms of Pompe disease (PD), caused by acid alpha-glucosidase (GAA) deficiency accompanied by lysosomal glycogen in muscle. Although gene therapy with adeno-associated virus (AAV) vectors to replace GAA could treat PD early in life, its benefits are expected to be transient. We tested gene therapy and CRISPR/Cas9 mediated gene editing for PD with two AAV vectors containing: 1) a human GAA transgene with a CMV enhancer/CB-actin promoter (CB) flanked by mouse genomic DNA from the Gaa intron 1/exon 2 boundary (Donor), and 2) a CB-SaCas9 transgene and a guide RNA that targets the intron 1/exon 2 boundary in Gaa (CRISPR). Recombinant AAV9 vectors were administered at 12 of age to infant Gaa knockout mice. For gene therapy, mice received Donor only (1E+13 vg/kg). For genome editing, mice received the Donor (1E+13 vg/kg) and CRISPR (2.6E+12 vg/kg)) or high dose CRISPR (2.6E+13 vg/kg). Tissue collection at 12 and 60 weeks was performed for skeletal muscles, diaphragm, heart, and liver for GAA activity and glycogen content analyses. A transgene integration assay and next-generation evaluated genome editing. Muscle function testing revealed that mice treated with the Donor vector had improved performance, in comparison with vehicle treated mice. The GAA activity assay revealed a significant increase in the skeletal muscles of Donor and Donor + CRISPR vector groups to greater than wildtype levels in both the 12-week and 60-week groups, indicating stable expression of the Donor vector in the heart and skeletal muscle for >1 year. However, liver GAA activity was lower than wild-type levels, indicating that muscle expression was therapeutic. Donor vector DNA was quantified, revealing stable quantities of vector DNA in the heart and decreasing vector DNA in the liver. No significant differences in GAA activity were observed between the Donor and Donor+CRISPR groups. Glycogen content was significantly decreased in skeletal muscles and in the heart at 12 and 60 weeks. The integration assay revealed that CRISPR vector treated groups had a very low level of transgene integration at 12 weeks. Unexpectedly, treatment with the Donor vector alone revealed very low-level transgene integration at 60 weeks. The CRISPR vector had nuclease activity in transfected cells; however, next generation sequencing did not detect indels in treated mouse liver or heart even for the high dose CRISPR group, indicating a lack of nuclease activity in vivo. Anti-Cas9 antibodies were not increased, indicating a lack of immune responses. Thus, AAV vectors can transduce skeletal muscles, diaphragm, and heart, stably correcting GAA deficiency for greater than one year, in absence of genome editing. Transduction of non-dividing striated muscle early in life can stably treat PD, and should be considered for the treatment of infantile-onset PD.



405. Epigenomic Fingerprinting of Primary Immune Cells Defines Unique Cell Identities

Andrea L. Johnstone[1], Matthew R. Marunde[1], Danielle N. Maryanski[1], Carolina P. Lin[1], Ellen N. Weinzapfel[1], Liz Albertorio-Saez[1], Dughan J. Ahimovic[2], Michael J. Bale[2], Juliana J. Lee[3], Michael-Christopher Keogh[1], Immunological Genome Consortium (ImmGen)[3]

[1]EpiCypher, Durham, NC,[2]Weill Cornell Medicine, New York, NY,[3]Harvard Medical School, Boston, MA

Understanding immune cell differentiation is central to cell and gene therapy research. Regulation of chromatin structure drives many of these processes, leading to the widespread application of chromatin accessibility (ATAC-seq) and transcriptional (RNA-seq) profiling for immune cell characterization. However, these assays often fail to provide mechanistic insight and can lack the granular detail required to define rare and/or novel immune cells (e.g. exhausted T cells). Epigenomic features such as histone post-translational modifications (PTMs) and chromatin-associated proteins (e.g. transcription factors) mark distinct genomic compartments and regulate chromatin structure, gene expression, and cell function. Mapping these features provides a rich context to study cell fate and has great potential for discovering new biomarkers and drug targets. To better understand gene regulatory mechanisms and cell differentiation in the immune system, we aimed to build a comprehensive database consisting of reference epigenomic maps of over 100 unique primary mouse immune cells. Towards this end, we describe the generation of >2,200 epigenomic profiles from seven unique chromatin features (CTCF binding and histone PTMs associated with poised & active enhancers, active promoters, active gene bodies and repressed genes) in a limited number of FACS-sorted mouse immune cells using an automated CUT&RUN approach. By using defined nucleosome spike-in controls to confirm histone PTM antibody specificity and control for overall sample quality / assay success, the resulting datasets represent a gold-standard resource for the field. Bioinformatic analysis shows that chromatin features define unique cell identities, providing future avenues of research for applying these insights in novel cell & gene therapy approaches. Further, this high-throughput epigenomic mapping approach can be applied to address other challenges in cell & gene therapy research, including understanding gene regulation in differentiating iPSCs, mechanisms of epigenetic editors (e.g. dCas / CRISPRoff), defining the impact of chromatin states on gene editing efficiency, mechanisms of CAR T cell expansion, T cell exhaustion, and more.

406. Characterizing Insertional Oncogenic Risk of a Novel CD8-Targeted Fusosome for *In Vivo* CAR T Cell Generation

Matthew McNeill, Jeremy Kinder, Vasily Vagin, Amanda Lamoureux, Adam Johnson, Jason Lih, Nicolas Devos, Kutlu Elpek, Edward J. Rebar, Jagesh Shah, Terry J. Fry, David Shivak, Christina Chaivorapol

Sana Biotechnology, South San Francisco, CA

Introduction: *In vivo* CAR T cell therapies may offer key advantages over *ex vivo* therapies including reduction in manufacturing cost and time, broader patient access, and treatment durability. However, *in vivo* gene therapies require high on-target cell-specific delivery and sufficient transgene transduction to ensure product safety and efficacy. To overcome these challenges, Sana has tested SG299 fusosome, a self-inactivating lentiviral vector (LVV) pseudotyped with a CD8-targeting fusogen that enables CD8 T cell-specific delivery and integration of a CD19 CAR transgene *in vivo*. While SG299 uses the same LVV integration machinery as those in *ex vivo* CAR T cell therapies conventionally transduced with VSV-G pseudotyped LVV ("VSVG-LVV"), here we aim to assess insertional oncogenic risk by demonstrating that the genome-wide integration site (IS) profile of LVV in T cells from 6 donors is comparable to those observed in *ex vivo* clinical therapies. **Methods:** Activated CD8 T cells were transduced with SG299 or VSVG-LVV at 4 doses and harvested 4 days later. We evaluated vector copy number (VCN) per diploid genome (dg) and number of CAR+ cells. To gauge insertional oncogenic risk, we compared the IS profiles at 1 selected dose for preferential integration of SG299 LVV genome-wide and near or within known oncogenes. A specific dose was selected to enable a sufficient number of IS for analysis (> 10k IS per donor) while keeping VCN at < 5 copies to align with FDA guidance for CAR T therapies and companion *in vivo* studies. **Results:** At the selected dose, transduction of CD8 T cells yielded low intra-donor variability with highly correlated VCN and CAR+ cells across donors and vectors (VCN, $R^2 > 0.96$; CAR expression, $R^2 > 0.75$). Across donors, VCN was > 1.4 copies/dg and percent of CAR+ cells were > 33.8% (33.8 - 64.6% for SG299, 53.5 - 84.4% for VSVG-LVV) demonstrating sufficient transduction of CD8 T cells for IS analysis. Our assessment of insertional oncogenic risk yielded no evidence of preferential integration of SG299 LVV near or within 738 Cancer Gene Census (CGC) genes when compared with our VSVG-LVV data or Nobles et al. data from pre-infused cells collected from 58 patients across 4 *ex vivo* CAR T trials (all comparisons, $p < 0.01$). In addition, a genome-wide IS analysis did not identify *bona fide* preferential integration in any donor. While T cells from 2 donors had one nonoverlapping region on chr19 statistically enriched for SG299 LVV (FDR < 0.05), these regions were large (> 340 kbp), > 50 kbp from the nearest CGC gene, and nonrecurrent. Manual examination of the IS profile in this region showed that the enrichment may be due to artifacts and that the distributions across donors and LVVs were comparable. **Conclusions:** *In vitro* transduction of T cells with SG299 and VSGV-LVV resulted in comparable CAR transduction as measured by VCN and CAR expression, lack of recurrent evidence for preferential SG299 LVV integration across the genome, and comparable IS profiles to VSVG-LVV used in autologous therapies. These findings suggest SG299 likely carries comparable insertional oncogenic risk to *ex vivo* clinical CAR T products. While FDA recently announced an evaluation of T cell malignancy risk in patients treated with autologous therapies, Levine et al. indicate that the benefit of these CAR T therapies outweighs the risks given the low incidence of T cell malignancies (< 0.05%) and even lower numbers of CAR+ associated lymphomas. Given this, the IS profile of VSVG-LVV remains a reasonable comparator for oncogenic risk assessment. Together with prior studies demonstrating high specificity of SG299 (Shah ASGCT 2023, Chavan ASH 2023), these data demonstrate a desirable potency, specificity, and genomic safety profile that warrants continued development of the fusosome platform to generate CAR T therapies.

407. Somatic Mutation Tracking in Hematopoietic Stem Cell Gene Therapy Reveals Absence of Clonal Hematopoiesis

Francesco Gazzo[1,2], Andrea Calabria[1], Fabrizio Benedicenti[1], Maria Rosa Lidonnici[1], Samantha Scaramuzza[1], Francesca Tucci[1,3], Fabiola De Mattia[1], Sarah Marktel[1,3], Valeria Calbi[1,3], Francesca Fumagalli[1,3], Fabio Ciceri[3], Luigi M. Naldini[1,4], Alessandro Aiuti[1,3,4], Giuliana Ferrari[1,4], Eugenio Montini[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]Department of Electronics, Information and Bioengineering, Politecnico di Milano, Milan, Italy,[3]Pediatric Immunohematology and BMT, San Raffaele Hospital, Milan, Italy,[4]Vita Salute San Raffaele University, Milan, Italy

In hematopoietic stem cell Gene Therapy (GT), patients' Hematopoietic Stem and Progenitor Cells (HSPCs) are genetically corrected *ex vivo* and reinfused to reconstitute the entire hematopoietic system and provide therapeutic benefit. During this process, HSPCs are subjected to tremendous pressures to sustain high levels of proliferation until the hematopoietic reconstitution is complete. So far it is unknown if this likely stressful condition may result in the accumulation of somatic mutations which may trigger decay of the hematopoietic functions and increased risk of oncogenesis. The impact of prolonged and heightened HSPCs proliferation rates on cellular fitness and safety remains an open question in GT. Indeed, HSPCs are not comprehensively geno-protected from DNA damage accumulation in HSPCs during aging and/or in specific disease conditions as it has been observed in sickle cell disease and Fanconi Anemia.Here we performed an analysis of somatic mutations in exons of 40 genes involved in clonal hematopoiesis and myeloid cancer in 23 GT patients treated for metachromatic leukodystrophy (MLD, 15 <3 years old, 8 3-12 years old) and 9 for β-thalassemia (β-Thal, 6 pediatric and 3 adults >30 years old). We used genomic DNA from HSPCs cells prior infusion and peripheral blood mononuclear cells harvested at 2 years after GT and at the last available time point (2.5-7.5 years after GT). Sequencing reads correctly aligned on the targeted exon panel resulted in an average coverage of 4,400 and 4,300 reads/base in β-Thal and MLD patients respectively.The average mutation rate in the adult β-Thal patients was >2 fold higher than the rate measured in the pediatric patients (11.3 ± 11 vs 5.6 ± 3.5). Moreover, the average somatic mutation rates in adult and pediatric β-Thal patients were both significantly higher than in MLD patients (1.6 ± 0.72, p-value=0.0136 vs adult β-Thal, p-value=0.012 vs pediatric β-Thal, by Kruskal-Wallis test). None of the mutations were pathological or likely pathological. Most somatic mutations (85 out of 96) exhibited a Variant Allele Frequency of less than 2%. The average number of mutations in both the clinical trials remained consistent across all time points, showing no statistically significant variations. Considering that the sequenced genomic interval corresponds to 76,715 bps and that we analyzed a total of 8,100 equivalent genomes per patient, the resulting mutation rate in β-Thal patients was $1.21x10^{-8}$ mutations/bp, while MLD patients resulted in a mutation rate of $2.6x10^{-9}$ mutations/bp. Five mutations (4 in β-Thal and 1 in MLD) were found at more than one time point, but none showed a progressive increase in abundance, suggesting that these mutations did not confer a selective advantage to the mutated cell clones.Our work revealed that no somatic mutations known to drive clonal hematopoiesis or myeloid cancer, nor accumulation of somatic mutations, were found in our GT patients, indicating that the GT treatment was neutral in these conditions. However, the underlying disease in β-Thal patients resulted in a significantly higher mutation burden than MLD patients, a finding worth of deeper analysis and that supports long-term follow-up assessments of the clonal composition of the hematopoietic system in GT patients.

Vector Product Engineering, Development, and Manufacturing (excluding AAV)

408. Single and Low Dose Self-Replicating RNA Vaccine Provides Effective Immune Protection Against Rabies in Healthy Volunteers

Christian Maine[1], Shigeki Miyake-Stoner[1], Brandon Essink[2], Guillermo Somodevilla[3], Yaquelin Rodriguez[3], Andrew Geall[1], Nathaniel Wang[1], Parinaz Aliahmad[1], Zelanna Goldberg[1]

[1]Replicate Bioscience Inc, San Diego, CA,[2]Velocity Clinical Research, Omaha, NE,[3]Cordova Research Institute, Miami, FL

Despite the unprecedented success of mRNA vaccines for COVID-19, their widespread expansion to other infectious diseases is limited by unacceptable side effects at doses required for protection. Self-replicating RNA (srRNA) technology is a fully synthetic RNA modality that is able to amplify in the host cell due to the presence of a virally derived replication machinery. Thus, lower amounts of srRNA lead to exponential protein expression, which enables lower clinical dosing and better tolerated vaccines. Replicate Bioscience has developed novel, proprietary srRNA vectors with improved performance for a variety of therapeutic applications. To demonstrate the superior performance of our technology, we designed and manufactured RBI-4000, a proprietary srRNA vector encoding the rabies glycoprotein encapsulated in a lipid nanoparticle. Rabies was selected as a target for this clinical validation as the general population is exposure-naïve, in contrast to influenza or SARS-CoV-2 where pre-existing exposure may confound a clean interpretation of the vaccine-generated immune responses. Additionally, an easy to interpret immune surrogate of protection has been defined by the WHO, where titers of rabies virus neutralizing antibodies (RVNA) ≥ 0.5 IU/mL are protective. RBI-4000 was evaluated in a Phase I clinical trial (NCT06048770). Healthy adults, age 18-45 and seronegative for rabies antibodies were recruited at two clinical sites in the U.S. Sequential cohorts (n=18 subjects) were dosed IM with 0.1, 1, or 10 micrograms (mcg) of RBI-4000 in a 2-dose prime-boost schedule or prime only for the highest dose. An active control cohort of 12 subjects received RabAvert, a commercial vaccine, per the recommended schedule. Enrollment is complete, and the study is ongoing. Interim analysis demonstrated that all doses were well tolerated, with no DLTs, SAEs, or safety signals. Acute reactogenicity was limited to grade 1 or 2 events, with a single exception of a time-limited grade 3 fever reported by 1 subject in the mid dose group, at

the prime dose only. Acute reactions generally resolved within 3-4 days post-injection with no treatment or limited use of OTC oral NSAIDs. Local reactogenic events were more common than systemic and increased in a dose-dependent manner. RBI-4000 was immunogenic at all dose levels tested, with RVNA achieving WHO-defined protection levels in over 70% of subjects at the 0.1 mcg dose after two doses. At 10 mcg, protective RVNA titers were observed at close to 100% following a single dose. RBI-4000 is the first clinical demonstration of next-generation srRNA technology. The favorable safety profile combined with strong immunogenicity demonstrates an orders of magnitude advance in the therapeutic window of efficacy: tolerability over traditional mRNA vaccines. The increased therapeutic window enables broader applications of our platform to more complex infectious disease targets, oncology applications, and protein replacement therapeutics as well as being better able to address acute pandemic manufacturing requirements.

409. Kinetics-Based Assembly of Shelf-Stable PEI/DNA Particles for Viral Vector Production

Jinghan Lin[1], Yizong Hu[2], Di Yu[1], Hai-Quan Mao[1]

[1]Johns Hopkins University, Baltimore, MD,[2]Massachusetts Institute of Technology, Boston, MA

Introduction. Current methods for producing adeno-associated virus (AAV) vectors predominantly involve the transfection of plasmid DNA (pDNA) and poly(ethyleneimine) (PEI) particles into HEK293 cells. The quality of the pDNA/PEI particles plays a crucial role in achieving efficient AAV vector production, which is primarily determined by the initial mixing of pDNA and PEI solutions. However, precisely controlling the assembly of pDNA/PEI complexes in a homogenous manner has been challenging, risking the reproducibility of AAV production[1]. In this study, we developed a kinetics-based approach to assemble shelf-stable pDNA/PEI particles at controlled size ranging from 200 to 1000 nm with a high degree of uniformity. Through this approach, the assembly is extended, rendering the mixing process less time-sensitive and allowing for increased flexibility in the mixing process. **Methods.** To formulate pDNA/PEI nanoparticles, pDNA and PEI were pre-diluted into different buffers. The buffers were designed to a specific pH and ionic strength to control the assembly of pDNA/PEI nanoparticles to larger sizes. The DNA and PEI working solution were then mixed to initiate an instantaneous assembly. The size growth of the nanoparticles was stopped by adding a stabilization buffer to the total pDNA/PEI solution. The transfection efficiency of these particles was assessed by examining luciferase pDNA expression in HEK293T and HEK293F cells. **Results.** This kinetics-based method generated pDNA/PEI particles with good uniformity regardless of mixing rates from different complexation methods. The size control was achieved by tailoring pH and ionic strength of the mixture. Specifically, citrate ions were found to facilitate the growth of the pDNA/PEI particles with a lower needed concentration than phosphate buffer to induce similar growth rate. It also improved the uniformity of the particles, indicated by a much lower polydispersity (**Fig. 1A**). The assembled pDNA/PEI particles remained stable on the bench for at least 4 h at room temperature and after freeze-thaw cycles, indicated by the stable sizes of the particles as well as comparable PDI values (**Fig. 1B**). In addition, transfection efficiency of the pDNA/PEI nanoparticles prepared by this method was tested on HEK293T and HEK293F cell lines. The luciferase expression levels in both cell lines were increased with the larger size particles, with the peak transfection efficiency obtained at 600 nm (**Fig. 1C**), suggesting that the transfection efficiency of pDNA/PEI particles can be tailored in a size-dependent manner. **Conclusions.** This kinetics-based assembly method for synthesis of pDNA/PEI particles offers distinct advantages such as high DNA concentration, controllable assembly kinetics, high scalability, and high shelf stability and storage stability upon freeze-thaw cycle. The final DNA concentration in such pDNA/PEI particles can be tuned up to 200 μg/mL of pDNA. This method also eliminates the need for specific mixing equipment, as the assembly of pDNA/PEI particles was primarily controlled by assembly buffers with specific pH and ionic strength. The particles can be stabilized at different desired sizes, remaining stable both on benchtop and cryopreserved at -80 °C. These controlled-sized pDNA/PEI particles exhibit efficient transfection capabilities, indicating their great potential as an effective delivery system for viral vector production. **References.** [1] Y. Hu, *et al.*. ACS Nano, 13(9): 10161-10178 (2019).

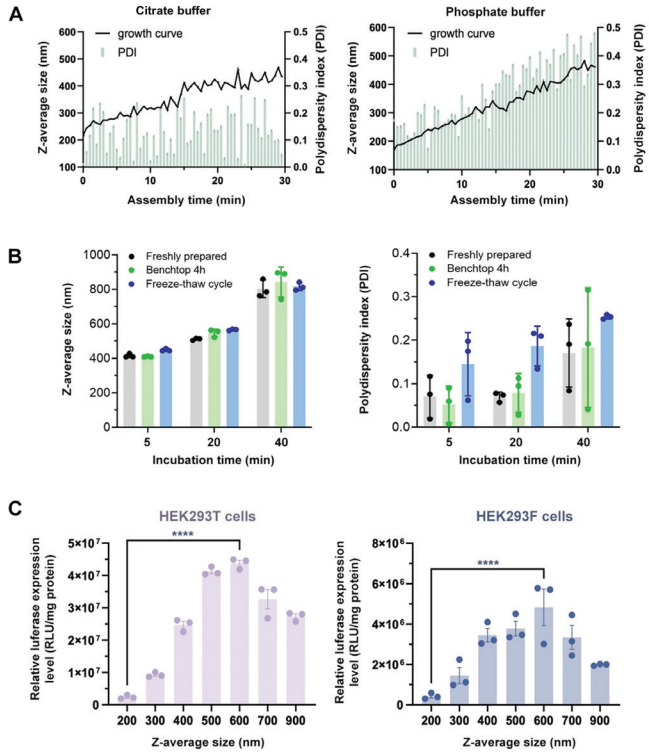


Figure 1. (A) Assembly of pDNA/PEI particles using citrate buffer or phosphate buffer. (B) Z-average particle size and PDI of pDNA/PEI particles for freshly prepared pDNA/PEI particles or stored on the benchtop for 4 h at room temperature or after one freeze-thaw cycle after mixing with cell culture medium for 5, 20, and 40 min. (C) Luciferase transfection efficiency of different size pDNA/PEI particles in HEK293T adherent cells at 24 h or HEK293F suspension cells at 48 h after transfection.

410. Machine-Designed Synthetic 3' UTRs Significantly Increase mRNA Stability

Elise D. Flynn[1,2], Görkem Garipler[1], Emily Hoelzli[1], Uri Laserson[1], JB Michel[1], Alyssa Morrow[1], Meimei Shan[1], Ashley Thornal[1]

[1]Patch Biosciences, New York, NY,[2]Authors are displayed alphabetically, New York, NY

Using mRNA as a therapeutic has received enormous attention in the last few years, but the inherent instability of the molecule remains a

hurdle to achieving long-lasting therapeutic levels of protein expression. We have engineered synthetic 3' UTR sequences that greatly increase mRNA stability, resulting in prolonged protein expression *in vivo* for potential use in mRNA therapeutics. **Approach**: Our approach involves a massively parallel reporter assay (MPRA) that measures the half-life of tens of thousands of distinct mRNA designs in a single experiment. This data allows us to train large machine learning models, which guide the design of synthetic 3' UTR sequences predicted to enhance mRNA stability. By synthesizing and testing large pools of candidate 3' UTRs, we continuously refine our machine learning models. Through multiple design-build-test iterations, our platform identifies numerous 3' UTR sequences with improved mRNA stability. **Results**: In total, we have evaluated over 200,000 unique 3' UTRs across various cellular contexts with half-lives up to 20 hours. Several selected 3' UTR sequences were validated by formulating them as mRNA-LNPs and injecting them systemically into mice. Whole-body bioluminescence imaging at multiple timepoints over a two-week period confirmed that by four to seven days after the injection, our 3' UTRs were driving 30-100-fold higher protein output compared to the human hemoglobin beta 3' UTR sequence, a commonly used benchmark. Furthermore, our UTRs extended the threshold levels of protein expression by up to an additional 6 days. Notably, these 3' UTRs demonstrated successful translation across different species, cell types, delivery modalities, and associated coding sequences (GFP and luciferase). We believe that our 3' UTRs, along with our sequence discovery platform, have the potential to significantly enhance a wide range of mRNA therapeutics.

411. Generation of Off-the-Shelf CAR Gamma Delta T Cells Using CRISPR and AAV, a New Era in Cell Therapy

Genesis Snyder[1], Alexia Martin[1], Noushin Saljoughian Esfahani[1], Colin Maguire[1], Dean A. Lee[2], Meisam Naeimi Kararoudi[3]

[1]Nationwide Children's Hospital, Columbus, OH,[2]Nationwide Children's Hospital, Canal Winchester, OH,[3]Nationwide Children's Hospital and Ohio State University, Columbus, OH

Introduction Autologous alpha/beta (αβ) chimeric antigen receptor (CAR) T cells have been successful in treating hematologic malignancies. However, production of these cells is expensive and has led to a high rate of production failure. On the other hand, using donor αβ T cells causes lethal graft versus host disease, thus limiting their accessibility. Here we propose to genetically engineer Gamma Delta (γδ) T cells as an alternative source of T cells to increase their accessibility while providing a potent off-the-shelf CART therapy which does not cause GvHD. However, gene editing of γδT cells has been challenging and to the best of our knowledge there is no report on CRISPR/AAV gene editing of these cells. Given biological similarities between NK cells and γδT cells and our extensive experience with genome editing in NK cells, we hypothesized that growing γδT cells on feeder cells expressing mbIL21 and 41bbL enhances the DNA repair machineries. This would allow efficient CRISPR gene targeting, and site directed CAR insertion delivered by AAV vectors in γδT cells. This is particularly important since the efficacy of αβCAR-T cells generated by lentiviral transduction, has been correlated with random insertion site of the CAR DNA into the genome of T cells. Utilizing a site directed CAR gene insertion via CRISPR and AAV would minimize random insertion of the CAR. To test our hypothesis, we expanded peripheral blood primary γδT cells and electroporated them with Cas9/RNP targeting CD38 and CD70. The electroporated cells were transduced with CD38CAR and CD70CAR constructs delivered by AAV6. The CD38KO/CD38CAR γδT and CD70KO/CD70CAR γδT could overcome CAR-mediated fratricide and showed enhanced antitumor activity against hematological and solid tumors. The expanded γδCAR-T cells could be expanded and cryopreserved to clinically relevant numbers. We believe that this technology would offer an unlimited source of adaptive cell therapies for cancer and potentially other diseases such as autoimmune disorders. *Methods* Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coats (n=4). γδT cells were isolated from the PBMCs utilizing StemCells EasySep kit. Following their isolation, the cells were expanded using irradiated feeder cells (mbIL21 and mb41BBL expressing K562) and kept in culture medium that was supplemented with 100IU of IL2 every 48 hours. Expansion was repeated every 7 days. On day 21 of expansion, γδT cells were electroporated with Cas9/RNP and transduced with AAV6 (75K MOI) delivering CAR DNAs equipped with homology arms for the targeting sites. To confirm the KO and CAR expression, 2 days post transduction, flow cytometry was performed. The γδCAR-T cells were further expanded for three weeks. Calcein and InCucyte were used to measure cytotoxicity against the T-ALL, glioma and neuroblastomas. *Results* Our results showed successful proliferation of both freshly isolated and genetically modified γδT cells to clinically relevant numbers. Flow cytometry was able to confirm the loss of CD38 expression (efficiency was 99.4% ± 0.5% (mean ± SD, n=2)) CD 70 (efficiency was 97.7% ± 0.5%, n=3) and the expression of our CD38CAR construct (efficiency was 65.5% ± 10.2%, n=2) and CD70CAR (efficiency was 50.2% ± 10%, n=3). Both Calcein assay and InCycute showed enhanced killing efficiency of the CAR γδT cells. *Conclusion* We believe that our current strategy provides a method for generating novel, allogenic, off-the-shelf CART cell therapies that are accessible, economic and versatile for several cancer types.

412. Lentiviral Vector Packaging and Producer Cell Lines Yield Titres Equivalent to the Industry-Standard Four-Plasmid Process

Matthew Tridgett[1], Marie Mulet[1], Sherin Parokkaran Johny[1], Maria Ababi[1], Meenakshi Raghunath[1], Richard Parker-Manuel[1], Matthew Peckett[1], Jade Kent[1], Louise Holland[1], Robert Asatryan[1], Louise Montgomery[1], Tsz Lung Chow[1], Ryan Beveridge[1], Ieva Salkauskaite[1], Sarah Dowding[1], Heloísa Berti Gabriel[1], Maolin Li[2], Dan Yi[3], Ping Zou[4], Corinne Branciaroli[1], Weimin Valenti[1,5], David Chang[1,5], Keith Meaney[1], Maria I. Patrício[1], Qian Liu[1]

[1]OXGENE, Oxford, United Kingdom,[2]WuXi Advanced Therapies, Wuxi, China,[3]WuXi Advanced Therapies, Shanghai, China,[4]WuXi Advanced Therapies, Wuxi, United Kingdom,[5]WuXi Advanced Therapies, Philadelphia, PA

Lentiviral vectors are widely used in cell and gene therapies. However, large-scale plasmid production, testing and transfection all complicate and increase the cost of this therapeutic modality. To address this challenge, we have developed clonal lentiviral vector packaging and

producer cell lines by stable integration of lentiviral genetic constructs. This reduces or eliminates plasmid transfection during vector manufacturing. Flask-scale production testing with various therapeutic cargo genes - including anti-CD19 CAR, anti-BCMA CAR and Factor VIII - revealed consistency in the performance of the packaging and producer cell lines compared to the industry-standard four-plasmid transfection method. For example, production titres when encoding anti-CD19 CAR were 2.2 $\times 10^7$ TU/mL with the transient system, 7.2 $\times 10^7$ TU/mL with the packaging cell line, and 7.6 $\times 10^7$ TU/mL with the producer cell line. Production by clonal stable cell lines remained high for three months of continuous culture. Moreover, the producer cell lines remained free of replication-competent lentivirus throughout stability testing, highlighting the safety of this production approach. In addition, we have scaled up production with the clonal packaging cell line to ten litres and have plans to scale up further to fifty-litre bioreactor scale. Finally, we have created a GMP-grade master cell bank of the clonal packaging cell line. By decreasing the dependence on plasmid transfection, while achieving lentiviral vector production consistent with the industry-standard four-plasmid method, the cell lines developed here can reduce manufacturing costs and operational risk, thus improving manufacturability and enabling patient access to lentiviral vector-based cell and gene therapies.

413. Establishment of a Scalable Stable Lentivirus Manufacturing Process in Suspension Perfusion Mode

Holger Laux[1], Maximilian Klimpel[1], Nikki Indresh Lal[1], Marta Arrizabalagacascallana[2], Vicky Pirzas[1]

[1]CSL Behring Innovation GmbH, Marburg, Germany,[2]UCL, London, United Kingdom

Relevance to Theme Selected: Enabling lentivirus manufacturing in suspension perfusion mode for viral-based gene therapies using stable producer cell lines. **Impact/Novelty:** Implementation of a scalable lentiviral vector perfusion process using an ultrasonic cell separation system with increased perfusion rates. **Introduction:** Production of lentiviral vectors for clinical gene transfer trials is currently mainly done via transient expression. This is challenging due to the high cost of GMP-grade plasmids, the labour-intensive nature of a transient process as well as the lower reproducibility and scalability. To facilitate clinical grade lentiviral vector production, we have developed a state-of-the-art stable suspension packaging cell lines in the past. We have now significantly improved the suspension perfusion process using an ultrasonic cell separation system with optimized perfusion conditions and developed an efficient tailor-made DSP process to purify and sterile filter LVVs. **Methods/Approach:** We have established a HT small scale perfusion system (AMBR250) using an ultrasonic cell separation equipment. With this system we have performed a variety of suspension perfusion runs to identify the best conditions, which were confirmed at 2 L bioreactor scale and the LVV material was used to improve the LVV downstream process (purification, concentration and sterile filtration). **Results:** The producer cell lines are expressing high infectious titer. Conditions have been optimized to improve LVV stability, maintain high cell viability for at least 2 weeks, and increase the ratio of infectious to non-infectious particles. We have also identified more gentle culture conditions using an AMBR250 perfusion system with an ultrasonic cell retention device. **Conclusion:** Scalable production of LVVs for commercial and clinical use is a hurdle due to traditional transient production methods. We show in this work that we are capable to express and purify LVVs at high infectious titer over a period of 2 weeks using stable suspension producer cell lines and have significantly improved the process using state of the art HT perfusion systems.

414. Stable Lentiviral Producer and Packaging Cell Lines Achieve > 1E8 TU/mL

Adam Carcella, Jeremy Gam, Brianna Jayanthi, Michelle Chang, Omar El-Ghouch, Paulena Lieske, Manali Desai, Jeff McMahan, Luis Gonzalez, Kevin D. Smith, Martín Carcamo

Asimov, Boston, MA

Chimeric Antigen Receptor T-cell (CAR-T) therapy is one of the most successful types of immunotherapy to date. This success has led to ex vivo engineered cell therapies broadening to larger indications, such as solid tumors, increasing the need for scalable technologies. Lentiviral vectors have emerged as the dominant tool of choice to genetically modify immune cells due to their capacity to transduce both dividing and non-dividing cells, ensuring long-term stable transgene expression with a reliable safety profile. The current industry standard for lentiviral production relies predominantly on transient transfection of HEK293 cells which has issues with scalability, batch variability, and a high manufacturing cost largely due to the large-scale GMP plasmids required. To address these inherent shortfalls of transient transfection-based lentiviral production without sacrificing the speed to clinical and commercial stages, Asimov has developed an inducible stable packaging and producer cell line system.Asimov's LV Edge platform consists of both packaging and producer monoclonal suspension HEK293T-based, inducible cell lines encoding all the genetic elements needed for VSV-G pseudotyped lentiviral vector production. The system integrates synthetic biology with hyperactive transposase-based genomic integration to attain high copy numbers of lentiviral and transgene components under stringent inducible regulation. Both cell lines are generated through a cell line development (CLD) process with best-in-class monoclonality assurance technology, ensuring robust and reproducible performance. Our packaging cell line is a ready-to-transfer platform with stably integrated essential viral genes, permitting lentiviral production from induction of the integrated packaging components and a single-plasmid transfection encoding the transgene of interest. Generation of a producer cell line takes 4 months from transfection to research cell bank availability. Our CLD process eliminates the need for the costly GMP-grade DNA generation, and uses a novel, high-throughput clone screening approach to only progress the top performing clones. Put together, our model-guided process achieves high-E8 TU/mL titers at harvest from producer cell lines and greater than 1E8 TU/mL titers from our off-the-shelf, ready-to-transfect packaging cell line across multiple therapeutic transgenes with commercially available media and feeds. Both the packaging and producer HEK293T cell lines exhibit robust growth profiles and high viability, similar to the parental cell line, after the insertion of the lentiviral components. In addition, long-term stability studies have shown the packaging and producer cell lines to be stable without antibiotic selection. In conclusion, Asimov's LV Edge platform has the potential to shift the industry standard beyond

the current four-plasmid transient transfection system toward a more scalable and cost-effective approach, making engineered cell therapies more accessible to patients.

Targeted Gene and Cell Therapy II

415. KRAS Mutant Gene Editing Abolishes Tumor Growth *In Vivo* and Overcomes Acquired Resistance to KRAS^{G12C} and KRAS^{G12D} Small Molecule Inhibitors

Neil Desai[1], Audrey Grunenberger[2], Veronica Guzman-Gonzalez[2], Léa Cabrera[2], Elodie Czuba[2], Virginie Faure[3], Veronique Josserand[3], Gilles Di Vita[1,2]

[1]AADIGEN, Pacific Palisades, CA,[2]DIVINCELL, Nimes, France,[3]IAB, Grenoble, France

KRAS codon12 mutations are among the most commonly observed mutations in pancreatic, colorectal and lung cancers. Recently, KRAS^{G12C} inhibitors (sotorasib,adagrasib) have been approved. However, small molecule KRAS inhibitors are limited by adaptive resistance, mainly associated with the emergence of secondary KRAS mutations, activation of feedback pathway and high amplification of KRAS^{G12C} allele. KRAS^{G12D} inhibitors (MTRX-1133 and INCB161734) have recently entered clinical development. We have developed a potent strategy combining in vivo gene editing with tumor-selective peptide nanoparticles, to impair cancer cell proliferation by directly targeting mutations at codon-12 of KRAS oncogene and to overcome acquired resistance to KRAS^{G12C} and KRAS^{G12D} inhibitors. ADGN-121, ADGN-122, ADGN-123 are gene-editing complexes containing mRNACAS9 and sgRNA targeting specifically KRAS^{G12D}, KRAS^{G12V} or KRAS^{G12C} respectively, complexed with proprietary peptide-based nanoparticles to promote tumor targeted delivery upon systemic administration. We demonstrated that ADGN-121,122 and 123 selectively silenced KRAS^{G12D}, KRAS^{G12V} and KRAS^{G12C}, respectively, in colorectal, pancreatic and lung cancer cells resulting in inhibition of cell proliferation (IC50:10-30 nM) and phosphorylation of ERK and AKT. We showed that only two IV-administrations of ADGN-121 containing gRNA^{G12D} are required to abolish Panc1 tumor growth in a dose dependent manner with strong tumor regression at 1.0mg/kg. Similarly, only two IV administrations of ADGN-122 containing gRNA^{G12V} abolished SW403 tumor growth with strong tumor regression at 1.0 mg/kg. We demonstrated a synergistic combination between ADGN-121/Abraxane in pancreatic carcinoma and between ADGN-122/Capecitabine in colon Adenocarcinoma. No effect on tumor growth was observed with nonspecific gRNA. ADGN treatments were well tolerated, with no signs of clinical toxicity detected after single or repeat administration. ADGN-123 containing gRNA^{G12C} can overcome sotorasib/adagrasib acquired resistance in vitro. ADGN-123 blocks the proliferation (IC50 : 10-20 nM) of cells with secondary KRAS$^{G12C/R68M}$ and KRAS$^{G12C/Y96D}$ mutations or with KRAS in a permanent active state and effectively inhibits ERK phosphorylation. ADGN-121 containing gRNA^{G12D}/mRNACas9 can overcome MRTX1133 resistance in vitro. ADGN-121 can effectively inhibit the proliferation (IC50 : 10-15 nM) of ASPC-1 MRTX-1133R and PANC-1 MRTX-1133R cells with high level of KRAS^{G12D}-GTP active state. No resistance was detected after ADGN-123 or ADGN-121 treatments. Our study provides a proof-of-concept that ADGN can be applied to target driver mutations of cancers *in vivo* and permanently disrupt the oncogenic alleles, leading to major tumor regression. ADGN-121, and ADGN-122 were effective in invivo targeting selectively the two major KRAS mutations in G12D and G12V, which are present in 4.2% and 3.6% of all cancer patients respectively. Importantly, ADGN-123 and ADGN-121 constitute a potent alternative strategy to overcome resistance associated to small molecule inhibitors of KRAS^{G12C} and KRAS^{G12D}

416. A High-Avidity, CD8-Co-Receptor-Independent, HPV-Specific TCR Coupled with a TGF-β Chimeric Switch Receptor Enhances Polyfunctional Profiles and Anti-Tumor Activities in HPV-16/52-Associated Cancers

Susanne Wilde[1], Tao Jin[2], Nina Kallin[1], Lu Yang[2], Ying-Yin Chao[1], Alexandra Wagner[1], Luis F. Olguín-Contreras[3], Wolfgang Uckert[4], Barbara Wollenberg[5], Ulrike Protzer[3], Yi Sun[2], Jingxian Liu[2], Ke Zhang[4]

[1]SCG Cell Therapy, Planegg, Germany,[2]SCG Cell Therapy, Shanghai, China,[3]Institute of Virology, Technical University Munich, Munich, Germany,[4]SCG Cell Therapy, Singapore, Singapore,[5]Department of Otolaryngology, Head and Neck Surgery, School of Medicine, Technical University Munich, Munich, Germany

Background: Treatment of human papillomavirus (HPV)-associated cancers with TCR-T cells targeting the HPV E7 viral antigen has demonstrated promise in early clinical proof-of-principle studies but was limited by tumor-derived suppressive mechanisms. Using our proprietary GianT™ platform, we selected a natural HLA-A*02:01-restricted TCR from a donor diagnosed with an HPV-positive oropharyngeal squamous tumor via high-throughput TCR isolation and characterization processes. We further combined expression of the HPV-specific TCR with a TGF-β-receptor-4-1BB immunoswitch (HPV TCR-TB) to transform the effect of TGF-β, a key immunosuppressive cytokine, into a co-stimulatory signaling trigger to combat the tumor microenvironment. Methods: T cells were genetically modified with a lentiviral vector encoding an HPV $E7_{11-19}$-specific, HLA-A*02:01-restricted TCR and a switch receptor (TB) composed of the TGF-β receptor II exodomain fused to the endodomain of 4-1BB. HPV-TCR T cells +/- TB were produced in a fully closed and automated system, and were analyzed for TCR expression, functional avidity, TCR recognition motif, CD8-dependency, recognition of different HPV genotypes, target-dependent cytotoxicity, proliferation, cytokine secretion and long-term activity *in vitro* as well as for anti-tumor efficacy *in vivo*. Further, HPV-TCR-TB T cells were analyzed for potential alloreactivity and off-target recognition. Results: HPV-E7-specific T cells displayed a high avidity for the cognate peptide in a nanomolar range. The TCR was functional in both $CD8^+$ and $CD4^+$ T cells without adding the CD8-co-receptor, leading to secretion of inflammatory cytokines IFN-γ and TNF-α and killing of HPV^+ target cells. HPV TCR-TB T cells showed less or no upregulation of pSMAD2/3, maintained a more memory-like population, a lower exhaustion phenotype and sustained their functionality in a TGF-β rich environment. During sequential *in vitro* co-culture assays, TCR-T cells without TB eventually failed to eliminate

tumor cells, while HPV TCR-TB T cells maintained their ability to expand and kill tumor targets in the presence of TGF-β. Further, HPV TCR-TB T cells displayed no alloreactivity against the most common class I HLA molecules covering the Asian, American and European population, no reactivity against similar human self-antigen peptides presented by HLA-A*02:01, and no reactivity against primary or iPSC-derived HLA-A*02:01-positive cells. Moreover, *in vivo* studies showed efficient control of xenogeneic tumor cell growth in different tumor models, including SCC090, CaSki-E7 and HepG2-E7 xenotransplanted CDX mice. HPV TCR-TB T cells, both at low and high doses of 0.7E7 and 2E7 TCR^+ T cells/mouse were able to inhibit the growth of tumors via a single intravenous injection. Expansion of T cells could be detected in the following weeks while no side effects were observed. Conclusions: CD8 co-receptor independent, natural high affinity HPV TCR-TB T cells exhibited high specificity, potency and a favorable safety profile for the treatment of HLA-A*02:01-restricted HPV-16- and HPV-52-positive solid tumors. The TGF-β-receptor-41BB immunoswitch significantly enhanced T cell functionality upon exposure to TGF-β, driving sustained TCR anti-tumor activity. These data support the ongoing clinical investigation in HPV-associated cancers.

417. Hostile Takeover: Diagnosing Cancer from within Using a Cancer-Activated Genetic Construct as a Novel Imaging Platform for NSCLC Detection

Mohammed Goryawala[1], Hung-Yu Henry Lee[1], Ling Tong[2], Trupti Patil[1], Alex Harwig[1], Chloe Xia[1], Suthara Ramachandran[1], Xiaobin Wu[1], Dariusz Wodziak[1], Badriprasad Ananthanarayanan[1], Nadege Morisot[1], Dean W. Felsher[2], Tim Sproul[1], David Suhy[1]

[1]Earli, Inc, Redwood City, CA,[2]Stanford University, Palo Alto, CA

Early detection and localization of primary cancers has been shown to improve clinical outcomes and overall survival. While new screening techniques such as a liquid biopsy may indicate the presence of cancer and possibly provide the tissue of origin, they cannot pinpoint the exact location of cancer and its metastases. Furthermore, current imaging techniques are limited to cancers that endogenously express a certain gene product. Alternate imaging approaches using ^{18}F-FDG rely on increased metabolic rates of tumors vs. surrounding non-tumor tissue, often resulting in high background and inability to detect low-metabolic tumors. Earli is developing a highly sensitive platform that is designed to use systemically administered cancer-activated DNA construct with engineered promoter to usurp dysregulated cancer pathways and force the tumor to produce a synthetic biomarker to detect and localize the malignancy while remaining transcriptionally silent in normal adjacent tissues or benign lesions.EARLI-207 is a DNA nanoplasmid with a cancer-activated promoter to drive the selective expression of a functionally inactivated somatostatin receptor 2 (SSTR2fi) gene product as a cell surface reporter. Abrogation of internalization and signal transduction allows the safe use of SSTR2fi as an ectopically expressed PET reporter gene (PRG). Following formulation in a proprietary lipid nanoparticle (LNP) and intravenous administration, EARLI-207 is designed to preferentially transfect tumors with transcriptional activation within cancer cells resulting in selective cell-surface expression of $SSTR2_{fi}$ that can be imaged with FDA-approved and clinically validated SSTR2 PET tracers such as ^{68}Ga-DOTATATE. For preclinical development, a DNA nanoplasmid (EARLI-206) that drives the selective expression of Firefly Luciferase (FLuc) whose response can be measured using Bioluminescence Imaging (BLI) was also developed.To ascertain the sensitivity of the system, H1299 human lung cancer cells engineered to express 900 mRNA copies of the SSTR2 transgene (H1299-SSTR2) were injected into mice and revealed a discernable PET signal with as little as 31,000 cells. Mixed cell titration studies using subcutaneous (SQ) tumors with H1299-SSTR2 and non-expressing H1299-wt cells suggest tumors with transfection rates between 0.1% and 1% H1299-SSTR2 cells had a significant PET signal, compared to tumors with 100% H1299-wt. Furthermore, intratumoral (IT) dose titration of EARLI-204 (CAG-$SSTR2_{fi}$) reveals that dose levels sufficient to transfect ~1-2% of tumor cells as assessed by IHC results in the transcript production of approximately 300-400 copies of SSTR2 mRNA per cell. We continue to use these parameters as benchmarks to develop of a series of novel LNP formulations for the intravenous delivery of the EARLI-204 DNA nanoplasmid. Finally, IV dosing of EARLI-206 in SQ and intralung (orthotopic) tumor models results in cancer-activated expression of FLuc in both SQ and lung tumors with 100-fold increase ($p < 0.05$) BLI signal as compared to PBS-dosed mice. Ongoing experiments with EARLI-207 aim to show a similar response in the PET system with the expectation that tumors as small as 4 mm will be identifiable. The results demonstrate an effective molecular imaging platform for drug discovery ranging from in vivo screening to building PK-PD-efficacy relationships and enabling human dose projection for the Earli PET localization drug. The benchmarks and findings established with this preclinical imaging pipeline are currently being evaluated in large animals such as dogs and pigs, and will be translated to humans in planned clinical trials.

418. Development of Inhalable Polymeric Nanoemulsions for Delivery of Gene Silencing Therapeutics in Lung Tumors

Kasturi Siddhanta, Ling Ding, David Oupicky

Department of Pharmaceutical Sciences, University of Nebraska Medical Center, Omaha, NE

Lung cancer is one of the most common and deadliest cancers globally, accounting for the highest number of cancer-related deaths, with a 5-year survival rate of around 20%. While anti-PD-L1 monoclonal antibody immunotherapy holds promise in combating various cancers, studies suggest its efficacy may be compromised by factors such as poor lymphocyte infiltration and the emergence of an immunosuppressive microenvironment. RNA interference via siRNA/miRNA presents novel possibilities in gene silencing therapeutics, particularly lung cancer, with inhalation delivery offering a targeted, non-invasive approach to directly address specific genetic factors in the lung. We hypothesized that immune check point inhibition using siPD-L1/miR-34a cocktail delivered via inhalation route can enhance anticancer effects through miRNA's global regulatory role and minimizing systemic exposure. We have developed an emulsion polyplex (EP) as inhalable delivery system for lung cancer treatment, which can deliver si/miRNA encapsulated in PAMD-C@PFOB nanoemulsions (PAMD-C: polymeric AMD3100-Cholesterol, a copolymer for CXCR4 inhibition, PFOB: perfluorooctylbromide). PAMD-C was synthesized by Michael

addition followed by preparation of PAMD-C@PFOB oil-in-water nano emulsions (1% v/v) using ultrasonication. The formulation had a size of 170 nm and zeta potential of +20 mV which enabled successful binding of anionic si/miRNA to the nanoemulsion via electrostatic interactions. Gel retardation assay showed that at w/w ratio ≥ 2, the RNA was completely bound by the nanoemulsions. Effective CXCR4 targeting by PAMD-C@PFOB in U2OS cells (expressing GFP-tagged CXCR4) was confirmed by CXCR4 redistribution assay. Flow cytometry performed on transfected H1975 cells showed 60% double positive populations of labelled RNA and emulsion, indicating their successful uptake in the formulation. Lung metastatic breast cancer model was generated by IV injection of luciferase-tagged 4T1 breast cancer cells in Balb/c mice. Bioluminescent imaging done at 6 h and 24 h after nebulized delivery of the EPs to the mice revealed a five-fold higher lung distribution compared to other major organs (Figure 1). H&E staining confirmed that the EPs carrying anti-PD-L1 siRNA and miR-34a significantly reduced tumor burden. When compared with placebo, the EP treatment also prolonged median survival from 20 to 50 days. Immunofluorescence staining on the resected tumors showed increased CD8+ T cell infiltration, calreticulin exposure, and cytotoxic cytokine production. Flow cytometry showed reduced populations of immunosuppressive cells like Tregs and MDSCs. ELISA tests revealed lower levels of immunosuppressive cytokines like IL-10 and TGF-β upon EP administration. The inhalation platform developed here enables effective pulmonary delivery of si/miRNA to treat lung cancer by improving siRNA internalization, endosomal escape, lung penetration, and biodistribution compared to conventional polyplexes. CXCR4 antagonism and PD-L1 gene silencing was achieved, thus inhibiting important pathways involved in lung cancer pathology. EPs developed in this study remodeled the tumor microenvironment to enhance anti-tumor immune response, achieving significantly longer survival. In conclusion, this proof-of-concept study demonstrates the potential of using PAMD-C@PFOB to improve the efficacy of pulmonary siRNA therapeutics via inhalation delivery.

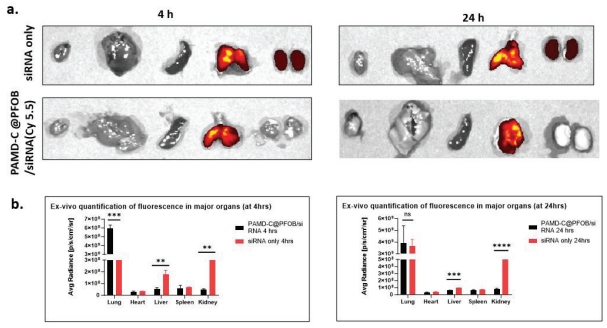


Figure 1. Biodistribution of EPs in 4T1 induced lung tumor model. (a) *Ex vivo* fluorescence imaging of major organs after nebulized administration of Cy3-PAMD-C@PFOB/Cy5.5-siRNA EPs at 4h and 24 h **(b)** *Ex vivo* quantification of fluorescence distribution in major organs at 4 h and 24 h post nebulized administration (n =3 , mean ± SD, Student's t-test). Significance was indicated as *p<0.05, **p<0.01, and ***p<0.001.

419. Discovery of Tumor Reactive TCRs and Their Cognate Antigenic Targets via High-Throughput Functional Screening

Candace Perullo, Jinyu Zhu, Yifan Wang, Kimberly M. Cirelli, Qikai Xu, Alexander Cristofaro, Amy Virbasius, Emily Miga, Rachel Lent, Shoshana M. K. Bloom, Jackson Lirette, Kenneth L. Jahan, Livio Dukaj, Jin He, Ryan Kritzer, Joel Sher, Chandan Pavuluri, Jenna LaBelle, Sida Liao, Cagan Gurer, Gavin MacBeath

TScan Therapeutics, Waltham, MA

Introduction: T cell receptor engineered T-cell therapies (TCR-T) are a promising modality for cancer treatment because unlike CAR-T cells, TCR-T cells can target both intracellular and extracellular tumor antigens. Tumor infiltrating lymphocytes (TILs) can be a source of tumor specific TCRs to fuel target discovery efforts; however, anti-tumor TCRs are rare [1] and most TILs are bystanders specific to other pathogens [2-3]. To identify targets of TILs that are cancer specific with a favorable safety profile, we developed a high-throughput reactivity screening method to identify TCRs from the TIL repertoire that target shared cancer specific antigens. The targets for these tumor reactive TCRs can then be rapidly identified using TScan's proprietary TargetScan platform. Methods: Primary CD8+ T cells were engineered to express a library of 2,439 TCRs derived from the lesions of 13 melanoma patients. The T cell pool was co-cultured with seven melanoma cell lines with patient matched HLAs and a normal cell line control. T cells expressing the activation induced markers CD69 and CD137 were sorted and sequenced to identify the TCRs of the activated T cells. Individual TCRs were selected for validation based on strong enrichment when co-cultured with multiple cell lines that express the same HLA allele. TCRs with confirmed reactivity to multiple cancer cell lines were then screened using TargetScan, wherein their cognate antigens were identified. Results and Conclusions: 95 TCRs were identified in the high-throughput melanoma screen and selected for validation. 48 of these TCRs were validated when tested individually and 24 were screened using TargetScan. 21 out of 24 were successfully deorphanized and found to target therapeutically relevant shared antigens restricted to A*02:01, including MLANA, Tyrosinase, and MAGE-C2. Using melanoma-derived TILs, we have established that the high-throughput reactivity screen to TargetScan pipeline can be used to identify tumor-reactive TCRs against shared tumor antigens and this approach will be expanded to other cancer indications to allow for the discovery of new targets of TCR-T therapy. References: 1.Scheper, W., Kelderman, S., Fanchi, L.F., Linnemann, C., Bendle, G., Rooij, M.A.J. de, Hirt, C., Mezzadra, R., Slagter, M., Dijkstra, K., et al. (2019). Low and variable tumor reactivity of the intratumoral TCR repertoire in human cancers. Nat Med *25*, 89-94. 2.Oliveira, G., Stromhaug, K., Klaeger, S., Kula, T., Frederick, D.T., Le, P.M., Forman, J., Huang, T., Li, S., Zhang, W., et al. (2021). Phenotype, specificity and avidity of antitumour CD8+ T cells in melanoma. Nature *596*, 119-125. 3.Simoni, Y., Becht, E., Fehlings, M., Loh, C.Y., Koo, S.-L., Teng, K.W.W., Yeong, J.P.S., Nahar, R., Zhang, T., Kared, H., et al. (2018). Bystander CD8+ T cells are abundant and phenotypically distinct in human tumour infiltrates. Nature *557*, 575-579

420. CRISPR-Cas9 Genetic Targeting of Metastatic Pancreatic Cancer

Kirsten Bowland[1], Jiaying Lai[2], Selina Shiqing K. Teh[1], Alexis Bennett[1], Alyza Skaist[3], Yan Zhang[3], Elizabeth Thompson[1], Sarah J. Wheelan[4], Ralph H. Hruban[1], Rachel Karchin[2], Christine A. Iacobuzio-Donahue[5], James R. Eshleman[1]

[1]Dept. of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD,[2]Johns Hopkins University, Baltimore, MD,[3]Johns Hopkins University School of Medicine, Baltimore, MD,[4]National Institutes of Health, Bethesda, MD,[5]Memorial Sloan Kettering Cancer Center, New York, NY

Metastatic cancer affects millions of people worldwide annually and is the leading cause of cancer-related deaths. Most patients with metastatic disease are not eligible for surgical resection, and current therapeutic regimens have varying success rates, some with 5-year survival rates below 5%. Here we test the hypothesis that metastatic cancer can be genetically targeted by exploiting somatic single base substitution mutations that occur as part of normal aging prior to transformation. These passenger mutations are targetable because ~10% of them form novel tumor-specific "NGG" protospacer adjacent motif (PAM) sites targetable by CRISPR-Cas9. We studied five patients with pancreatic cancer (PC) who underwent a rapid autopsy to determine areas of evolutionary conservation that were maintained throughout all metastases in each patient's cancer. Using whole genome sequencing validated by high-depth capture sequencing, we found that that 90% of PAM targets were maintained between primary carcinomas and metastases overall. We identified rules that predict PAM loss or retention, where PAMs located in heterozygous regions in the primary tumor can be lost in metastases (private loss of heterozygosity (LOH)), but PAMs occurring in regions of LOH in the primary tumor were universally conserved in metastases. Using multitarget sgRNA which cut at a known number of sites in the human genome, we determined a relationship between the number of CRISPR-Cas9 cuts and growth inhibition in PC cells. We found that 8-10 target sites in non-coding regions were sufficient to induce >95% cancer cell death. Furthermore, only 2-3 targets were required to achieve a comparable level of cell death in the presence of a DNA double strand break inhibitor. Finally, using cocultures of PC cells transduced with sgRNA targeting multiple tumor specific PAMs, we demonstrate selective cell killing of three different PC cell lines. Importantly, deep sequencing of patient-matched normal lymphocytes lacking the tumor-specific PAMs treated with the same sgRNA array exhibited no editing at the targeted loci. This work establishes a bioinformatic platform for the discovery of tumor-specific CRISPR targets in the context of metastatic disease and demonstrates selective killing of cancer cells while sparing the patient's normal cells. These data establish the proof of principle for a gene therapy approach targeting metastatic disease.

421. CRISPR-Cas9 for Selective Targeting of Somatic Mutations in Pancreatic Cancers: A Novel Cancer Gene Therapy Approach

Selina Shiqing K. Teh[1], Kirsten Bowland[1], Alexis Bennett[1], Eitan Halper-Stromberg[1], Alyza Skaist[2], Jingyao Ma[3], Yining Zhu[3], Sarah Wheelan[2], Hai-Quan Mao[3], Nicholas J. Roberts[1], James R. Eshleman[1]

[1]Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD,[2]Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, MD,[3]Institute of NanoBioTechnology, Johns Hopkins University, Baltimore, MD

Introduction Somatic mutations are desirable targets to selectively eliminate cancer, yet most are found within noncoding regions. We have adapted the CRISPR-Cas9 gene editing tool as a novel, cancer-specific killing strategy by targeting the subset of somatic mutations that create protospacer adjacent motifs (PAMs). PAMs evolutionally allow bacterial cells to distinguish between self and non-self DNA for Cas9-induced double strand breaks. We hypothesized that a large number of somatic 5'-NGG-3' PAMs that arose from single base substitutions (SBSs) existed in pancreatic cancers (PCs) and other tumor types (e.g. NCG -> NGG), and these novel PAMs could serve as CRISPR-Cas9 targets for selective cancer cell elimination. **Methods** For method development, whole genome sequencing (WGS) data of paired tumor-normal (T-N) samples from three PC patients were put through PAMfinder, a software designed for somatic PAM discovery, to identify novel PAMs. PAMs with >95% variant allele frequency (VAF) and that produced sgRNAs with minimal off-target activity (based on CRISPOR) were Sanger sequenced. To determine prevalence of somatic PAMs in different tumor types, 591 T-N subtracted variant call files from ICGC were analyzed. To investigate target selectivity and specificity of patient-specific sgRNAs designed based on novel PAMs, we set up co-cultures to detect potential selective cytotoxicity using flow cytometry, while performing deep sequencing and WGS on sgRNA-transduced clones to study on- and off-target activities. To demonstrate *in vivo* efficacy, we screened 1080 lipid nanoparticle (LNP) formulations for *in vitro* transfection efficiency using luciferase and mCherry reporter assays to identify the optimal LNPs for *in vivo* delivery. **Results** On average, 417 somatic PAMs per PC case were found (9.2% of total SBSs), >99% of them were located in non-exonic regions, and 97% of the selected subset were Sanger confirmed. Analysis of ICGC data revealed medians of 478.5 and 430.5 somatic PAMs per tumor in two PC cohorts (N=174 combined), 2790 in lung (N=29), and 3235.5 in esophageal (N=388) cancer cohorts. We demonstrated 69-99% selective cell death of three targeted PC cell lines (Panc10.05, TS0111, Panc480) using 4-9 sgRNAs designed based on their somatic PAMs. Sequencing revealed on-target activity in the targeted cell line, and no Cas9 activity in either the patient's normal cells or an irrelevant cancer transduced with a multi-sgRNA expression vector containing 7 Panc480-specific sgRNAs. Off-target analyses on sgRNA-transduced clones found no evidence of off-target events. Finally, using Panc10.05, we identified the top performing LNP formulations with distinct helper lipids, and performed subsequent screening in monolayer cell culture and organoids of human pancreatic tumor cells to further identify top performing LNP formulations for *in vivo* screening. **Conclusions** Our results showed that adult solid tumors harbor hundreds, if not thousands, of somatic PAMs, and these PAMs could serve as selective

and specific novel CRISPR-Cas9 targets to selectively kill cancer cells. This study demonstrates the potential of CRISPR-Cas9 as a novel and selective anti-cancer strategy, and supports the genetic targeting of adult cancers.

Nonviral Approaches for Therapeutic Applications

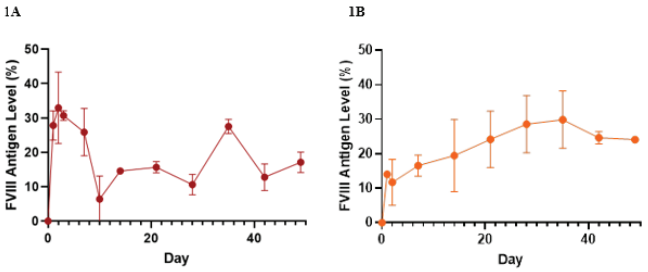
422. Optimization of Fluoroscopy Guided Ultrasound Mediated Gene Delivery in Canines for Sustained FVIII Expression

Savannah M. Lawton[1], Ting-Yen Chao[1], Feng Zhang[2], Masaki Kajimoto[1], Brian Hayes[3], Keith Loeb[3], Timothy C. Nichols[4], Carol H. Miao[5]

[1]Seattle Children's Research Institute, Seattle, WA,[2]University of Washington, Seattle, WA,[3]Fred Hutchinson Center Research Center, Seattle, WA,[4]UNC at Chapel Hill Francis Owen Blood Rsch. Lab., Chapel Hill, NC,[5]Seattle Children's Research Institute, Seattle, WA

Hemophilia A (HA) is an x-linked genetic condition that results in the faulty production of factor VIII (FVIII), a blood coagulation protein. Previously, we performed ultrasound mediated gene delivery (UMGD) of a hepatocyte specific human FVIII (hFVIII) plasmid into normal canines and generated sustained hFVIII expression between 7-15% over 60 days in 2 of 4 treated canines. In this study, we are attempting to further increase FVIII expression levels with a universal hFVIII plasmid, opposed to the previously used hepatocyte specific hFVIII plasmid in normal canines. Additionally, we will compare the difference in transfection efficiency between two liver lobes in the same canine that were each treated with a different ultrasound condition. We aim to optimize the ultrasound treatment parameters and plasmid for experiments on HA canines. A 14 mm x 20 mm balloon catheter was inserted into the hepatic vein via jugular vein access of two canines, and a mix of a high expressing hFVIII plasmid driven by the CAG promoter and RN18 microbubbles (MBs) were injected. Simultaneously, the ultrasound transducer, H114, was placed transcutaneously over the liver and was treated using a pulsed US treatment. In each dog, two lobes were treated, the left lateral lobe (LLL) and right lateral lobe (RLL) with 4 injections of the pDNA and MBs each. The individual lobe was treated for 2 minutes with the US transducer for each injection, for a total treatment time of 8 minutes. In canine 1 (C1) the LLL was treated with 1500 W and the RLL with 750 W. In canine 2 (C2) the LLL was treated with 750 W, and the RLL with 1500 W. Blood was collected on D1, 2, 3, then weekly for approximately 2 months, and subjected to ELISA to measure hFVIII activity. CBC and whole blood chemistry was also run on collected blood. C1 was sacrificed on D60, and the liver was harvested and sectioned into 1cm x 1cm sections. Copy number analysis was run on sections from both treated lobes, as well as the untreated lobes to analyze transfection with differing US conditions. Representative sections were selected from each liver lobe and subjected to H&E staining and reviewed by a certified pathologist. C2 is scheduled for sacrifice and liver collected on D90. Both canines had notable hFVIII expression over the two months followed. C1 had initially high hFVIII values of 30-42% which decreased slightly following day three but remained stable around 20% (Figure 1A). The expression for C2 continued to increase steadily for a month, and currently is stabilizing around 25-30% (Figure 1B). Copy number analysis from C1, showed significantly higher plasmid copies in the RLL. Interestingly, none of the canines developed species-specific anti-human FVIII antibodies. Both dogs had very minor elevation in the AST/ALT levels on day one, but these levels decreased to normal by day three. No other parameters from CBC and whole blood chemistry analysis showed any significant changes in either dog. This data suggests that the use of the CAG-F8X10 plasmid and differing ultrasound conditions further improved the technology of fluoroscopy guided UMGD in canines. Furthermore, these results suggest a promising therapeutic outcome if applied in HA canines.

Figure 1. Human FVIII expression levels in treated Canine 1 (1A) and Canine 2 (1B) blood plasma following UMGD



423. Intrathecal Administration of Lipid Nanoparticles to Silence Pain-Associated Genes by CRISPR Interference

Thomas J. Payne[1], Raquel Tonello[2], Jeffri S. Retamal[2], Paulina D. Ramírez-García[2], Nigel W. Bunnett[2], Colin W. Pouton[1]

[1]Monash Institute of Pharmaceutical Sciences, Parkville, Australia,[2]New York University, New York, NY

Chronic pain is inadequately treated by opioids and non-steroidal anti-inflammatory drugs, which have life threatening side effects. Lipid nanoparticle (LNP)-encapsulated mRNA can be delivered systemically to manipulate expression of disease-associated genes. However, systemically-administered LNPs do not efficiently penetrate the central nervous system due to their large size and are thus unsuitable for therapeutic targeting of the central mechanisms of pain. We investigated the therapeutic effects of intrathecally (IT) administered LNP formulations encapsulating mRNA to silence pain-associated genes using CRISPR interference (CRISPRi). LNPs were formulated comprising a lipid formulation analogous to the FDA-approved product *Onpattro*. To determine whether IT administration of LNPs can effectively control gene expression in the spinal cord, sensory ganglia and brain, LNPs were used to encapsulate nanoluciferase (NanoLuc) mRNA. LNP-NanoLuc mRNA or unencapsulated NanoLuc mRNA was injected IT into the lumbar spinal cord of mice. After 24 or 48 h, spinal cord, brain and dorsal root ganglia (DRG) were collected to measure NanoLuc protein. After administration of LNP-NanoLuc mRNA, NanoLuc expression was detected in the lumbar DRG and spinal cord, with similarly high levels of expression in the thoracic and cervical spinal cord, hind brain, mid brain and forebrain (Fig. 1). In contrast, after administration of unencapsulated NanoLuc mRNA, expression was detected only at the site of administration (lumbar spinal cord and

DRG). To determine whether LNP-mRNA formulations can be used for the treatment of pain, LNP formulations were used to down regulate the gene encoding adaptor associated kinase 1 (AAK1), a therapeutic target for chronic pain. AAK1 mediates dynamin-dependent retrieval of synaptic vesicles from the central terminals of primary sensory neurons and is thus required for sustained release of neurotransmitters in nociceptive circuits of the dorsal horn of the spinal cord. A dCas9 mRNA CRISPRi construct with either a single-guide RNA (sgRNA) targeting the promoter of the mouse AAK1 gene or a non-targeting sgRNA negative control (NC) were encapsulated into LNPs. A model of post-operative pain was studied in which an incision was made on the plantar surface of the paw of anesthetized mice. LNP-dCas9 AAK1 or LNP-dCas9 NC was administered by IT injection. Paw withdrawal responses to stimulation of the plantar surface with von Frey filaments or radiant heat were used to assess mechanical allodynia or thermal hyperalgesia, respectively. In mice treated with LNP-dCas9 NC, robust mechanical allodynia and thermal hyperalgesia were apparent for 96-168 h. LNP-dCas9 AAK1 prevented both mechanical allodynia and thermal hyperalgesia for 96 h (Fig. 2). Withdrawal responses of the unoperated contralateral paw and rotarod latency were unaffected by LNP-dCas9 NC or LNP-dCas9 AAK1, suggesting that normal motor function was unaffected. These results suggest that the IT administration of LNP encapsulating CRISPRi formulations can be used to silence pain-associated genes, offering a much-needed non-opioid therapy for the relief of chronic pain.

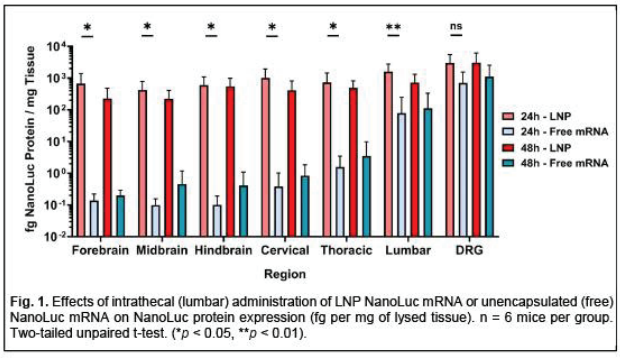


Fig. 1. Effects of intrathecal (lumbar) administration of LNP NanoLuc mRNA or unencapsulated (free) NanoLuc mRNA on NanoLuc protein expression (fg per mg of lysed tissue). n = 6 mice per group. Two-tailed unpaired t-test. (*$p < 0.05$, **$p < 0.01$).

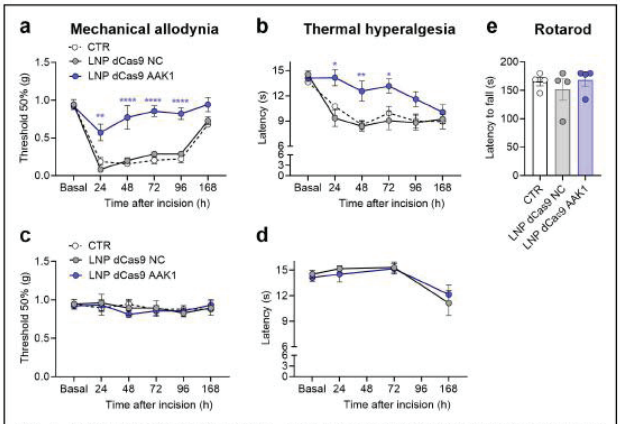


Fig. 2. Nanoparticle-encapsulated AAK1 mRNA prevented postoperative pain. Mechanical allodynia (**a, c**) and thermal hyperalgesia (**b, d**) of the plantar incision (ipsilateral; **a, b**) and non-incision (contralateral; **c, d**) measured 24 – 168 h after intrathecal injection of LNP dCas9 AAK1 or LNP dCas9 NC. Locomotor activity (rotarod) in naïve mice 48 h after treatment (**e**), n=4-6 mice per group. Mean±SEM. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 vs. CTR. 2-way ANOVA, Sídák multiple comparisons test.

424. Focused Ultrasound- and Long-Circulating Nanoparticle-Mediated Nucleic Acid Delivery and Genome Editing in the Brain

Gijung Kwak[1], Angad Grewal[2], Griffin Mess[2], Haolin Li[1], Hanseok Ko[3], Richard J. Price[4], Amir Manbachi[2], Justin Caplan[2], Betty Tyler[2], Jung Soo Suk[1,2]

[1]Department of Neurosurgery, University of Maryland School of Medicine, Baltimore, MD,[2]Department of Neurosurgery, Johns Hopkins University School of Medicine, Baltimore, MD,[3]Institute for Cell Engineering, Johns Hopkins University School of Medicine, Baltimore, MD,[4]University of Virginia, Charlottesville, VA

Successful translation of nanoparticles (NPs) for nucleic acid-based therapy of brain diseases, including brain cancers and neurodegenerative diseases, requires safe and efficient nucleic acid delivery to the targeted disease area within the brain. Stereotaxic convection-enhanced delivery has been explored to achieve therapeutic delivery to the target site in the brain, but the procedure of invasive intracranial injection can cause adverse effects, including infection, edema, and neuron damage. To address this limitation, systemic brain targeting has been investigated in clinical and preclinical studies. However, systemically administered NPs inevitably encounter the blood-brain barrier (BBB), which is the highly challenging barrier established throughout cerebral capillaries that limits trans-vascular NP transport into the brain. The BBB penetrable adeno-associated virus serotype 9 (AAV9)-based brain gene therapy, Zolgensma®, was approved by the FDA, but AAV9 highly accumulated in the liver, heart, and muscle as well. Here, we introduce a two-pronged strategy, comprising BBB-opening focused ultrasound (FUS) and long-circulating poly(ethylene glycol)-coated poly(beta-amino esters) (PEG-PBAE) NPs, that enables systemic nucleic acid delivery to the brain. FUS applied to a predetermined coordinate within the brain induces cavitation of systemically administered microbubbles (MBs) passing through the cerebral capillaries to open the BBB in a targeted, transient, and reversible manner. In parallel, our nucleic acid-loaded PEG-PBAE NPs stably circulate in bloodstream and efficiently penetrate the brain tissue. PEG-PBAE polymers were complexed with plasmid DNA (pDNA) or messenger RNA (mRNA) to form NPs (A and B). PEG-PBAE NPs exhibited excellent colloidal stability in relevant physiological environments, including artificial cerebrospinal fluid and whole mouse serum (C and D). Systemically administered PEG-PBAE NPs carrying pDNA or mRNA exhibited stable circulation in the bloodstream for up to 4 hours, a time window of FUS/MB-mediated BBB opening (E, F, and G). Accordingly, the combination of PEG-PBAE NPs + FUS/MB resulted in efficient NP accumulation at the FUS-applied target site within the brain while exhibiting minimal off-target accumulation (H and I). Freshly prepared and serum-incubated PEG-PBAE NPs showed comparably widespread transgene expression in the brain, underscoring that the ability of our NPs to penetrate the brain tissue was retained (J and K). FUS/MB + PEG-PBAE NPs carrying reporter pDNA or mRNA achieved efficient reporter protein production in astrocytes and neurons at the FUS-applied site (L, M, N, and O). Likewise, FUS/MB + PEG-PBAE NPs carrying mRNA encoding CRISPR-associated protein 9 (Cas9) and sgRNA targeting loxP-flanked STOP cassette in the Ai9 mice chromosome (P) resulted in efficient tdTomato expression, indicating successful gene editing, in astrocytes and neurons at the FUS-applied

site (Q, R, and S). Overall, FUS/MB + PEG-PBAE NPs provide a highly versatile means to enable efficient site-specific therapeutic nucleic acid delivery and gene editing in the brain.

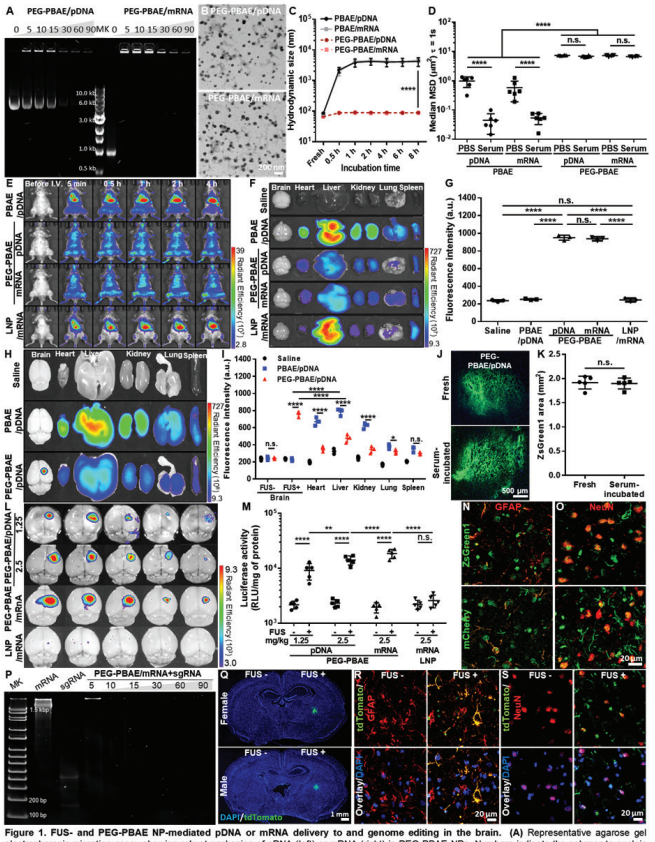


Figure 1. FUS- and PEG-PBAE NP-mediated pDNA or mRNA delivery to and genome editing in the brain. **(A)** Representative agarose gel electrophoresis migration assay showing robust packaging of pDNA (left) or mRNA (right) in PEG-PBAE NPs. Numbers indicate the polymer-to-nucleic acid weight ratios. MK: molecular weight marker. **(B)** Representative transmission electron micrographs of PEG-PBAE NPs carrying pDNA (top) or mRNA (bottom). Scale bar = 200 nm. **(C)** Colloidal stability of PBAE and PEG-PBAE NPs carrying pDNA or mRNA incubated in aCSF at 37 °C for up to 8 hours. **(D)** Median MSD values of PBAE and PEG-PBAE NPs carrying pDNA or mRNA in PBS or whole mouse serum. MSD is a square of distance traveled by an individual particulate matter within a predetermined time interval (*i.e.*, time scale; $\tau = 1$ s) and thus is directly proportional to the particle diffusion rate. **(E)** Representative whole-body fluorescence images of live animals intravenously treated with PBAE NPs, PEG-PBAE NPs, or LNPs carrying Cy5-labeled pDNA or mRNA over time. **(F)** Representative fluorescence images of major organs harvested at 4-hour post-administration from the animals in Figure 1E. **(G)** Quantification of Cy5 fluorescence intensity of the serum collected at 4-hour post-administration of NPs from the animals intravenously treated with PBAE NPs, PEG-PBAE NPs, or LNPs carrying Cy5-labeled pDNA or mRNA. **(H)** Representative fluorescence images and **(I)** Cy5 fluorescence intensity of brains and other major organs harvested at 4-hour post-administration from the animals treated with FUS on the right striatum and intravenously received saline or PBAE or PEG-PBAE NPs carrying Cy5-labeled pDNA. **(J)** Representative fluorescence images showing reporter ZsGreen1 expression in the brain sections at the infusion site within healthy mouse brain striatum 48 hours after the intracranial CED of freshly-prepared or serum-incubated PEG-PBAE NPs carrying pDNA encoding ZsGreen1 (0.1 mg/kg pDNA). Blue: nucleus; Green: ZsGreen1. **(K)** Image-based quantification for area of ZsGreen1 expression. **(L)** Bioluminescence images of brains harvested at 48- or 24-hour post-administration from animals intravenously treated with PEG-PBAE NPs carrying pDNA or mRNA encoding luciferase or LNP carrying mRNA encoding luciferase (1.25 or 2.5 mg/kg pDNA or 0.5 mg/kg mRNA) and subsequently received a FUS treatment on right striata. **(M)** Quantification of luciferase activity in the homogenized hemispheres from the brains in Figure 1L. Representative confocal images showing reporter ZsGreen1 or mCherry production in FUS-treated areas of **(N)** GFAP- and **(O)** NeuN-stained brains harvested at 48- or 24-hour post-administration from animals intravenously treated with PEG-PBAE NPs carrying pDNA or mRNA encoding ZsGreen1 or mCherry (2.5 mg/kg pDNA or 0.5 mg/kg mRNA), respectively, and subsequently received a FUS treatment on right striata. Green: ZsGreen1 or mCherry; Red: astrocyte (GFAP) or neuron (NeuN). **(P)** Repersentative TBE gel electrophoretic analysis showing the successful packing of Cas9 mRNA and sgRNA 298 in PEG-PBAE NPs. Numbers indicate the polymer-to-nucleic acid weight ratios. MK: molecular weight marker. **(Q)** Representative confocal images of the brains harvested at 5-day post-administration of NPs from female (upper) and male (bottom) Ai9 mice intravenously treated with PEG-PBAE NPs carrying Cas9 mRNA and sgRNA 298 (0.5 mg/kg mRNA and sgRNA each) and subsequently received a FUS treatment on right striata. Blue: nucleus; Green: tdTomato. Representative confocal images showing reporter tdTomato production in untreated or FUS-treated areas of **(R)** GFAP- and **(S)** NeuN-stained brains from the animals identically treated as in Figure 1Q. Blue: nucleus; Green: tdTomato; Red: astrocyte (GFAP) or neuron (NeuN). n.s.: no significance, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ (one-way ANOVA).

425. Supramolecular Assembly of Polycation/mRNA Nanoparticles and *In Vivo* Monocyte Programming

Leonardo Cheng[1,2,3], Yizong Hu[1], Stephany Yi Tzeng[1,3], Jordan J. Green[1,2,3], Hai-Quan Mao[1,2,3]

[1]Johns Hopkins University, Baltimore, MD,[2]Institue for NanoBioTechnology, Baltimore, MD,[3]Translational Tissue Engineering Center, Baltimore, MD

Statement of Purpose: Circulating monocytes and their antigen-presenting cell (APC) descendants (macrophages, Mo-dendritic cells) are important therapeutic targets. Monocytes, with their great abundance, estimated to be nearly 20-fold higher than conventional dendritic cells, present an underexplored opportunity to leverage the innate immune system against target diseases. However, there is a lack of methods to controllably assemble mRNA particles of sizes that specifically target phagocytes. Exploiting the assembly kinetics of mRNA particles provides a potential route to engineer submicron (200-1000 nm) particles and explore size-dependent delivery mechanisms. Here, we develop a new kinetic assembly process for controlled assembly of submicron particles from biodegradable poly(beta-amino ester) (PBAE) and mRNA. We report preferential uptake and intracellular delivery of 400-nm particles in mouse monocytes, which differentiate into APCs and elicit potent tumor-specific immune activation. *Methods:* The PBAE was synthesized and formulated into nanoparticles (NPs) with mRNA according to our reported method.[1] Submicron particles were prepared by controlling the ionic strength and pH of the buffer with colloidal stability to obtain particles of defined size. Their *in vitro* transfection efficiencies were assessed in bone marrow-derived macrophages (BMDMs). Select particles loaded with luciferase-encoding mRNA (mLuc) were administered intravenously (*i.v.*) into Balb/c to evaluate transgene expression level *in vivo* by In Vivo Imaging System (IVIS). Cre recombinase-encoding mRNA were administered *i.v.* into Ai9 mice to assess cell type-specific transfection *in vivo*. Particles with effective monocyte transfection were loaded with control (mLuc) or ovalbumin (mOVA) encoding mRNA with/without CpG ODN1826 (ODN), a Toll-like receptor 9 agonist, and administered to C57BL/6 mice to evaluate anticancer immune activation in a melanoma model. *Results:* Submicron PBAE/mRNA particles were formulated by kinetic assembly and stabilized between 100 and 900 nm (Fig. 1A-B). Compared to conventional 100-nm NPs, 400-nm particles transfected BMDMs more effectively at high doses *in vitro* (Fig. 1C). After *i.v.* injection, larger particles achieved have higher overall transgene expression than 100-nm NPs with most expression located in the liver and spleen (Fig. 1D). 400-nm particles achieved the highest transfection in monocytes and monocyte-derived phagocytes compared to other particle sizes in the spleen and liver after *i.v.* injection (Fig. 1E-F). In an OVA-expressing B16 melanoma model, 400-nm particles elicited significantly more OVA-specific T cells in the tumor and draining lymph node than 100-nm NPs (Fig. 1G). *Conclusion:* Kinetic assembly of PBAE/mRNA particles enables the controllable and scalable production of stable submicron (200-1000 nm) particles. 400-nm submicron particles effectively delivered mRNA and adjuvant to phagocytes, particularly circulating monocytes, *in vivo*. Transfected circulating monocytes can be programmed for activation as antigen-presenting cells leading to antigen-specific T cell activation.

References: 1. Hu, Y, *et al.* ACS Nano, 2019, *13* (9), 10161.

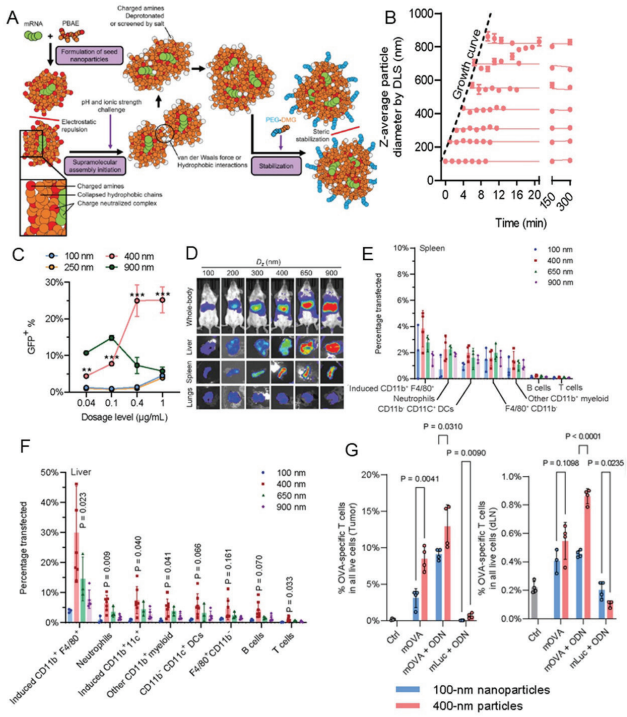


Figure 1. (A) Schematic of kinetic assembly. (B) Linear growth of submicron particles and stabilized size upon growth arrest. (C) Transfection efficiency of BMDMs by particles *in vitro*. (D) *In vivo* mLuc transgene expression live imaged *in vivo* by IVIS. (E-F) *In vivo* transfection efficiency in the spleen (E) and liver (F) after *i.v.* injection of Cre mRNA-loaded particles. (G) OVA-specific T-cells in the tumor and draining lymph node after *i.v.* injection of mOVA/ODN-loaded 400-nm particles.

426. Non-Viral Targeted Laser Delivery of Multi-Characteristic Opsin Genes for Treatment of Geographic Atrophy

Samarendra K. Mohanty[1], Sanghoon Kim[2], Subrata Batabyal[2], Michael Carlson[2], Amir Singh[1], Adnan Dibas[2], Najam Sharif[1]

[1]Nanoscope Therapeutics Inc, Dallas, TX,[2]Nanoscope Technologies LLC, Bedford, TX

Purpose: Vision loss in end-stage age-related macular degeneration (AMD) patients, particularly due to geographic atrophy (GA), is linked to the loss of photoreceptors. Highly photosensitive MCO expression in higher-order retinal neurons restores light sensitivity to the retina in patients with permanent photoreceptor loss. Non-virally delivered Multi-Characteristic Opsin (MCO) optogenetic therapy may be a promising treatment strategy for retinal degenerative diseases. Optical Coherence Tomography (OCT)-guided laser-delivery of MCO plasmids achieves spatial targeting of GA lesions while avoiding healthy regions of the peripheral retina. **Method:** To achieve targeted delivery of MCO, we integrated a near-infrared (NIR) laser beam with an OCT imaging beam to locate retinal lesions. A mixture of MCO plasmids and gold nanorods (GNRs) was intravitreally injected into multiple animal models. Upon laser micro-irradiation, the GNRs cause localized enhancement of laser intensity leading to the formation of nanopores on the targeted cell membranes, thus permeabilizing the membrane to enable precise and site-specific delivery of MCO-plasmids. The efficacy of laser-delivered MCO was evaluated in retinal degeneration (*rd*) rodent models by immunohistochemistry (IHC) and electrophysiology. The safety and toxicity of this delivery method were evaluated in wild-type rodents and African green monkeys using electrophysiology, OCT, intraocular pressure (IOP) monitoring, and IHC. **Results:** Expression of MCO in the retina led to long-term expression up to 1 yr. Enhanced electroretinography (ERG) and visually evoked cortical activities in *rd* animals were observed, which correlated with MCO expression. No observed adverse effect level (NOAEL) was determined for GNR, plasmids, and laser doses. No noticeable decline in ERG, retina thickness (via OCT), increase in cell death, or increase in immune cell response (via IHC) was observed in the therapeutic dose range in wild-type rodents and monkeys. Robust MCO delivery through the internal limiting membrane (ILM) in monkeys, as visualized by fundus imaging of the reporter (mCherry) fluorescence, led to functional improvement, measured by redERG, attributed to MCO's broadband activation spectrum. **Conclusion:** The safety and efficacy of non-viral spatially targeted laser-delivery of MCO observed in multiple animal models offer an opportunity for efficient and targeted therapeutic gene expression in specific retinal cells within GA lesions. This establishes the utility of the method for translational applications while avoiding immune rejection and adverse events typical of virally delivered gene therapies.

427. Unraveling a Multifactorial Host Immune Response to Intramuscular Electrotransfer of DNA-Encoded Antibody Therapy

Debby Thomas[1], Jenny Sprooten[2], Jannes Govaerts[2], Pascal Merchiers[3], Maarten Dewilde[1], Abhishek Garg[2], Kevin Hollevoet[4], Nick Geukens[4]

[1]Laboratory for Therapeutic and Diagnostic Antibodies, KU Leuven, University of Leuven, Leuven, Belgium,[2]Laboratory of Cell Stress & Immunity, KU Leuven, University of Leuven, Leuven, Belgium,[3]Biomerpa BV, Kasterlee, Belgium,[4]PharmAbs, the KU Leuven Antibody Center, KU Leuven, University of Leuven, Leuven, Belgium

DNA-based antibody gene transfer aims to administer antibody-encoding nucleotides, rather than the antibody protein, utilizing a non-viral plasmid DNA (pDNA) vector. This strategy aims to achieve sustained *in vivo* antibody production, addressing challenges inherent to conventional antibody production and delivery. Previously, we demonstrated preclinical proof of concept for diverse DNA-encoded monoclonal antibodies (mAbs) and nanobodies delivered in muscle or tumor using electroporation (EP). We also gained insights into pDNA fate post-injection. The present pharmacokinetic (PK) profile of mAbs expressed in vivo in mice, after intramuscular (IM) EP, is typified by a gradual increase in the initial week(s), culminating in peak plasma levels after 3-4 weeks, followed by a decline of 50% after about 6 weeks, to roughly 90% after several months. Maintaining plasma levels at peak concentrations for an extended duration is highly desirable for this approach to broaden its application potential.In this study, we aim to delineate the host immune response in mouse muscle following IM pDNA EP and identify factors directly linked to expressed mAb PK. To achieve this, knockout (KO) models and immune phenotyping are employed, utilizing a validated pDNA encoding a murine 4D5 anti-HER2 mAb. Earlier experiments demonstrated that mAb PK

in athymic nude and RAG1 KO mice did not significantly differ from wild-type (WT) mice, suggesting that T and B cells, including antigen-presenting pathways, do not drive the decline in mAb expression. To assess the impact of the innate immune response, we evaluated 4D5 PK in IFNAR1 KO mice, which lack type-I interferon receptor function. After IM p4D5 EP, plasma mAb levels showed no significant difference between IFNAR1 KO and WT mice throughout the 12-week follow-up. This finding diminishes the likelihood that DNA sensing, including cGAS/STING, affects mAb PK. Concurrently, we ran flow cytometry panels on WT mice muscle, harvested one week post-IM EP. Compared to control muscle (including untreated, vehicle-injected, and EP only), infiltrated T cells showed more of the effector phenotype and dendritic cell (DC) muscle infiltration was increased. NK cell-mediated cytotoxicity was considered, but muscle NK cell infiltration was limited and did not increase after pDNA EP. This suggests that cytotoxic T-cell responses, facilitated by DC-based antigen presentation, may target mAb-producing cells. However, this does not appear to impact mAb kinetics based on earlier observations in RAG1 KO mouse model.In addition, while assessing the IM macrophage population upon treatment, we found that the percentage of pro-inflammatory macrophages (M1) increased, while the anti-inflammatory macrophages (M2) population remained unchanged. This data, supported by an increased DC infiltration, implies that phagocytic antigen-presenting cells play a role in the decreased mAb production. Potentially, cells transfected with mAb-encoding pDNA increase their "eat-me signals" due to cell stress (EP, protein overproduction, ER stress, etc.), attracting phagocytic cells. Ongoing work investigates whether modulating phagocytosis impacts mAb kinetics. In conclusion, this work started to unravel a multifactorial host immune response triggered by IM electrotransfer of a DNA-encoded mAb. As we elaborate on these observations, factors demonstrably associated with mAb kinetics will be of particular interest, as modulating these responses could allow for a more robust and prolonged *in vivo* mAb expression.

428. Sustained Endogenous FVIII Expression in Hemophilia A Mice is Achieved through *In Vivo* CRISPR/Cas9 mRNA LNP Gene Editing

Chun-Yu Chen[1], Xiaohe Cai[1], Carol H. Miao[1,2]

[1]Center for Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA,[2]Pediatrics, University of Washington, Seattle, WA

Small nucleotide mutations including small deletion/insertion, missense, nonsense, and splicing site mutations in the human FVIII gene result in severe hemophilia A (HA) in approximately 47% of patients. Among these, frameshift mutations contribute to around 15.2% in severe HA patients. CRISPR/Cas9-mediated indel (Insertion and deletion) mutations can facilitate the correction of frameshift-induced pre-termination codons to restore missing FVIII protein expression in affected HA patients. Moreover, advancement of lipid nanoparticle (LNP) technology provides an *in vivo* gene delivery approach. Our goal is to optimize the delivery efficiency of Cas9 mRNA-encapsulated LNPs targeting the native site of FVIII gene synthesis, liver sinusoidal endothelial cells (LSECs), for efficient correction of FVIII gene mutations. Immunodeficient HA mice (NSG HA) with a 5 bp deletion causing a frameshift mutation in FVIII exon 1 was established and served as the gene editing animal model. We encapsulated Cas9 mRNA with sgRNA into LNPs and intravenously deliver the LNPs to NSG HA mice. After treatment with Cas9 mRNA/sgRNA LNPs, average gene editing rates of 15% in LSECs and 57% in hepatocytes were observed. Among the gene-edited cells, over 60% of the frameshift mutations in FVIII gene exon 1 were corrected by CRISPR/Cas9 induced in-frame indel mutations. Additionally, NSG HA mice generated sustained expression of averagely 3% functional FVIII for 26 weeks following Cas9 mRNA/sgRNA LNPs treatment. To enhance LSEC-targeting ability, we incorporated mannose ligands on LNPs targeting mannose receptors on LSECs. We developed a novel MC3 LNP formulation, incorporating mannose-conjugated PEG for specific LSEC targeting. The efficacy of this mannose-conjugated MC3 LNP in LSEC targeting was assessed, with immunostaining and luciferase assays demonstrating a marked improvement in targeting efficiency toward LSECs. All the data suggested that our LNPs effectively delivered gene editing agents to induce indel mutations in both hepatocytes and LSECs *in vivo*. Cas9 mRNA/sgRNA LNPs specifically target and correct indel mutations in mutant FVIII exon 1, leading to the permanent correction of the mutant FVIII gene in LSECs and achieving therapeutic levels of FVIII expression in NSG HA mice. We continue to enhance the delivery and targeting efficiency of LNPs toward LSECs to to increase endogenous FVIII expression post gene editing in NSG HA mice.

Challenges to Immunological Responses to Therapeutic Interventions

429. Intricate Play Between Different Cell Types is Critical for CD8$^+$ T Cell Response Against AAV Encoded Transgene Product in Liver

Sandeep Kumar[1], Moanaro Biswas[1], Jyoti Rana[2], Di Cao[3], Barry J. Byrne[4], Ype De Jong[5], Roland W. Herzog[6]

[1]Pediatrics, Indiana University, Indianapolis, IN,[2]Pediatrics, IUPUI, Indianapolis, IN,[3]Pediatrics, Indiana University School of Medicine, Indianapolis, IN,[4]Pediatrics, University of Florida, Gainesville, FL,[5]Weill Cornell Medicine, New York, NY,[6]Pediatrics, IU School of Medicine, Indianapolis, IN

Regulatory approval for multiple adeno-associated virus (AAV) based gene therapy products highlight the scope of AAV gene therapy. Immunogenicity to the AAV capsid and its encoded transgene product remain major concerns. It is well established that the innate sensor toll like receptor (TLR) 9 and its downstream adaptor molecule, myeloid differentiation primary response 88 (MyD88) are critical for detection and initiation of immune responses against the capsid and transgene product. AAV vectors lacking unmethylated CpG motifs (TLR9 ligand) are currently used in clinical studies. Recently, in a murine model of AAV liver gene transfer we demonstrated that cellular responses to the transgene product are independent of TLR9-MyD88 sensing and instead rely on interleukin 1 receptor 1 (IL-1R1) activation by cytokines IL-1α and IL-1β. Further, we demonstrated that these cellular responses are mediated by multiple cell types including plasmacytoid dendritic cells (pDCs), Kupffer cells (KCs), and cross presenting XCR1$^+$ DCs.

During the first 10 days, pDCs were the principal IL-1 producing cells that infiltrated AAV transduced livers. Subsequently, we noticed the emergence of IL-1 producing neutrophils, which could also be found in the co-clusters. In the present study we sought to identify potential roles of neutrophils and B cells in mediating transgene specific cellular responses following AAV liver gene transfer. We pre-treated a group of wild type (WT)-C57BL/6 mice (n=8) with neutrophil depleting antibody (α-Ly6G) and utilized μMT$^-$ mice (n=8), which lack mature B cells. Mice were intravenously injected with a previously identified immunogenic dose (1x10^{9}vg/mice) of hepatotropic AAV8 vector that encoded ovalbumin (AAV8-OVA) under the control of ubiquitous promoter elongation factor 1-α (EF1-α). Four weeks after vector administration, mice were bled to analyze peripheral blood mononuclear cells (PBMCs) by flow cytometry for OVA specific CD8$^+$ T cells using a class I MHC tetramer (H2-Kb -SIINFEKL). As expected, 50% of mice in the isotype treated group developed OVA-specific CD8$^+$ T cells. Surprisingly, none of the μMT$^-$ mice and only 25% (2/8) mice from α-Ly6G antibody treated group developed OVA-specific CD8$^+$ T cells. We hypothesized that B cell depletion may mitigate transgene product specific CD8$^+$ T cell response. We treated a group of WT-C57BL/6 mice (n=8) with two doses (3 days apart) of α-CD20 antibody (8mg/kg) intravenously prior to AAV8-OVA administration. Four weeks following vector administration PBMCs were analyzed for OVA specific CD8$^+$ T cells. Here, again 50% of control animals had circulating OVA specific CD8$^+$ T cells whereas only 12.5% (1/8) mice in α-CD20 treated group had CD8$^+$ T cells against OVA. B cell depletion with anti-CD20 has been successfully used in patients to prevent TMA that results from complement activation upon high-dose systemic AAV delivery. Previously, using the same experimental approaches, we found that B cells were not required for CD8$^+$ T cell responses against AAV capsid. It is therefore surprising that B cells may play a role in CD8$^+$ T cell responses to the transgene product that are initiated locally within the liver, the underlying mechanism of which remains to be elucidated.

430. Immune Modulation via Expansion of Treg Cells by THOR-834 Leads to Enhanced rAAV Transgene Expression

Mincheol Park, Lavesh A. Gwalani, Mona Motwani, Margaret Hennessy, Zhengyu Luo, John Bladon, Robert Jackson, Jie Bu, Christian Meuller, Sourav R. Choudhury

Genomic Medicine Unit, Sanofi, Waltham, MA

Gene therapy using recombinant adeno-associated viral (rAAV) vectors is a promising approach for treatment of variety of genetic disorders. However, despite clinical successes, challenges persist. Host adaptive immune responses can generate cytotoxic T lymphocytes (CTL) and, as has been reported in some clinical trials, loss of transgene expression over time. Immune mediated dorsal root ganglion (DRG) toxicity also poses a risk. Regulatory T cells (Tregs) play a key role in suppressing CTLs and maintaining homeostasis and self-tolerance. Our goal was to exploit this suppressive capacity of Tregs to control AAV-mediated CTL responses, avoid potential AAV toxicity, and promote the persistence of transgene expression. Interleukin-2 (IL-2) treatment activates Tregs, however, recombinant IL-2 is not specific to Tregs and is rapidly cleared. THOR-834 is a synthetic PEGylated form of IL-2 with enhanced Treg specificity and a longer half-life. We hypothesized that treatment with THOR-834 could promote expansion of Tregs, thus minimizing AAV-mediated CTL responses. To test our hypothesis, we developed a mouse model of AAV immunogenicity. We delivered rAAV via intramuscular injection and observed CD8 T cell expansion, an increase in the effector memory CD8 T cell population, capsid- and transgene-specific cytotoxic T-cell responses, and long-term loss of transgene expression. Mice that received a single subcutaneous injection of THOR-834 alongside the rAAV exhibited expansion of Tregs, reduction of CD8 T cells including effector memory CD8 T cells, and maintenance of transgene levels for at least 10 weeks. In addition, we tested THOR-834 in a rat model of DRG toxicity where we also observed suppression of CD8 T cell proliferation and of effector memory CD8 T cell responses. These results suggest that THOR-834 has the potential to address the challenge of AAV immunogenicity. Finally, to explore the clinical potential of our strategy, we extended our studies to non-human primates. In cynomolgus macaques, co-treatment with a single dose of THOR-834 led to Treg expansion and enhanced transgene levels following rAAV gene delivery thereby confirming that THOR-834 dampened rAAV-mediated immune responses in NHPs as well. These studies lay the groundwork for a new strategy which harnesses Tregs to suppress adaptive immune responses to AAV and enhance the efficacy of rAAV-based gene therapy.

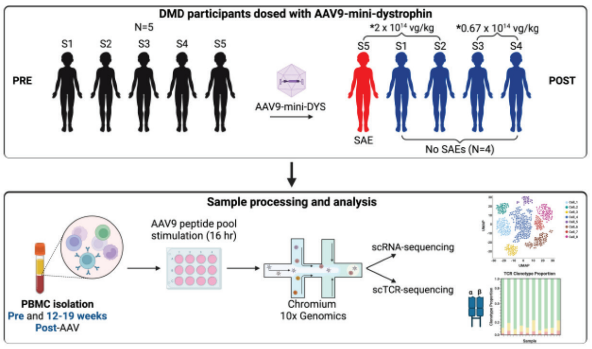
431. Single-Cell and TCR Analysis from Duchenne Muscular Dystrophy Patients Treated with AAV9 Mini-Dystrophin Reveals an Expansion of Human Herpesvirus-Specific T-Cells in a Patient That Exhibited Thrombotic Microangiopathy

Micahel Emami[1], Mark A. Brimble[2], Alejandro Espinoza[3], Jane Owens[4], Thomas A. Lanz[5], Philip K. Farahat[6], Matteo Pellegrini[7], Courtney Young[8], Paul G. Thomas[2], Armando Villalta[6], Stefan A. Schattgen[2], Melissa J. Spencer[1]

[1]Department of Neurology, UCLA, Los Angeles, CA,[2]Host Microbe Interactions, St Jude Children's Research Hospital, Memphis, TN,[3]Department of Human Genetics, University of California, Los Angeles, Los Angeles, CA,[4]Rare Disease Research Unit, Pfizer, Cambridge, MA,[5]Drug Safety Research and Development, Pfizer, Groton, CT,[6]Department of Physiology and Biophysics, University of California, Irvine, Irvine, CA,[7]Department of Molecular, Cell and Developmental Biology, University of California, Los Angeles, Los Angeles, CA,[8]MyoGene Bio, San Diego, CA

Duchenne Muscular Dystrophy is a major disease target for AAV-mediated gene therapy, with several approaches at various stages of clinical implementation. However, high systemic doses, often at, or exceeding 1x10^{14} vg/kg, are necessary to achieve sufficient skeletal muscle transduction. With this approach, there is a risk of immunogenicity leading to serious adverse events (SAEs), with thrombotic microangiopathy (TMA) being a particular clinical concern. A better understanding of the AAV-host immune response is needed before AAV-based delivery can be used to treat all patients with DMD. Here, using patient peripheral blood mononuclear cells (PBMCs) we performed single-cell RNA-sequencing of the transcriptome and T-cell receptors (TCR). Five patients had samples collected before, and after (12-19 weeks), treatment with an AAV9-microdystrophin.

Of these five patients, two received a dose of $6.7x10^{13}$ vg/kg and three received $2.x10^{14}$ vg/kg. One patient within the higher dose group experienced TMA shortly after administration. Cell frequencies remained relatively stable between the collected timepoints; however differential gene expression analysis showed upregulation of interferon response genes in several cell types. Since T-cell receptors that respond to a particular antigen in a given HLA-context also share similar, definable, features, we looked for the presence of TCR clonotype groups (TCR clumps) that were expanded after treatment. We identified two groups of related TCR sequences that appeared across two individuals with shared HLA-alleles in post-treatment samples. These same TCR sequences were not observed in the pre-treatment samples. These TCRs could be directly related to the AAV administration; however, it was not possible to determine their specificity. Interestingly, we also observed two separate clumps that were expanded in frequency in the post-treatment sample of the patient that exhibited TMA. Through literature database similarity matching, all the clonotypes within these clumps were identified as being reactive to known A02 restricted human-herpesvirus (HHV) reactive epitopes: one clump specific for an epitope within CMV pp65 (NLVPMVATV) and the other for EBV BLMF1 (GLCTLVAML). These data provide insight into the mechanistic basis of human immune-AAV interactions and lay a foundation for an improved understanding of why TMA arises in only a subset of individuals receiving high-dose systemic AAV.



432. Adeno-Associated Virus Serotype 8 Efficiently Transduces Multiple Antigen-Presenting Cells in the Liver

Di Cao[1], Sandeep Kumar[1], Moanaro Biswas[1], Maite Munoz[1], Bridget Brian[1], Junping Zhang[1], Dongsheng Duan[2], Weidong Xiao[1], Roland W. Herzog[1]

[1]Herman B Wells Center for Pediatric Research, Indiana University, Indianapolis, IN,[2]Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO

Low immunogenicity associated with AAV is one of the features that led to its development as a preferred vector for *in vivo* gene delivery. A potential reason for the low immunogenicity of AAV has been proposed to be their inefficiency in transducing professional antigen-presenting cells (APCs). A post-entry block has been proposed as an impediment to dendritic cell (DC) transduction. However, prior studies performed *in vitro* experiments or analyzed lymphoid organs such as spleen or lymph nodes. Such approaches are not without limitations. *In vitro* studies lack the complexity and physiological context of living organisms, limiting their ability to capture systemic interactions and responses observed in *in vivo* studies. Analysis of splenocytes misses events in the target organ of gene transfer. In addition, the sparse interception of time points poses challenges in identifying the optimal detection window if expression is transient. Even if certain APCs participate in immune response induced by the AAV vector, the reporter transgene (e.g., a fluorescent protein) may undergo degradation within APCs before detection, thereby eluding the capture of the fluorescent signal. In pursuit of comprehensively elucidating if AAV vectors are capable of transducing APCs, we employed the Cre reporter tool strain Ai14 mice. These mice are conditional reporter mice that harbor LoxP-Stop-LoxP-tdTomato cassette under the control of CAG promoter. In the presence of Cre recombinase, the LoxP-flanked stop codon will be excised, thereby leading to sustained and robust cytoplasmic tdTomato expression. We intravenously injected Ai14 mice with $2x10^{11}$vg/mouse of an AAV8 vector that encoded Cre recombinase under the control of cytomegalovirus (CMV) enhancer/promoter. To assess the transduction profile of AAV, tdTomato expression in different cell populations of liver and spleen was analyzed by flow cytometry on days 3, 7, 10 and 14 post vector administration (n=4/time point). To our surprise, robust tdTomato expression was observed in the liver as early as day 3 post vector administration with continual increase over time. Besides hepatocytes (up to 86% on day 14), tdTomato expression was also observed in a variety of APCs, including Kupffer cells (KCs), monocytes/neutrophils, plasmacytoid dendritic cells (pDCs), DCs (DC1 & DC2), B cells, and endothelial cells with highest expression in DC2 (78%) followed by monocytes/neutrophils (~74%) and KCs (64%). tdTomato expression in pDCs, DC1, B cells and endothelial cells ranged from 10-35%. In contrast, tdTomato expression profile in the spleen was different and much more modest. Amongst splenocytes, the highest expression of tdTomato was observed in monocytes (4.3% on day 14), followed by DC1 and DC2 (each ~1%), whereas other cell types had undetectable expression above the background. To assess vector distribution between liver and spleen, we quantified AAV genome copy number using droplet digital PCR. Vector genome copy numbers were 20-40 folds higher in the liver compared to the spleen on days 3-14. In contrast to AAV8, AAV1 vector transduced only ~6% of hepatocytes and showed much lower transduction of immune cells in the liver (2-4% of B cell, macrophages, and DC2 by day 14). In the spleen, ~1% of monocytes were transduced, with no evidence for transduction of other APCs. In conclusion, AAV vectors may efficiently transduce a variety of intrahepatic APCs in a serotype-dependent manner, which challenges traditional thinking about AAV-immune system interactions. Based on a wealth of prior studies, we suspect that transgene expression in these cells is transient and may not necessarily interfere with tolerance induction via hepatic gene transfer.

433. Artificial miRNA Delivered through AAV Induces Dorsal Root Ganglion Toxicity in Cynomolgus Macaques and C57BL6 Mice

Shih-Ching Joyce Lo, Zachary Hawley, Ingrid Pardo, Maria Zavodszky, Kyle Ferber, Denitza Raitcheva, Casey Fergal

Biogen, Cambridge, MA

Artificial miRNAs targeting superoxide dismutase 1 (SOD1) expressed from adeno-associated virus (AAV) vector have been investigated as

a therapeutic approach to treat amyotrophic lateral sclerosis (ALS) patients who carry toxic gain of function mutations within the gene. AAV-induced dorsal root ganglion (DRG) toxicity has limited the development of gene replacement approaches but has yet to be reported for RNAi AAV-based therapies. Here, we report an AAV9-based artificial miRNA that induces DRG toxicity in non-human primates (NHPs) and provide evidence that this phenomenon can be recapitulated within C57BL6 mice. Histopathological analysis showed that NHPs and mice develop AAV-induced DRG toxicity, including DRG neuron degeneration and necrosis, and nerve fiber degeneration that were associated with increases in cerebrospinal fluid (CSF) and serum phosphorylated neurofilament heavy chain (pNF-H). Furthermore, RNA-seq analysis of DRGs after AAV-treatment showed that several pathways were preserved between NHPs and mice, including increases in innate/adaptive immune responses and inflammatory pathways, and decreases in mitochondrial- and neuronal-based genes. Finally, specific endogenous miRNAs were upregulated in the DRGs of both NHPs and mice after AAV treatment. Increased expression of these endogenous miRNAs can be identified within the CSF of NHPs, and significantly correlates with pNF-H, implicating these endogenous miRNAs as a potential biomarker of AAV-induced DRG toxicity.

434. Understanding the Immune Response to Central Nervous System-Directed Adeno-Associated Virus Vectors

Ashley Harkins, Amanda Tutto, Katelyn Sylvia, Meghan Blackwood, Motahareh Arjomandnejad, Acadia Kopec, Allison Keeler

Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA

Immune responses to adeno-associated virus (AAV) gene therapies in human clinical trials remain a hinderance to their widespread application and have not been well predicted by preclinical studies in animals, particularly the induction of capsid-specific T cells and loss of transgene expression. Even less is known about immune responses to AAV in the central nervous system (CNS), a unique immune environment and a challenging location to study responses. In a CNS-directed AAV gene therapy clinical trial, our group observed induction of systemic capsid-specific immune responses and increases in CXCL10 in the cerebrospinal fluid suggesting an inflammatory response and potential recruitment of immune cells to the CNS. It is important that we understand the effects of direct administration of AAV to the CNS for both the peripheral and local immune responses. We sought to explore the immune response to AAV in a mouse model to measure immune responses in the CNS. We bilaterally injected the thalamus of C57BL6 mice with PBS, AAV8, or AAVrh32.33, which has previously been shown to induce capsid-specific T cells in mice. Both capsids delivered the reporter genes secreted embryonic alkaline phosphatase (SEAP), an immunogenically inert transgene, or Ovalbumin (OVA), a potent stimulator of the immune system. Animals were followed for 6- (established response) or 12-weeks (late response) post-injection. IFNγ ELISPOT assays were run to measure peripheral capsid-specific T cell responses. At 6 weeks, 2 of 3 animals in the AAVrh32.33-SEAP group were positive, indicating a capsid-specific T cell response. While we expected animals in the AAVrh32.33-OVA group to exhibit a capsid-specific response as well, it is possible that the immunogenic properties of the transgene skewed the T cell response towards a transgene-specific response. Surprisingly, at 6 weeks, 2 of 4 animals in the AAV8-SEAP group were ELISPOT positive. Hemophilia patients treated with AAV8 developed capsid-specific T cells in the peripheral blood, but AAV8 has not previously been described to elicit a T cell response in mouse models. At 12 weeks, animals in all groups were ELISPOT negative, indicating that circulating capsid-specific T cells are too few to detect at this timepoint. Despite positive ELISPOT responses at 6 weeks, AAVrh32.33 and AAV8-SEAP treated animals exhibit stable serum SEAP expression over time. Vector genomes are higher in the brain in both groups compared to the liver suggesting serum expression may come from the CNS rather than the liver. Forthcoming histology will provide insight into transgene expression in the collected tissues. Vector genomes do not decline from 6 to 12 weeks, suggesting clearance of transduced cells was not observed, even in OVA treated animals. At 6 weeks, AAVrh32.33 injected animals, regardless of transgene, exhibit significantly higher percentages of CD3+ T cells and CD8+ T cells in the brain compared to the PBS group, measured by flow cytometry of immune cells isolated from perfused brains, indicating T cell infiltration in the CNS. Additionally, both groups have noted increases of CD4+CD25+FoxP3+ T regulatory cells (Tregs) in the brain compared to PBS treated, although the OVA group had a higher percentage of Tregs. At 12 weeks, cellular infiltration in the brain is lesser compared to 6 weeks, but the Treg population remains elevated compared to PBS animals, indicating potential long lasting Treg residency after AAV injection in the brain. Upcoming work will include repeating these studies in a mouse model predisposed to neuroinflammation as many patients receiving AAV gene therapy exhibit disease-related neuroinflammation and could have exacerbated responses to direct administration. Understanding the immune response to AAV gene therapy in CNS-directed therapies will allow us to develop safer, more effective gene therapies.

435. *In Vivo* Delivery and Protection by Engineered Synthetic DNA-Encoded Influenza H1HA Head-Directed Monoclonal Antibodies in Mice

Abigail Rose R. Trachtman[1], Jacqueline D. Chu[1], Jillian Eisenhauer[1], Kaitlyn Flowers[1], David Custodio Zegarra[1], Laurent M. Humeau[2], Trevor RF Smith[2], David B. Weiner[1], John Kash[3], Ami Patel[1]

[1]The Wistar Institute, Philadelphia, PA,[2]Inovio Pharmaceuticals, San Diego, CA,[3]Viral Pathogenesis and Evolution Section, Laboratory of Infectious Diseases, NIAID, NIH, Bethesda, MD

Monoclonal antibody (MAb) therapy is revolutionizing modern medicine, for cancer, and autoimmune treatment among others with more than 200 MAbs approved for clinical use or under review worldwide. For infectious disease, monoclonal antibodies received initial emergency use authorization during the COVID-19 pandemic and the recent licensure of nirsevimab for respiratory syncytial virus prophylaxis opens a new paradigm for mAb delivery against infectious diseases. Routine mAb delivery for seasonal infections like influenza A viruses could provide a significant public health benefit. However, the global potential of MAbs remains limited due to developability limitations, manufacturing costs, delivery route challenges, and the

need for cold chain storage. In vivo gene delivery platforms, including synthetic plasmid DNA vectors can be engineered to encode antibody heavy chain and light chain genes for direct delivery. Synthetic DNA is simple to manufacture, temperature-stable, and DNA-encoded antibodies (DMAbs) are therefore a promising alternative modality for in vivo delivery of protective mAb.Influenza viruses cause acute respiratory illnesses and are a significant cause of morbidity and mortality in all age groups, particularly young children and older adults, and influenza-related illnesses have returned to pre-COVID pandemic levels. As an approach to influenza prophylaxis, we engineered 11 different synthetic DMAbs encoding human IgG1 monoclonal antibodies targeting multiple epitopes on the H1N1 influenza A virus hemagglutinin (HA) protein globular head. DMAbs were sequence optimized and then tested for their ability to express in vitro. Then, in vivo DMAb expression was evaluated following delivery of multiple doses (25-100ug) to BALB/c mice (n=5/group), with peak serum levels reaching >20ug/mL at the highest dose. Serum binding and neutralization activity against A/H1N1/pdm2009 lineage viruses was detected, identifying 2 DMAbs with potent activity comparable to a previously published DMAb, DMAb-2-12C. These top 2 DMAbs were next administered to DBA/2 mice (n=15 mice/group, 6.25-25ug doses) alone and in combination, achieving expression levels between 1-5ug/mL by day 5 post-administration. The mice were challenged intranasally on Day 6 post-administration with 10LD50 of H1N1 A/California/07/2009 X179A. Protection against weight loss and mortality was observed for all DMAb treatment groups, including at the lowest doses. Interestingly, the lungs histopathological analysis and immunohistochemistry (IHC) staining indicated dose-dependent interstitial pneumonia and presence of influenza NP antigen. Lesions and positive IHC staining were only seen in negative control groups and low dose DMAb groups, with groups receiving higher doses of DMAb not showing any lesions.Taken together, our data demonstrate that synthetic DNA H1HA head-directed monoclonal antibodies are functional and offer protection in mice against lethal H1N1 pdm2009 infection, even at low doses. Our data are supportive towards translation of this approach to larger animal models with consideration for further translational development. DMAb delivery offers important advantages for seasonal influenza prevention including long-term expression, potentially facilitating access to significant US. and global populations.

Poster Abstracts

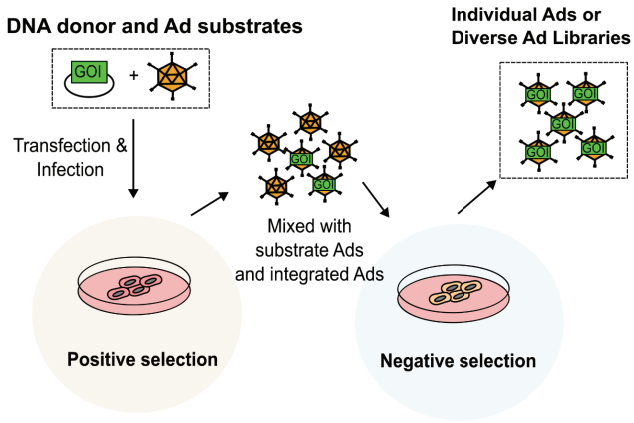
Wednesday Posters: Viral Vectors

436. FastAd- A System for the Rapid Generation of Single Adenoviruses or Complex Adenoviral Vector Libraries

Shao-Chia Lu[1], Yi-Yuan Lee[2], Felix Andres[1], Daniel Moyer[3], Michael A. Barry[4,5]

[1]Division of Infectious Diseases, Department of Medicine, Mayo Clinic, Rochester, MN,[2]Department of Computational Biology, Cornell University, Ithaca, NY,[3]Immunology Track, Mayo Clinic Graduate School of Biomedical Sciences, Mayo Clinic, Rochester, MN,[4]Department of Immunology, Mayo Clinic, Rochester, Mayo Clinic, Rochester, MN,[5]Department of Molecular Medicine, Mayo Clinic, Rochester, MN

Adenovirus (Ad) vectors are highly effective gene delivery systems for *in vivo* applications. However, the standard process for constructing recombinant Ads is laborious and time-consuming, limiting rapid development, such as responding quickly to a healthcare emergency, or for high throughput applications. Here, we introduce the FastAd system, for the direct generation of recombinant Ads in living mammalian cells. In this system, recombinant DNA from plasmids or PCR products are knocked into infectious "receiver" viruses using positive and negative selections with three site-specific recombinases. This approach bypasses all traditional adenovirus cloning and rescue steps. With the FastAd system, the production time was shortened from a traditional 2-month timeline to 9-10 days for large scale production of CsCl-purified viruses. For example, we generated a replication-defective Ad expressing SARS-CoV-2 spike protein, a single-cycle Ad expressing influenza NP and a conditionally replicating Ad expressing murine OX40 ligand. Additionally, the FastAd system was also used to generate diverse barcode, gRNA and cDNA libraries. For example, following transfection of cells at a T25 flask scale, the system was able to generate a library containing over 3 million unique clones, verified by next-generation sequencing (NGS). Furthermore, the FastAd system was further used to capture a comprehensive CRISPR genome-wide sgRNA library within a 9-day production timeline. This Ad-gRNA library underwent multiple passages within cells, enabling the identification of essential host factors that effectively suppress the adenovirus life cycle. In summary, the FastAd system represents a simple and versatile tool for the rapid generation of individual recombinant adenoviruses as well as the production of complex Ad libraries.



437. An Integrase Deleted Retrovector with Persistent Payload Expression without Integration Suitable for Prophylactic and Therapeutic Applications

Bradford Steele, Jacqueline Fischer-Lougheed, Stephanie Lees, Cecilia Roh, Robert Johnson
GenVivo Inc, San Marino, CA

Integration of transferred genes into a host cell via retrovectors provides a clear advantage for duration of transgene expression over transitional or episomal vectors. However, activation and inactivation of genes by insertional mutagenesis or the deregulation of an oncogene are potential detrimental outcomes. We previously described non-integrating MLV vectors (IDRV) made by alanine mutations across three key acidic residues. All 7 mutants successfully exhibited early strong protein expression from the mRNA vector payload comparable to vectors with a functional integrase. The vector described here utilized *gagpol* (GP) with a complete deletion of the integrase gene (dINT). The particle titer of dINT vector was not affected by the deletion when compared to wildtype (WT) vector, in contrast to the report by Lai et al (2001, J Virol). Using our modified HSV-TK (v-eTK) suicide gene construct as the payload, a vector made with the deleted integrase was compared to the WT vector and several IDRV vectors. Experiments were performed to ascertain: 1) the level of v-eTK expression over time in transduced A375 melanoma cancer cells; 2) the amount of cell kill in the presence of the prodrug ganciclovir (GCV) in A375 and other cancer cell lines; and 3) the lack of integration of the v-eTK into the host genome. Flow cytometry data demonstrated stable expression of v-eTK over time in A375 transduced with the WT vector, while cells transduced with the dINT vector showed high expression at early time points followed by a steady decrease. Of note, v-eTK levels were still measurable at 14 days; thus as noted below is due to persistence of v-eTK protein rather than gene integration. Interestingly, the IDRV vectors tested exhibited lower v-eTK expression at all times despite similar transduction titers. The v-eTK/GCV cell kill activity in A375 transduced with the dINT v-eTK vector was comparable to that observed with a functional integrase for up to 7 days. Decreased yet detectable cell kill activity was measured in the dINT vector group at 14 days consistent with persistent protein expression. Other cancer lines were also tested with the dINT vector. In each cell line, there was variable but significant cell kill at day 3 (D3) and in most cases, detectable cell killing at D10. The degree of the bystander effect in some of the cancer cell lines may explain the differential in the lower percentage of cells transduced vs the high cell kill. Relative Psi copies (packaging signal of the vector) were compared with vector copy numbers (VCN) (measured from LTR sequences) from extracted DNA by ddPCR. The DNA extraction method includes all genomic DNA plus cDNA made from the reverse transcription of the vector payload. The dINT vector-transduced cells showed measurable Psi and VCN copies at D1 which decreased to near or below the limit of detection for the assays by D7, consistent with the early appearance and later disappearance of cDNA which is not integrated. This compares with the Psi and VCN copies from the WT vector transduced cells which decreased from D1 to D3 and were constant thereafter at significant levels, consistent with integration. We have constructed dINT vectors which are capable of transducing cancer cells which display protein expression comparable to the WT at early time points and in many cell lines demonstrate limited expression for over 2 weeks. This extended protein expression in the absence of integration will be useful for certain applications, while avoiding the downsides of integration and improve on the safety of the vectors. In applications requiring transient expression of a certain time period such as targeted combination immunotherapy, gene editing delivery, and therapeutic and prophylactic vaccines, and given the low natural immunity to the vector, the dINT may have future widespread applicability.

438. Engineering T4 Bacteriophage Nanoparticles: A Next-Generation Paradigm for Effective Vaccine Development

Jarin Taslem Mourosi, Ayobami Awe, Jingen Zhu, Venigalla B. Rao
Biology, Catholic University of America, Washington, DC

The T4 bacteriophage nanoparticle has emerged as a novel and promising platform for the development of vaccines against infectious diseases, as well as a large capacity vector for gene therapies. Its inherent safety, stability, and natural properties akin to adjuvants contribute to the potential for creating effective, long-lasting, and cost-efficient multivalent vaccines that are accessible to global communities. The head of the T4 bacteriophage is decorated with two non-essential proteins: the small outer capsid protein (SOC) and the highly antigenic outer capsid protein (HOC), both of which are well-positioned for displaying foreign antigens. While T4 can efficiently express and display its own proteins, foreign proteins are not expressed abundantly due to factors influencing expression, including protein size and folding kinetics. We hypothesize that modulating the transcription and translational elements in the T4 genome can enhance foreign protein expression and presentation. As a proof of concept, we incorporated an antigenic subunit, the receptor binding Domain III (DIII) of the envelop protein of Dengue viruses 1 & 2 and of Zika virus into the phage genome. Both Dengue virus (DENV) and ZIKA virus (ZIKV) pose significant global health threats because the current live attenuated DENV vaccine is not sufficiently effective, and no licensed ZIKV vaccine is yet available. The DIII gene was fused to SOC and inserted into the genome through CRISPR engineering. Further, we created a mini library of constructs by varying codon usage (T4 codon vs *E. coli* codon), promoter type (early/later promoters), riboswitch-like regulatory elements, ribosome

binding sites, and terminators. Comprehensive screening of this mini library revealed that engineered phage with a strong late promoter of the tail sheath structural gene (gp18), or deletion of a 11 bp riboswitch-like regulatory element from the upstream of SOC open reading frame, exhibited a ~10-fold increase in the vaccine antigen compared to the control construct where the SOC native promoter was utilized. This means that the engineered T4 phages are decorated with a high density of a target vaccine antigen belonging to a eukaryotic viral envelope protein and can be directly employed as a vaccine without any chemical formulation. This approach could lead to development of effective and versatile next generation vaccines, particularly for rapid generation and deployment of stable, protein-based, vaccines against emerging and pandemic pathogens.

439. Unraveling the Mystery of CAV2 Tropism: Characterization of Canine Adenovirus Type 2 Virus Infection

Payal Agarwal[1], Theresa Higgins[2], Madison D. Hogans[3], Will P. Kretzschmar[3]

[1]Scott Ritchey Research Center & Department of Pathobiology, Auburn University, Auburn University, AL,[2]Auburn University, Auburn, AL,[3]Scott Ritchey Research Center, Auburn University, Auburn University, AL

Adenoviral (Ad) vectors are the most commonly used gene therapy vectors. The expression pattern of the cell surface receptors used by Ad determines the virus tropism. The natural tropism of adenovirus can be modified to deliver genes to Ad refractory cells. Canine adenovirus 2 (CAV2) is a canine adenovirus with a known ability to infect human cells, including neurons and ovarian cancer cells. The CAV2 genome is well characterized, but the cell surface receptors involved in CAV2 transduction are unknown. The identification of these receptors and using them to alter Ad tropism will help enhance adenovirus-based vectors as augmented gene delivery tools. Identification of new cell surface receptors will also help understand the basic biological mechanism of CAV2 infection, further expanding knowledge with respect to both this virus and this class of virus. CAV2 has been erroneously grouped with Ad5 and was believed to infect cells through the CAR receptor. However, we have shown that CAV2 infects cells refractory to Ad5, such as canine lymphoma cell lines 17-71 and OSW and canine mast tumor cell line MPT1. We have also shown that CAV2 infection is not correlated with cell surface CAR expression. CAR knockdown inhibited the Ad5 infections but did not affect CAV2 infections. Using a pulldown assay, Sulfo-SBED-based affinity assay, we identified DMBT1 & DSG1 as putative CAV2 receptors. Being a non-human viral vector, CAV2 holds significant potential in translational medicine. Our work has demonstrated that receptors involved in attachment and internalization of CAV2 can be DMBT1 and DSG1.

440. Easy and Efficient Lentiviral Transduction with a Macroporous Transduction Sponge

Thomas P. Quinn, Mei Fong, Lily Lee, Adrian Martin, Myn Uddin, Michael Haugwitz, Andrew Farmer

Takara Bio USA, San Jose, CA

The advancement and approval of engineered cellular therapies using lentiviral vectors are revolutionizing the treatment landscape for diseases resistant to traditional therapeutics. To optimize lentiviral transductions and mitigate inefficiencies, various strategies have been employed, such as "spinoculation" and the use of reagents (polybrene, Retronectin) to enhance cell-virus contact or modify cellular responses during transduction (rapamycin, prostaglandin E2). Despite their efficacy, many enhancers have limitations due to their bioreactivity impacting cell behavior. Moreover, lentiviral transductions in standard cell culture vessels face challenges as the virus must traverse considerable distances before reaching cells, leading to significant viral wastage due to insufficient transport and virus degradation. Microfluidic systems, by spatially constraining cells and virus to small volumes, address these biotransport issues inherent in standard cell culture systems. However, previous applications of microfluidics for lentiviral transduction necessitated special chips and hardware to facilitate the flow of cells and virus through microfluidic channels. To overcome these requirements, we developed a macroporous 3D alginate sponge, eliminating the need for additional hardware. The microfluidic sponge is made from calcium-crosslinked alginate, a GMP-compliant, FDA-approved biomaterial with high biocompatibility and low toxicity. The crosslinked alginate undergoes a gentle cryogelation process, yielding a sponge (5mm radius) with uniform pore sizes ranging from 20 to 300nm. The simple workflow involves a mixture of cells and virus applied to the sponge for absorption, followed by a short incubation of 1hr, media addition, and harvesting 24 hours later. The release of transduced cells is facilitated by adding a chelating release buffer that depolymerizes the sponge, ensuring the efficient release of transduced cells. Sponge-based transductions demonstrate flexibility and can accommodate a wide range of cell numbers (from 1e5 to 1e7) and types, transduction volumes (50-150 µl), and incubation times (4 hours to overnight). This straightforward workflow is designed to maximize transduction efficiency across a diverse array of cell targets, including both suspension and adherent cell types. Our alginate sponge demonstrated efficient transduction of over 15 cell targets, including human primary T cells, CD34+ HSCs, NK cells, and adherent cell lines, with high viability. Sponge-based transductions are consistent, with a coefficient of variation (%CV) of less than 15%. The innovative ex-vivo lentiviral transduction sponge is user-friendly, scalable, and compatible with diverse cell types, providing efficiencies equal to or surpassing conventional protocols.

441. Refined Expression of 3' End Long-Noncoding RNA Using Lentiviral Vectors

Liping Zhang, Jiali Yang, Hua Gao, Jia Xu, Wei Mao, Guodong Jia, Weiran Shen, Yang Xinglin

R&D, OBiO Technology (Shanghai) Corp., Ltd., Shanghai, China

Lentiviral Vectors (LVs) have proven effective for delivering genes of interest to target cells, but the delivery of long non-coding RNA (lncRNA) poses challenges. Many lncRNAs terminate with polyadenylation (polyA) signals, leading to disruptions in LV genome integrity during vector production. The conventional LV genome includes expression regulatory sequences, such as WPRE, which may interfere with lncRNA if termination is imprecise, introducing unnecessary and disruptive sequences. Additionally, certain lncRNAs undergo auto-processing during maturation, resulting in the truncation of the LV genome. To address these challenges, we implemented a reverse genome setting combined with the V-Pac system for lncRNA expression via LV. The V-Pac system incorporates a short CuO

regulatory element inserted upstream of the lncRNA coding region and the expression of CymR protein in the packaging cell line. CymR binds to CuO, inhibiting lncRNA expression. Once the LV vector is used for transduction in target cells without CymR, efficient lncRNA expression is achieved. Our study compared various polyadenylation signals for vector yield, revealing that SV40 polyA V2 yielded the best performance. Different LVs expressing various lncRNAs were produced and tested, demonstrating that our system could generate significantly higher LV yields. Notably, vectors constructed with a reverse expression cassette exhibited enhanced potency in terms of lncRNA functions related to inhibiting or promoting cell migration. These findings highlight the advantages of the V-Pac system combined with a reverse expression genome design for packaging lncRNAs using LVs.

442. Chimeric Ad5/3 and Ad5/21 for Improved Tropism of Human Airway Epithelial Cells

Lei Lei, Patrick L. Sinn
University of Iowa, Iowa City, IA

Background: Adenoviral (Ad)-based vectors are extensively used gene therapy tools. To date, human Ad type 5 has been the predominant choice for preclinical and clinical trials using Ad-based viral vectors in the airways. The fiber's C-terminal globular domain node (knob) binds to host cell receptors that mediate adenovirus infection. Human Coxsackie and Adenovirus Receptor (CXADR) is the principle receptor for most species C adenoviruses, including Ad5; whereas, Ad3 and Ad21 are species B adenoviruses that reportedly use DSG2 and CD46 as cellular receptors. Here we generated chimeric Ad5-based vectors that have its fiber/knob replaced with the corresponding region from Ad3 or Ad21. We ask if the chimeric vectors have altered receptor usage and determine the impact on cell-type specific transduction of primary human airway epithelial cells. **Methods**: Ad5, Ad5/3, and Ad5/21 (each expressing an mClover reporter gene) were used to transduce primary human airway basal cells, as well as fully differentiated primary human airway cultures. Transduction efficacy was determined by microscopy and flow cytometry. To confirm the cellular receptors of the chimeric Ad vectors, we constructed three CHO-tet-on stable cell lines expressing hCXADR, hCD46, or desmoglein-2 (hDSG2), respectively. Receptor cDNAs were cloned into a piggyBac transposon, and transfected CHO-tet on cells alongside a transposase expression plasmid. Puromycin was used to select positive cells. Doxycycline induces receptor gene expression. The receptor usage of Ad5, Ad5/3, Ad5/21was then determined by microscopy and flow cytometry. **Results**: Ad5/3 and Ad5/21 showed enhanced transduction of human airway basal cells as compared to Ad5. Over the course of 3 weeks following transduction, the basal cells grew into a well-differentiated, pseudostrified epithelial layer suggesting that transduction didn't impact cell differentiation. For each vector, secretory cells were the main cell type transduced, followed by ciliated cells, then basal cell. Transduction of fully differentiated airway cell cultures with Ad5/3, Ad5/21 and Ad5, were overall similar; however, a trend toward increased basal cell transduction was observed with Ad5/3 or Ad5/21 (over Ad5) which is consistent with higher basal cell expression of DSG2 and CD46 as determined by scRNA-seq.As expected, Ad5 used CXADR as its cellular receptor. Ad5/3 primarily used DSG2 as its receptor however some affinity for CD46 was observed. Ad5/21 primarily used CD46 as its receptor and showed little affinity for DSG2 or CXADR. **Conclusion**: This study suggests that Ad5/3 and Ad5/21 use DSG2 and CD46 receptors, respectively. The use of chimeric Ad5/ species B vectors may be a strategy to alter cell tropism and improve delivery of gene editing tools to airway progenitor cells for life-long correction of lung disease. **Acknowledgements**: This study is supported by NIH R01 HL-133089 funding.

443. Engineering of a Nucleic Acid Platform to Augment RNA Mediated Therapy

Debora Mazzetti, Marco Mineo, Ludovica Gaiaschi, Michal Nowicki, Pierpaolo Peruzzi
Brigham and Women's Hospital, Boston, MA

Background: RNA-mediated interference has been proven over the years to be biologically effective, yet its translational benefits in oncology have been lagging. This is mainly due to the fact that its efficiency is generally not at par with that of small molecules, nor is its ease of use. However, RNA constructs, in particular non coding transcripts, have the potential to offer a high degree of customization that make them superior to drugs in terms of achieving focused oncogene multitargeting, a progressively recognized necessity in cancer treatment. Methods: Taking advantage of the genetic structure of microRNA genes, we have engineered artificial RNA sequences which are recognized by the cell microprocessor as bona fide microRNA transcripts, but are cleaved into smaller components. The design allows to retrofit each sequence with multiple microRNAs, microRNA sponges and RNA aptamers, each with specific targeting functions. The net result is a relevant expansion of their interference capacity. Results: Three sequences of progressive structural complexity were designed and biologically validated in HEK293 cells as a proof of principle. Each showed retained ability to be processed by the cells into desired clusters of microRNAs (CL3 sequence), an additional sponge sequence against oncogenic miR-21 (CL3a21 sequence), and an aptamer directed against NFkB (CL3a21ap65). The targeting specificity of each sub-component of the sequence was confirmed by RTqPCRT, Western blotting and a luciferase reporter assay. A functional analysis of the biological effects of the three different sequences was performed in primary , patient-derived, glioblastoma cell lines after lentiviral mediated expression of each sequence. Adding the antimiR21 function to CL3 significantly reduced growth (clonal ability, migration potential and tumorigenicity) in vivo. Cell proliferation demonstrates a substantial decrease of nearly 50%. CL3a21 displayed a reduction of 60% in comparison to CL3 and 80% in comparison to the negative control in regards to migration and invasion properties. Similarly the clonal potential was significantly reduced in CL3a21 cells. Conclusion: Our study provides proof of principle and feasibility for designing artificial RNA constructs based on the genetic scaffolds of microRNAs. This strategy offers a significantly augmented capacity for tumor multitargeting by RNA-based interference. References: Bhaskaran V, et al. The functional synergism of microRNA clustering provides therapeutically relevant epigenetic interference in glioblastoma. Nat Commun; 10(1):442. doi: 10.1038/s41467-019-08390-z (2019). Bhaskaran V, et al. Design, engineering and cloning of artificial microRNA clusters for gene therapy applications. Nat Protoc; 14(12):3538-3553 (2019). Ebert, M., Neilson, J. & Sharp, P. MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. Nat Methods 4, 721-726 (2007). https://doi.org/10.1038/nmeth1079. Friedmann-Morvinski D, et al.

Targeting NF-κB in glioblastoma: A therapeutic approach. Sci Adv; 2(1): e1501292. doi: 10.1126/sciadv.1501292 (2016). Wurster SE & Maher LJ Selection and characterization of anti-NF-κB p65 RNA aptamers. RNA 14, 1037-1047 (2008)

444. Anellovector Derived from Human Retinal Pigment Epithelium Delivers and Expresses a Therapeutically Relevant DNA Payload in the Retina of Nonhuman Primates

Parmi Thakker, Alexander Auld, Vimal Vaidya, Nolan D. Sutherland, Maciej Nogalski, Isabella Gold, Hyun Jung Jun, Geoffrey Parsons, Tuyen Ong, Ashley Mackey, Chris Wright

Ring Therapeutics, Cambridge, MA

Anelloviruses are a highly diverse family of commensal ssDNA viruses that are a principal constituent of the human virome. These viruses have been found across many vertebrates and demonstrate broad tropism to multiple organs and tissues in humans. Moreover, they naturally evade the immune system, can persist for many years, and importantly do not appear to cause any known pathology in humans making anelloviruses an attractive tool for the delivery of genetic medicine. An anellovirus originally discovered and sequenced from the retinal pigment epithelium (RPE) of a human patient was vectorized to contain a CMV-eGFP or CMV-Aflibercept payload. In the first nonhuman primate (NHP) study of an anellovector (ANV.X.CMV-eGFP), we observed DNA delivery to the eye and mRNA expression that was comparable to AAV2 via IVT administration. In additional NHP studies, we administered an ANV.X-eGFP vector at higher doses as well as a vectorized therapeutic payload (ANV.X.CMV-Aflibercept). We are also evaluating the impact of redosing ANV.X.CMV-Aflibercept via the IVT route. These studies are the first demonstration of therapeutic payload delivery with an ANV vector and represent an initial investigation into the potential use of this novel platform to treat eye diseases such as wet AMD. This ANV platform could have several advantages compared to current gene therapies. A substantial proportion (up to 50%) of patients on gene therapies in development have failed injections (e.g., lack of Aflibercept expression) or require rescue medications due to loss of efficacy. Therefore, in addition to a high tissue specific and potency, a gene therapy vector such as ANV that has the potential to be re-dosed, could be a more effective, longer lasting, and tolerable treatment option than current standard of care.

445. Engineering Gene Therapy Vector Properties in the Baculovirus

Tanya Jain, Lucas Brown, Gang Bao

Rice University, Houston, TX

The CRISPR-Cas9 gene editing system has been widely researched over the last two decades, and viral methods are commonly used for the delivery of gene therapies such as CRISPR. Current common viral delivery methods include lentivirus and adeno-associated virus. These can efficiently deliver DNA into living systems, but have relatively small packaging capacities-5 and 8 kilobases, respectively. However, much larger gene circuits than this must be developed to create editing systems that afford more control over their induction and function. This research aims to use baculovirus, an insect-virus with a comparatively large carrying capacity, to deliver inducible, targeted gene therapies. As an insect virus, baculovirus poses unique challenges when used within mammalian systems, namely low transduction, inactivation by the complement system, and transient gene expression. This work focuses on the process of producing pseudotyped baculovirus containing proteins to enhance transduction, immune protection, and targeting. Genes for the pseudotyped proteins were placed downstream of the p10 insect promoter in the engineered baculovirus genome using standard molecular cloning techniques. Baculovirus was then produced, purified, and tested *in vitro* on various cell lines-including HEK293T cells, HEPA-1,6 cells, C2C12 muscle cells, and Jurkats-using GFP as a reporter for transduction, as well as *in vivo* in C57BI6 mice using a firefly luciferase reporter. Both *in vitro* and *in vivo*, vesicular stomatitis virus protein-G (VSV-G) has been determined to improve transduction efficiency. With VSV-G, a three-fold increase in transduction was observed in HEK293T and C2C12 cells, and a 30-fold increase was seen in HEPA-1,6 cells. Decay accelerating factor (DAF) also appears to provide protection from the complement portion of the immune system in experiments performed with mouse serum containing the complement system. *In vivo*, while pseudotyping with VSV-G and DAF each independently increased baculovirus transduction, adding both proteins to baculovirus together led to the highest transduction-approximately a 10-fold increase as compared to wild type-and expression that persisted for a month. Ongoing efforts are focused on the engineering and production of pseudotyped baculovirus containing proteins to enhance targeting and transduction in primary T-cells. The level of control afforded to clinicians and researchers by the large DNA capacity of baculovirus can be used to implement small-molecule inducible systems for temporal control of therapeutic gene expression, or autonomous cell-state sensing mechanisms to monitor and respond to CAR T-cell activity *in vivo*.

446. Papillomavirus as a Novel Viral Vector for Gene Therapy of Olmsted Syndrome

Francesco Diversi, Elisa Mazza, Fernanda de Castro Reis, Paul Heppenstall

SISSA, Trieste, Italy

Olmsted syndrome is a rare dominant genetic skin disease caused by a gain of function point mutation in the Transient receptor potential vanilloid-3 (Trpv3) gene. TRPV3 is a non-selective cation channel sensitive to non-noxious warm temperatures (31-39°C) mainly expressed in keratinocytes and sensory neurons. The mutation renders the channel hyperactive, thus increasing intracellular calcium levels; this leads to hyperproliferation and defects in the maturation of basal keratinocytes, invasion of immune cells, and chronic itch and pain. Olmsted syndrome, given its well-defined etiology, stands out as a promising target for gene therapy. Here we have asked whether we can reverse the disease phenotype of Olmsted syndrome by disrupting gene expression in a cell type-specific manner. In vivo gene therapy of the epidermis is particularly challenging: AAVs, the most common viral vectors, are not efficient in targeting keratinocytes, meanwhile, lentiviruses have been exclusively employed ex-vivo on reconstructed skin equivalents due to their capability to integrate into the host genome. We reasoned that we could employ papillomavirus (PV) as a viral vector: it has a natural tropism towards keratinocytes, its double-

stranded DNA cargo remains episomal, and it can package up to 8 kilobases - a much larger capacity than AAVs. We used papillomavirus to encapsidate SaCas9 with a gRNA to disrupt Trpv3 locus. A disrupted Trpv3 should be preferable over the mutated gene since genetic ablation of Trpv3 in mice leads to only mild phenotypes such as a thinner stratum corneum and an altered wavy fur. We transduced both a keratinocyte cell line and mouse primary keratinocytes with PV-SaCas9; nearly 60% of the alleles showed indel formation ten days after transduction. Next, we evaluated the functionality of the disrupted gene via FURA-2 calcium imaging. One week after the transduction of PV-SaCas9, keratinocytes showed a significant reduction in their response to carvacrol, a TRPV3 agonist. Furthermore, we reconstructed skin equivalents starting from PV-treated primary keratinocyte to prove its applicability to autologous transgenic skin transplants. Hence, we have demonstrated that papillomavirus can be engineered to efficiently deliver a gene of interest to keratinocytes, resulting in a promising new candidate for skin gene therapy.

447. Development of Adenoviral Vectors That Transduce Purkinje Cells and Other Cerebellar Cell Types in the Cerebellum of a Humanized Mouse Model

Emre Kul, Uchechi Grace Okoroafor, Amanda Dougherty, Lauren Palkovic, Hao Li, Paula Valino-Ramos, Leah Aberman, Samuel Young

University of Iowa, Iowa City, IA

Hereditary cerebellar ataxias are a group of monogenetic conditions due to gain or loss of function of genetic mutations resulting in debilitating impairments in movements, cognition, behavior and premature deaths. Cerebellar Purkinje Cell (PC) dysfunction and degeneration have been identified as a hallmark of these ataxias. Although much is known about causative mutations and their link to PC degeneration, no therapy exists to address the root cause of hereditary cerebellar ataxias. Viral vector mediated gene therapy is an attractive strategy to treat hereditary cerebellar ataxias as it has the potential to treat the root cause of the disease by restoring gene function. However, many of the associated gene encode large cDNAs that exceed the packaging limit of adeno-associated viral vectors (AAV) and lentiviral vectors. Helper-dependent adenoviral vectors (HdAd) with a large packaging capacity of around 36kb has the potential to deliver these large genes, making it a promising viral vector for hereditary cerebellar ataxia gene therapy. However, the commonly used 1st generation Ad vector to make HdAds are a Group C adenovirus which use the Coxsackie-adenovirus receptor and do not transduce PCs. To overcome this roadblock, we created chimeric 1st generation Ad vectors that utilize the human CD46 receptor (hCD46) to infect and transduce cells. Using a "humanized" hCD46 mouse model, we demonstrate these Ad vectors transduce PCs and other cerebellar cell types. Since the tropism and transduction properties of Ad vectors are dependent of the Ad capsid proteins, these chimeric 1st generation Ad vectors open new avenues for HdAd gene therapy approaches for cerebellar disorders and for multiple neurological disorders.

448. Paramyxovirus-Like Particles for Therapeutic Delivery of Proteins to Cells

Srijana Adhikari, Santosh Panthi, Phuong Tieu Schmitt, Anthony P. Schmitt

Veterinary and Biomedical Sciences, The Pennsylvania State University, State College, PA

Virus-like particles (VLPs) have the potential to safely deliver functional proteins to cells for therapeutic purposes. However, foreign proteins do not naturally package into VLPs. We previously discovered that paramyxovirus NP proteins contain short packaging sequences near their C-terminal ends that can be fused with foreign proteins, allowing the foreign proteins to participate in viral assembly and package efficiently into VLPs. These VLPs in turn are naturally capable of delivering the cargo to the target cell interiors. Using this approach, we have generated paramyxovirus VLPs loaded with different cargos, including Cre recombinase. These VLPs can deliver biologically active cargo to target cell cytoplasm and nuclei. We tested multiple paramyxoviruses as VLP production platforms, including parainfluenza virus 5 (PIV5) and Nipah virus (NiV), and successfully achieved efficient cargo packaging and delivery in each case. We also tested a series of altered PIV5 M mutant proteins that have enhanced virus assembly capacity. These resulted in significantly improved VLP production and a corresponding increase in the delivery of functional Cre into target cell nuclei. Together, these findings lay the groundwork for the use of paramyxovirus-like particles as safe and effective tools for delivering therapeutic proteins to cells and tissues.

449. Advancing Gamma Retroviral Vector Production: A Streamlined Process with One-Step Purification and Concentration Approach

Soodeh Baghaee Ravari, Josh Moore

Genezen, Technical Review, IN

Retroviral vectors (RVV), particularly Gamma retroviral vectors, are potent tools extensively utilized in cell therapy applications, offering the advantage of transferring larger transgenes for stable integration. The current manufacturing approach for RVV involves stable producer cell lines during upstream production, with minimal downstream processing due to the inherent instability of RVV envelopes. However, the development of producer cell lines adds a significant 3-4-month timeframe to the client's journey to IND/clinic. At Genezen, we have explored an alternative method for RVV production involving transient transfection, comparing three commercially available packaging cell lines that can produce higher transducing unit titers. Additionally, we implemented a high-speed centrifugation method for RVV concentration, addressing the prolonged duration and potential loss of vector titers associated with the traditional Tangential Flow Filtration (TFF) step. This study introduces a streamlined, one-step concentration method using high-speed centrifugation for RVV. Our findings demonstrate not only enhanced RVV stability but also scalability for late-stage and commercial vector productions. High-speed centrifugation allows for the concentration of viral titers in the range of 1e7-1e8 Transducing Units per milliliter (TU/mL) with a remarkable >60% overall recovery. Moreover, this purification method for RVV contributes to a reduction in overall production costs by eliminating the need for Anion Exchange Chromatography (AEX)

and TFF materials in the production process. The presented approach marks a significant advancement in RVV manufacturing, offering improved stability, scalability, and cost-effectiveness for applications in the gene therapy field.

450. Anellovectors: Expanding the Payload Capacity of Anellovirus-Based Vectors Beyond the Wild-Type Genome Capacity

Cato Prince, Nicole Boisvert, Silin Liu, Simeon Springer, Lovepreet Chahal, Rajendra Boggavarapu, Waseem Raja, Chris Reardon, Emily Stead, Yong Chang, Tuyen Ong, Geoffrey Parsons, Joseph Cabral
Ring Therapeutics, Cambridge, MA

We recently reported the development of the Self-Amplifying Trans-complementation of a Universal Recombinant Anellovector (SATURN) production system for producing a functional novel gene therapy class, termed Anellovectors, based on the vectorization of human commensal anelloviruses. Anelloviruses are a highly diverse family of non-enveloped negative-sense single-stranded DNA (ssDNA) viruses that are a ubiquitous component of the human virome. Despite their ubiquitous presence in human populations, anelloviruses are apathogenic and evade induction of robust humoral responses, making them attractive candidates for the next generation of genetic medicines. Here, we report the development of an Anellovector cargo that is significantly larger than the wild-type anellovirus genome. Anelloviruses are the only truly negative-sense ssDNA viruses that infect eukaryotes. Notably, anelloviruses also lack the Rep proteins produced by other ssDNA viruses, pointing to a unique replication and packaging mechanism that may allow for the encapsidation of larger genomes. The Anellovector presented here is based on the Beta genera of human anelloviruses which have genome sizes of ~2.8-3.0 kb. Using vector genomes of increasing size for encapsidation, we have demonstrated that Anellovectors are capable of packaging vector genomes ranging from 2.0kb - 5.0kb, representing up to 65% beyond the wild-type genome capacity. A combination of qPCR and next-generation sequencing was used to verify that the encapsidated expanded cargo DNA is intact, circular, and single-stranded. Electron microscopy was used to verify that vectors containing expanded genomes were capable of forming the expected 30nm particles. The overall potential for Anellovector cargo capacity appears to be similar to or even exceed the ~4.7 kb cargo capacity of AAV. The payload capacity of viral vectors often drives their therapeutic potential. Expanding the payload capacity of the Anellovector builds upon its intrinsic characteristics of immune favorability and tropism in efforts to reach the broadest possible patient population in need of gene therapies.

451. Improvements in AAV and Lenti-Viral Vector Production Using the Mirus RevIt AAV Enhancer and VirusGen LV Transfection Reagent and Kit

Ngan Trinh, Kelly Geosits, Khan Umaer, Srivanya Tummala, Marguerite Campbell, Brian Tomkowicz
Viral Vector Research & Development, SK pharmteco - US, King of Prussia, PA

Transient transfection of three plasmids (rep, cap, and helper) in HEK293 cells facilitated by a transfection reagent is the most widely used method for AAV and other viral vector production. It has, in fact, been shown effective for all viral production platforms and is a regulatory-validated approach. Mirus Bio, a leader in transfection reagent technology, recently developed the TransIT-VirusGEN® Transfection Reagent in conjunction with two enhancers: RevIT™ for AAV and VirusGEN® LV Enhancer for lentiviruses. We sought to investigate the performance of these AAV and LV systems in comparison to other commonly used transfection reagents, as well as address how the addition of enhancers improves performance, regardless of the transfection reagent used. Our results indicate that both enhancers, when added at the appropriate time in the transfection workflow, greatly improve overall titers of virus, when compared to transfection conditions without enhancer. Not only was there an improvement in overall titers, but when using the RevIT Enhancer, we also noticed an increase in full particles across a panel of AAV serotypes. With both TransIT-VirusGEN® and the LV Enhancer currently available in GMP grade and the RevIT AAV Enhancer available for GMP use later in 2024, these reagents will undoubtedly provide an overall improvement for viral vector manufacturing.

452. Development of a Universal, GMP Ready, Clonal HEK293 Cell Line for the Manufacturing of Adeno Associated, Lenti, and Adenoviral Vectors

Marguerite Campbell[1], Kelly Geosits[1], Ngan Trinh[1], Khan Umaer[1], Srivanya Tummala[1], Thomas J. Burton[2], Kaitlyn Krewson[2], Ryann Russell[2], Brian Tomkowicz[1]
[1]Viral Vector Research & Development, SK pharmteco - US, King of Prussia, PA,[2]Process Development, SK pharmteco - US, King of Prussia, PA

Utilizing the well-established, adherent, Frank Graham HEK293 cell line as a starting point, we have successfully adapted cells, from a qualified master cell bank, to grow in animal component-free, suspension conditions, in shake flasks and bioreactors. In head-to-head studies comparing our CBM-HEK suspension-adapted versus 3 commercially available suspension HEK293 cell lines, we demonstrate comparable yields of AAV vectors, following plasmid triple transfection method for virus production. In order to establish a cell line amenable for use in GMP-grade manufacturing, we clonally printed CBM-HEK293 pool cells using the Cytena f.sight cell printing and CloneSelect imaging platform. Herein, we show full-traceability of clonal outgrowth from single-cell through scale-up and have identified select clones that perform equally well in head-to-head comparison studies versus other HEK293 cell lines for virus production. The lead clone 4G9, supports the robust production of all viral platform

tested: AAV, LVV, and Adenovirus. Importantly, the 4G9 clone can support the production across a diverse panel of all natural AAV serotypes evaluated (AAV1, 2, 5, 6, 8, 9) as well as Lenti-viral CAR-Ts. Establishment of a GMP master cell banks for use in large scale AAV manufacturing is underway with expected availability in 2024. The advancement of this production-ready clone represents a significant cost saving for viral vector manufacturing. With integrated process and analytical development as well as commercial scale manufacturing, SK pharmteco can streamline the entire manufacturing process.

Wednesday Posters: AAV Vectors - Virology and Vectorology

453. CRISPR Screen for rAAV Production Implicates Genes Associated with Infection

Sakshi Arora[1], Emily O'Driscoll[2], John Lang[1], Beverly L. Davidson[1], Ophir Shalem[1]

[1]Children's Hospital of Philadelphia, Philadelphia, PA,[2]Department of Genetics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

Recombinant adeno-associated virus (rAAV) vectors are an effective and well-established tool in the growing gene therapy field, with five approved AAV-mediated gene therapies already on the market and numerous more in clinical trials. However, manufacturing rAAV vectors is an expensive, timely, and labor-intensive process that limits the commercial use of AAV-mediated gene therapies. To address this limitation, we screened producer cells for genes that could be targeted to increase rAAV yield. Specifically, we performed a CRISPR-based genome-wide knockout screen in HEK 293 cells using an antibody specific to intracellular, intact AAV2 capsids coupled with flow cytometry to identify genes that modulate rAAV production. We discovered that the knockout of a group of heparan-sulfate biosynthesis genes previously implicated in rAAV infectivity decreased rAAV production. These findings were validated in a secondary screen performed in producer cells that lack AAV receptor (AAVR). Additionally, we identified several vesicular trafficking proteins for which knockout in HEK 293 cells increased rAAV yields that were further validated using RNP based approach. This approach yielded higher knockout efficiency and avoided the selection effect on cell growth and viability. In summary, our work presents new pathways that can be co-opted to improve rAAV production, with practical applications to the adoption of AAV-gene therapies more broadly. (S.A. and E.O. contributed equally to the work)

454. Towards rAAV Genome Delivery with Lipid Nanoparticles and *In Vitro* Assembled AAV Capsids

Marco T. Radukic[1], Dinh T. Le[2], Lucas Krause[1], Timo Krassuski[1], Kristian M. Müller[1]

[1]Cellular and Molecular Biotechnology, Bielefeld University, Bielefeld, Germany,[2]III. Department of Medicine, Medical Center Hamburg-Eppendorf (UKE), Hamburg, Germany

Production of recombinant AAV in cell culture is inflexible, expensive, and hard to scale. We demonstrate methods to disentangle AAV genome production from packaging, which maintain the function of ITR flanked transgenes while allowing for low cost and flexible manufacturing. First, we established the *in vitro* production of rAAV-mimetic DNA with ITRs but without bacterial remnants and without the AAV length limit. Such DNA was packaged in lipid nanoparticles (LNPs) with proven redosing option. To demonstrate the versatility of the approach, we additionally added *in-vitro* produced mRNA of Rep78 to the LNPs, which should drive transient expression of the integrase. Cas9 guided nanopore sequencing of genomes of respectively transduced cells indeed revealed AAV wild-type like genome integration. For certain applications, the scalability of this approach might democratize gene therapy. In a second line of experiments, we established recombinant VP capsid protein expression in *E. coli* and obtained capsids directly with AAV5 VP3 or from inclusion body purification and refolding with AAV2 VP3. We also recombinantly produced the four Rep proteins and tested their helicase and nickase activity. The components together with the ITR-DNA provide an excellent basis to study factors of a mix and match strategy for AAV encapsidation.

455. Factors Influencing the Level of Plasmid Backbone Impurities in Self-Complementary AAV Products

Holly Appleton[1], Mike Boyd[2], Dmitriy Lukashev[3], Ralf Schmid[4], Ge Tan[5], Edward Oakeley[5], Joel Jenzer[5], Samantha Baker[6], Jasmin Haegele[5], Huang Hon-Ren[6], Lisa D'costa[1], Taylor Pleines[1], Sophie Marshall[1], Binod Pandey[1], Mukund Patel[1], Fangfang Liu[7], Quyen Truong-Royce[7], Marianne E. Goodwin[8], Gunjan Mehta[1], Patrick Bastek[7], Chris Gully[1], Chunping Qiao[7]

[1]Technical Research and Development, Novartis Gene Therapies, Durham, NC,[2]Novartis Gene Therapies, Quincy, MA,[3]Novartis, Cambridge, MA,[4]Novartis, Norfolk, MA,[5]Novartis Gene Therapies, Basel, Switzerland,[6]Novartis Gene Therapies, Cambridge, MA,[7]Novartis Gene Therapies, Durham, NC,[8]Novartis Gene Therapy, Raleigh, NC

Recombinant adeno-associated viral vectors (AAV) have demonstrated tremendous potential as an effective gene delivery vehicle for treating a wide range of human diseases. However, current AAV production methods have certain limitations including the presence of capsid associated nucleotide impurities, which originate from plasmid backbones or host cell DNA raising valid safety concerns. Previous studies have reported that the incorporation of short-hairpin DNA sequences into the backbone of the AAV gene of interest (GOI) plasmid leads to a decrease in the occurrence of reverse-packaged genomes. In this study, we aimed to evaluate whether the inclusion of a hairpin structure adjacent to the wild-type (wt-ITR) within the backbone could decrease the level of plasmid backbone impurities in self-complementary AAV using AAV2 and AAV8 serotypes. A total of four AAV2 and three AAV8 self-complementary (sc) vectors were produced using AMBR250 bioreactors via triple plasmid transfection. The vectors were harvested 72 hours post transfection and subjected to AAVX purification. Neutralized AAVX eluates were submitted for ddPCR titer, ddPCR plasmid backbone impurity quantification, and

PacBio NGS. While some of the assays are still ongoing, our preliminary data indicates: 1. The presence of backbone impurities within sc-AAV was found to be associated with wild-type (wt)-inverted terminal repeats (ITR); namely, the quantity of backbone impurities detected on PacBio NGS increased as the distance to the wt-ITR decreased. 2. The presence of a hairpin structure on the sc-AAV GOI plasmid backbone did not result in a significant decrease in the amount of backbone packaging impurities via ddPCR assay. 3. There was also a significant disparity in the percentage of backbone impurities between sc-AAV derived from AAV2 and AAV8. Specifically, AAV2 exhibited a higher percentage of backbone impurities, approximately 2.6 times higher than that of observed in AAV8. This study will offer valuable insights into the production of safer and more efficient recombinant AAV vectors for clinical applications.

456. Tuning AAV Transgene Expression with Autogenous, Chemical-Based Control Systems

Sara R. Wasserman, Matthew L. Hirsch, Nathaniel A. Hathaway

University of North Carolina, Chapel Hill, NC

A common challenge for pre-clinical AAV gene therapies is insufficient transgene expression. However, high or repeated AAV dosing regimens increase immunogenic risk. Thus, an auxiliary method of activating AAV expression at the transcriptional level could be valuable to the development of more effective gene therapies. To chemically activate the expression of AAV, we have developed a proximity-based technology that uses heterobifunctional small molecule recruiters of endogenous transcriptional regulators. When combined with a small, synthetic zinc-finger based molecular scaffold, this bioorthogonal approach facilitates targeted recruitment of the transcriptional upregulator bromodomain-containing protein 4 (BRD4) directly to AAV genomes. We first identify a series of minimal, autogenous cassettes that can effectively regulate AAV expression while preserving ~3 kb for single-stranded therapeutic payloads. The lead regulatory cassette efficiently induces AAV expression, increasing transgene production over 30-fold in vitro. CUT&RUN-Sequencing reveals that AAV genomes in cells treated with the heterobifunctional recruiter are significantly enriched for BRD4 occupancy. Chemical control of AAV expression is shown to be effective across multiple human cell lines and transgene cassette configurations. Activation of AAV expression by this method is dose-dependent, resistant to washout, and can be chemically reversed within 72h. Future steps for the application of this technology in translational models of dose-sensitive diseases are discussed. This chemical method of tuning AAV expression may help circumnavigate the immunogenic risk of repeated AAV treatments by providing a controlled and reversable chemical strategy to increase the expression of single-dose AAV therapies.

457. The Impact of rAAV Genome Size on Vector Production in the Sf9-Baculovirus System

Zhaohui Yang[1], Clarissa Tjoanda[1], XuShan Wang[2], Shreya Ahuja[1], Brandon Zhuang[1], Andrew Pla[1], Mary Ng[1], Nicole Nasta[1], Jessica Cheng[1], Stuart Nelson[3], Garrett Daniels[1]

[1]Prevail Therapeutics, a Wholly Owned Subsidiary of Eli Lilly and Company, New York, NY,[2]Eli Lilly and Company, Indianapolis, IN,[3]Eli Lilly, New York, NY

Recombinant adeno-associated virus (rAAV) is an effective vector for gene delivery currently being used in multiple FDA-approved gene therapies and over 100 clinical trials for the treatment of genetic diseases. The increasing demand for high-quality rAAV vectors requires improved manufacturing. The Sf9-Baculovirus production system has great potential to meet such needs with its high yield, packaging efficiency, and scalability. Wild-type AAV has a single-stranded DNA genome of approximately 4.7 kb. Previous studies have shown that the packaged genome size can impact the characteristics of rAAVs produced from mammalian cell lines. In Sf9-produced AAVs, packaged genomes that are slightly bigger than wild-type length have been reported, which likely leads to increased vector heterogeneity. In order to systemically investigate the impact of genome size on the production of rAAVs in Sf9 cells, we evaluated the yield and quality of a series of vectors with a wide range of genome sizes. The transgene cassettes were designed to have identical components (promoter, reporter, polyadenylation signal, backbone) and only differ by the length of stuffer sequences, resulting in undersized, close to wild-type, and oversized AAV genomes. Using a panel of characterization assays including gel electrophoresis, next generation sequencing (NGS), analytical ultracentrifugation (AUC), and expression-based analyses, we observed heterogeneous vector populations that encapsidated undersized genomes in both self-complimentary (dimer) and single-stranded (monomer) forms of DNA, whereas close to wild-type and oversized genomes only exhibit single-stranded DNA. Interestingly, there was a remarkable decrease in vector potency *in vitro* as genome size gets bigger, whereas the empty vector ratio as well as the purity of packaged DNA were similar across all the vectors tested. The production yield measured by physical titer using droplet digital PCR (ddPCR) was not significantly affected by genome size. To further interrogate whether the decreased potency of vectors with larger genomes was due to decreased infectivity, additional assays will be carried out. Since the viral capsid protein VP1 is known to play a critical role in the infectivity of the virus, the relative ratio of VP1 will also be analyzed. Taken together, this work demonstrated that the genome size has great impact on Sf9-produced rAAV product quality. Our findings can help further optimize the design of transgene cassette to improve the production of fully functional and high-quality rAAVs.

458. Co-Packaged Genomic DNA in AAV Particles is Not Detected in Animal Tissues After Systemic Administration

Matthew Fuller, Lorelei Stoica, James Maratt, Zachary Sykes, Emily Agro, Elliott Wilion, Nicholas J. Richards, K. Reed Clark, Samuel C. Wadsworth

Ultragenyx, Somerville, MA

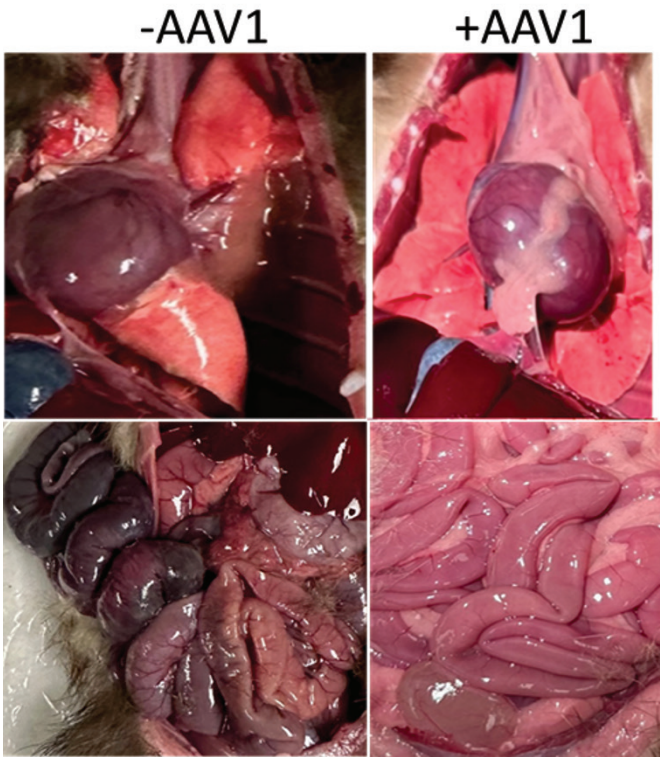
An adeno-associated virus (AAV) vector with a hu.37 capsid was developed for the treatment of hemophilia A. This AAVhu.37 vector, termed DTX201, carries a genome that encodes for expression of B-domain deleted human Factor(F)VIII. Preclinical characterization studies in hemophilia A mouse models and nonhuman primates with DTX201 have been published. The results of these studies, along with formal GLP safety studies, formed the basis for a clinical trial using DTX201 in subjects with severe hemophilia A. Because of the size of the hemophilia A population, and the likely requirement for high vector doses to achieve therapeutic benefit, a readily scalable, suspension, serum-free system was chosen for manufacturing of the DTX201 product. This system utilized a stable producer cell line (Pinnacle PCL) based on the HeLa S3 cell substrate. Advantages of the PCL platform for vector production include ease of scalable manufacturing, high vector yield and higher proportion of full length vector genome containing particles, as compared to other platforms. For the PCL platform, a potential perceived disadvantage is that the HeLa cell line was derived from a cervical carcinoma transformed by the genome of human papilloma virus type 18 (HPV18). To address this potential disadvantage, we characterized the presence of HPV18 sequences in DTX201 product via sensitive and quantifiable methods. We further examined whether transfer of HPV18 sequences could be observed in tissues of animals exposed to the DTX201 product. We demonstrate that HPV18 contaminants were detectable but not quantifiable in highly purified virus preparations. Importantly, HPV18 sequences were undetectable *in vivo* from mouse tissues extracted from multiple organs following intravenous delivery of DTX201. Taken together, these data support the safety of manufacturing high-quality gene therapy products such as DTX201 in PCLs derived from HeLa S3 cells.

459. AAV Gene Therapy Successfully Ameliorated Clinical Symptoms in Cystic Fibrosis G551D Ferrets

Cristian Ciobanu[1], William B. Guggino[2], Liudmila Cebotaru[1]

[1]Medicine and Physiology, Johns Hopkins University, Baltimore, MD,[2]Johns Hopkins University School of Medicine, Baltimore, MD

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis conductance regulator (CFTR). Developing a gene therapy for CF is still a critical need especially for patients who either are not approved for corrector therapy or do not tolerate it. One way to show definitively that gene therapy can be therapeutic is to rescue defective CFTR function in a relevant animal model that recapitulates, as closely as possible, human disease. To accomplish this, we used the ferret model bearing the G551D mutation which responds to the potentiator, VX-770, similar to humans. The goal was to assess whether dosing of AAV1/6-Δ27-264-CFTR vectors administered to G551D ferrets in the absence of proteasome inhibitors leads to widespread gene transfer, expression and phenotypic rescue. Most studies on CF gene therapy have targeted the lung. Here, we have taken a different approach targeted to treat several organs in a G551D ferret model of CF. We have chosen to use this model to study a distinctly different route of delivery whereby vector was both sprayed into the airway and injected systemically, testing the hypothesis that this dual route of delivery can transduce airways, lung, liver, pancreas, and ileumThe G551D ferrets were removed from VX-770 treatment 7 days before AAV treatment. 1 dose of 10^{11} vg of AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR was sprayed into the airways and injected intravenously into eight, 3-month-old male and female ferrets. 2-G551D and 2-wild-type ferrets were kept as controls. Animals were necropsied at 35 days post-instillation. AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR were detectable at $\geq 10^6$ vg/ug-genomic-DNA in trachea, bronchi, lung, ileum, liver and pancreas. mRNA expression was detected at $\geq 10^6$ copies/ug-genomic-DNA in trachea, bronchus, lung, pancreas and ileum and $\geq 10^5$ copies/ug-genomic-DNA in liver. Widespread CFTR protein expression was seen in the tissues of all the treated animals above levels measured in untreated wild-type and G511D ferrets. CFTR protein expression above untreated animal levels following a single dose of AAV1-Δ27-264-CFTR or AAV6-Δ27-264-CFTR suggests that transduction by AAV1 or 6 based vectors occurs in airways, liver ileum and pancreas of AAV treated G551D ferrets. The gross morphology of the treated ferrets resembled that of the normal ferrets compared to the untreated G551D ferrets. Minor amounts of mucous was present compared to untreated animals. The results are important in that we were able to transduce trachea, lung liver, pancreas and ileum despite the barriers associated with mucous plugging, AAV degradation and potential inhibitory factors in the sputum. Successful transduction of G551D ferrets suggests that systemic delivery of AAV may be the preferred route of delivery for a gene therapy for CF to reduce multiorgan disease. Transduction of the G551D ferrets in the organs affected in CF provide promise for rescue of multiorgan disease in CF.



460. Comprehensive Characterization of AAV Vectors' Genome Integrity Through Long-Read Sequencing

Ting Chen[1], Kirk Elliott[1], Amanda Zhang[2], Ayda Mayer[2], Lan Jin[1], Mi Shi[1], Olivier Danos[1], Ye Liu[1]

[1]Research and Early Development, REGENXIBO Inc., Rockville, MD,[2]Technical Operations, REGENXIBO Inc., Rockville, MD

Recombinant adeno-associated virus (AAV) is a promising *in vivo* gene delivery vector due to its safety and efficacy evaluated in current approved AAV products. It is of utmost importance to characterize the genome integrity of AAV vectors since defective vectors could compromise the effectiveness and safety of AAV gene therapy. In this study, we present a comprehensive methodology for the quantitative and visual assessment of packaged AAV genomes using the long-read sequencing technology. Our methodology utilizes the single-molecule real-time (SMRT) sequencing from PacBio for its high accuracy in analyzing AAV genomes. It accommodates sequencing data from both single-stranded AAV (ssAAV) and self-complementary AAV (scAAV). It is designed to facilitate accurate quantification of single molecules mapped to either vector sequence (i.e., transgene and ITR) or non-vector sequences (i.e., cis-plasmid backbone, trans plasmid, helper plasmid and host genome sequences). The entire analysis comprises several distinct features. 1. Long sequencing reads are aligned to a unified version of vector and non-vector sequences for each AAV sample, followed by graphic presentation of the AAV genome integrity using the Integrative Genomics Viewer (IGV) software. We further evaluate the ITR integrity and AAV genome truncation hotspots through graphical representation of read counts in each starting and ending alignment position throughout the AAV transgene region spanning from 5' ITR to 3' ITR. 2. A plot is generated to depict the distribution of lengths of reads that are aligned to the AAV transgene region. This enables us to assess whether the AAV genomes are primarily distributed at their expected full length or instead exhibit a specific concentration at other lengths (i.e., truncation or duplication). 3. We characterize full length AAV genomes as well as partial genomes that may consist of, for instance, 5' ITR and a partial transgene region or a partial transgene region followed by 3' ITR. We also calculate the compositions of non-vector sequences and chimeric reads arising from the recombination of vector and non-vector sequences. 4. Abundances of snapback genomes related to cis plasmid, trans plasmid, helper plasmid and host DNA sequences in the AAV population are quantified. 5. The ITR integrity is assessed by analyzing the composition of reads that contain both ITRs, 5' ITR only, 3' ITR only or no ITRs. Furthermore, we quantify ITR flip and flop configurations. We present the analysis of two ssAAV vectors expressing either a microRNA or an antibody fragment under the control of the CAG promoter. They exhibited 73%-83% full length genomes with 0.08%-0.14% non-vector sequence contamination. We also analyzed multiple scAAV vectors that were packaged into AAV8 or AAV9-variant capsids and expressed three antisense sequences fused with modified U7 small-nuclear RNA scaffold under the control of mouse U7 promoters. Our data showed 84%-85% full length genomes with 0.04%-0.16% non-vector sequence contamination in these scAAV vectors. The ssAAV vectors demonstrated flip and flop configurations (i.e., flip/flip, flip/flop, flop/flip, flop/flop) with ratios of approximately 1:1:1:1 and 1:1.5:1.5:1, whereas all the scAAV vectors presented a consistent ratio of approximately 4:1:1:4. This comprehensive analysis offers us a useful perspective on the quality of our AAV products, presenting valuable insights for further research and applications.

461. An Artificial Exonic Barcode System for AAV Capsid Selection

Matthew J. Burke[1], Xiufang Pan[1], Yongping Yue[1], Jin Han[1], Chady H. Hakim[1], Jun Li[2,3,4], Lei Jin[2,3,4], Shi-Jie Chen[2,3,4], Dongsheng Duan[1,5,6,7]

[1]Department of Molecular Microbiology and Immunology, University of Missouri, Columbia, MO,[2]Department of Physics, University of Missouri, Columbia, MO,[3]Department of Biochemistry, University of Missouri, Columbia, MO,[4]Institute for Data Science and Informatics, University of Missouri, Columbia, MO,[5]Department of Biomedical, Biological & Chemical Engineering, University of Missouri, Columbia, MO,[6]Department of Neurology, University of Missouri, Columbia, MO,[7]Department of Biomedical Sciences, University of Missouri, Columbia, MO

Capsid selection is a major determining factor for the efficacy and safety of adeno-associated virus (AAV) gene therapy. The short nucleotide (3 to 15 nucleotides) barcode system has been used to compare the transduction and expression of various AAV capsids using high-throughput sequencing and bioinformatic analysis. However, the short barcode system has some limitations. For example, the results cannot be validated by a different method. The system is unsuitable for *in situ* evaluation of transduction and expression at the single-cell level in tissues. Since the cDNA sequence and the gene sequence are identical, it is also impossible to rule out DNA contamination in the cDNA preparation completely. Further, different bioinformatic algorithms

may yield different outcomes. To overcome these limitations, we developed an artificial exonic barcode system. Specifically, we designed a set of artificial exons distinctive from the genomic sequences of commonly studied species, including humans, non-human primates, dogs, pigs, mice, rats, and rabbits. To accurately quantify the vector genome and the transcript copy number of the candidate AAV capsids, we designed and validated a series of exonic barcode-specific TaqMan qPCR assays. Using this system, we screened 11 AAV capsids in a mouse model of Duchenne muscular dystrophy and normal canines. Our results are highly consistent with the literature. The artificial exonic barcode system is a promising approach to identifying the best capsids for AAV gene therapy (Supported by NIH).

462. AAV Cell Surface Target Profiling Using Binding and Functional Cell Microarray Screening Technology

Claire Tebbutt[1], Beth Walker[1], Lucy Hanley[1], Clare Halliday[1], Elli Tudge[1], Lucinda Branton[1], Louis Sykes[1], Ruofan Wang[2], Giovanna Gayoso[2], Ashley Przygocki[2], Mark Aspinall O'Dea[1]

[1]Charles River Laboratories, High Peak, United Kingdom,[2]Charles River Laboratories, Rockville, MD

AAV (Adeno-Associated Viruses) vectors have emerged as a promising gene therapy. In 2019 > 200 clinical trials accounted for 24% of viral vector studies worldwide (1), however challenges remain in overcoming organ specificity, innate immunity, and dose-dependent toxicity (2). Here we describe a novel dual screening capability for AAV's, using the Charles River Retrogenix Cell Microarray technology, to enable identification of novel cell surface receptor interactions involved in virus sequestration and functional payload delivery. The Retrogenix Cell Microarray consists of over 6,500 full length human cell surface, secreted and heterodimeric proteins, over-expressed in HEK293 cells. Now expanded to include non-human plasma membrane proteins with ≤ 90% human homology in the extracellular domain, the array can be used to identify novel primary receptors, potential off-targets and assess in vitro / in vivo efficacy (3) and safety profiling (4) for gene therapeutics. Additionally, a functional screening method has been developed enabling AAV binding to specific cell surface receptors (5) and internalization (transduction) observed via reporter gene expression. WT AAV9 controls presented over X cell surface interactions when tested, allowing distinction between sequestration and transduction activity upon cell surface binding. Examples include KIAA0319L, a protein receptor required for AAV endocytosis, together with AAV attachment factors and co-receptors, such as fibroblast growth factor receptors (FGFR), heparan based molecules, solute carrier proteins, interleukins and neuropeptides. Functional and binding studies can facilitate candidate selection, demonstrate therapeutic specificity and de-risk future in vivo studies. This technology presents surface proteins in their natural environment, affording several advantages over conventional array technologies, including low false positive rates and high target specificity. AAV screening against Human and Non-Human arrays for potential off-target binding presents a unique strategy to help de-risk therapeutic candidates and provide supporting data for IND submissions. 1. Goswami R, et al. Gene Therapy Leaves a Viscious Cycle. Front Oncol.2019; 9:297. 2. Pupo A, et al. AAV vectors: The Rubik's Cube of Human Gene Therapy. Molecular Therapy. 2022; 30:12. 3. Freeth J, Soden J. New Advances in Cell Microarray Technology to Expand Applications in Target Deconvolution and Off-Target Screening. SLAS Discov. 2020; 25:2. 4. Shay T, et al. Human Cell Surface-AAV Interactomes Identify LRP6 as Blood-Brain-Barrier Transcytosis Receptor and Immune Cytokine IL3 as AAV9 Binder. BioRxiv pre-print. 2024. 5. Pillay S, et al. An Essential Receptor for Adeno-Associated Virus Infection. Nature. 2016. 530; 7588.

463. CBN-1100: A New and Differentiated Non-AAV Parvovirus Vector Designed to Overcome Current Gene Therapy Limitations

Sebastian Aguirre[1], Yoshifumi Hashimoto[1], Carolyn Pelletier[1], Melvys Valledor[1], Michael Rak[1], Yousuf Aqeel[2], Gillian Payne[2], Richard Matters[3], Shu-hao Liou[3], Joshua Merritt[3], Marc Abrams[4]

[1]Platform Development Team, Carbon Biosciences, Waltham, MA,[2]Analytical Development Team, Carbon Biosciences, Waltham, MA,[3]Process Development Team, Carbon Biosciences, Waltham, MA,[4]Carbon Biosciences, Waltham, MA

CBN-1100 is a new recombinant non-AAV parvovirus vector which overcomes many of the challenges associated with AAV parvovirus vectors: it offers ~1 kb more cargo capacity, minimal seroprevalence in humans, compelling systemic biodistribution and natural liver de-targeting. CBN-1100 leverages a wild type autonomous parvovirus capsid which is >80% divergent from all AAV capsids at the amino acid level. Among its advantages, the larger capacity offers the ability to deliver therapeutic transgenes and tissue-specific promoters which are out-of-reach for AAV. In this work, we demonstrate that CBN-1100 can efficiently encapsidate functional AAV2 ITR-flanked cassettes with an insert size of at least 5.5 kb, including large reporter genes and editing cassettes containing spCas9 and sgRNAs. CBN-1100 also demonstrates robust manufacturability; vector cell culture yields using our plasmid-based mammalian cell process are comparable to AAV industry standards at 40 liter bioreactor scale. Chromatography-free purification resulted in vector product with ~90% filled capsid by analytical ultracentrifugation and cryo-electron microscopy. Finally, we observed robust gene expression in several diverse human cell types, achieving transduction efficiencies of up to 90% with predictable kinetics and dose proportionality. The attributes of CBN-1100 suggest that viral vectors powered by non-AAV parvovirus capsids offer a path forward for numerous targets and indications that are undruggable using current gene therapy technology.

464. Chimeric and Rationally Designed Compact Promoters for Cardiomyocyte-Specific Gene Expression

Prasad R. Konkalmatt[1], Chris A. Reid[2], Melissa Van Pell[1], Samantha Jones[1], Ze Cheng[1], Joe Woods[1], Anastasiia Budan[1], Alex Aycinena[1], Mo Mandegar[2], Kathy Ivey[1]

[1]Tenaya Therapeutics, South San Francisco, CA,[2]Ascend GCTx, San Francisco, CA

Adeno-associated virus (AAVs) vectors have become among the preferred gene delivery vehicles for a broad range of human gene

therapy applications. AAVs have proven ability to transduce and provide durable gene expression in various organs and cell types, including cardiomyocytes (CMs), making them well suited for the treatment of genetic cardiomyopathies and other prevalent forms of heart diseases via gene replacement therapy as well as gene editing. The broad tissue tropism of AAV serotypes and their smaller packaging capacity calls for the development of shorter tissue-specific promoters to increase specificity in tissue targeting while maintaining a therapeutic levels of expression, potentially improve the safety profile, and enable AAV gene therapies for larger genes or sizeable genome editing constructs without exceeding the ideal packaging capacity of ~4.7 kb. As a novel strategy to develop a shorter CM-specific promoter, we created a library (n = ~1000) of 250 bp chimeric promoters and screened them for cardiac-specific gene expression. These chimeric promoters were constructed using the natural 500 bp proximal promoter regions of five genes that are preferentially expressed in the adult CM. The promoter chimeras fused to EGFP were independently screened for their magnitude of gene expression in human induced pluripotent stem cell-derived CMs (iPSC-CMs). The top twelve chimeric promoters were then packaged in AAV9 capsids and evaluated in mice and non-human primates for cardiac-specific expression versus a 400 bp cardiac specific promoter, hTNNT2. Sequence analysis of the top-performing chimeric promoter revealed enriched CM-specific transcription factor (TF) binding sites within the proximal region. Further trimming and introduction of additional CM-enriched TF binding-sites and known cis-regulatory elements resulted in expression comparable to the TNNT2 promoter when evaluated in mice, but with a much smaller footprint. As an alternative approach, we also used rational design starting with the proximal portion of the TNNT2 promoter to create short promoters with strong and CM specific expression. We began by identifying regions enriched for CM-specific TF binding in the TNNT2 proximal promoter from human, mouse, and chicken. Fusing the TF-enriched sequences with known TATAA box and transcription start site (TSS) elements resulted in <220 bp promoters that produced 0.57 ±0.08, 0.45 ±0.16, and 1.02 ±0.21-fold gene expression in the human iPSC-CMs compared to 400 bp hTNNT2 promoter. Overall, this strategic approach resulted in successful development of a suite of novel, shorter CM-selective promoters that produced varying degrees of gene expression in the mouse heart. These novel short promoters have the potential to facilitate creation of AAV cardiac gene therapies for larger genes of interest, as well as cardiac gene editing where extra space is needed for genome editing machinery, while maintaining CM specificity and without exceeding the packaging capacity of AAV vectors.

465. AAV9, AAVrh.10, and AAVrh.74 Exhibit Different Cell Type Tropisms in the Heart Following Systemic Delivery and AAV9 Mediates Superior Cardiomyocyte Transgene Expression in Murine and Non-Human Primate Models

Ze Cheng, Emilee Easter, Lindsey M. Rollosson, Emma Xu, Huanyu Zhou, Karl Doerner, JianMin Lin, Kathryn N. Ivey, Timothy Hoey, Laura M. Lombardi

Tenaya Therapeutics, South San Francisco, CA

Gene therapy is an emerging treatment option for both acquired and inherited cardiac disorders and targeting cardiomyocytes following systemic delivery is critical to enable gene therapy treatments for many cardiac conditions. Several natural AAV serotypes, including AAV9, AAVrh.10, and AAVrh.74, have been hypothesized to efficiently transduce cardiomyocytes *in vivo* and are being advanced in cardiac gene therapy programs. We, for the first time to our knowledge, studied heart transduction and transgene expression properties of AAV9, AAVrh.10, and AAVrh.74 head-to-head using cell type specific RNA reporters in both murine and NHP models. In mice, all three serotypes show similar transduction to the heart as measured by viral genome DNA load, however, AAV9 generates higher expression level of cardiomyocyte-specific RNA reporter transcripts than the other two serotypes manufactured in parallel on the same platform. We also performed a similar study with fibroblast-specific RNA reporter transgenes and the results show that, in contrast to cardiomyocyte transgene expression, all three serotypes mediate transgene expression in fibroblasts with similar efficiency in mice, highlighting the difference in their cell type tropisms. We further investigated the relationship between cell type specific transgene expression level and cardiac gene therapy efficacy by treating a cardiomyocyte-autonomous cardiomyopathy mouse model with the same gene editing based therapeutic cassette delivered by AAV9, AAVrh.10, and AAVrh.74 at the same dose. The degree of efficacy, which was highest with AAV9, correlated with *in vivo* cardiomyocyte transgene expression level and not with viral genome DNA load in the heart. Finally, we measured heart transduction and cardiomyocyte transgene expression of all three serotypes in Cynomolgus monkeys and observed similar trends as those achieved in mice, namely that AAV9 mediates the highest level of cardiomyocyte gene expression. Together, our work demonstrates that AAV9 more efficiently expresses transgene in cardiomyocytes following systemic administration compared to AAVrh.10 and AAVrh.74. Our results also highlight that it is crucial to use cell type specific reporter cassettes while performing comparison studies to support capsid selection for cardiac and other categories of gene therapy programs, as failing to do so could lead to the risk of misguided selection.

466. Designing Therapeutic Recombinant AAV Vectors Using *In Silico* Vector Modeling

Ethan Waple[1], Jennifer C. Gifford[1], Sarath Mandava[1], Nicholas Ketz[2], Alpha Diallo[2], Amicia D. Elliott[2], Adam S. Cockrell[1]

[1]Solid Biosciences, Charlestown, MA,[2]Form Bio, Austin, TX

When designing safe, efficacious, and cost-effective AAV gene therapies, early R&D seeks to determine the best vector configuration with the intended full-length genetic payload from thousands or even millions of possibilities. Genetic cargo packaged within a single production lot can be a heterogenous population of recombinant AAV vector particles comprising a combination of different molecular species. *In silico* vector modeling may support early identification of *cis* elements in genetic cargos that may shift vector productions to the desired homogenous population of therapeutic molecules. We used long read sequencing to profile the molecular species in early discovery research grade rAAV therapeutic vector productions which revealed the presence of intended full-length vector species as well as varying amounts of unintended molecular species including

vector truncations, chimeric molecules, molecules containing plasmid backbone sequences, and a variety of double-stranded vector molecules (i.e. snap-back genomes). *In silico* modeling of the rAAV vector using the FORMsight[AI] artificial intelligence platform revealed specific *cis* nucleotide elements in promoter and intron regions that exhibit high propensities for truncations to occur. Further, some *cis* nucleotide elements align with predicted secondary structures in the single-stranded rAAV DNA molecules. Modifying the promoters and introns resulted in decreased truncation propensity and decreased secondary structures. We hypothesize that long-read sequencing analysis of the modified rAAV vectors will validate a shift to a higher proportion of full-length single-stranded rAAV molecules packaged into vector particles, thereby resulting in a shift to a more homogenous population of vector particles containing therapeutic transgenes. Overall, utilizing *in silico* vector modeling to assess thousands and potentially millions of vector configurations prior to *in vitro* and *in vivo* testing could dramatically improve workflow efficiency resulting in a more cost-effective therapeutic for gene therapy applications.

467. Rational Engineering of the AAV P5 Promoter Improves Vector Purity

Stephen M. Winston[1], Mark A. Brimble[2], Isaiah Reeves[2], Yunyu Spence[1], Pei-Hsin Cheng[2], Christopher L. Morton[1], Andrew M. Davidoff[2]

[1]Department of Surgery, St. Jude Children's Research Hospital, Memphis, TN,[2]St. Jude Children's Research Hospital, Memphis, TN

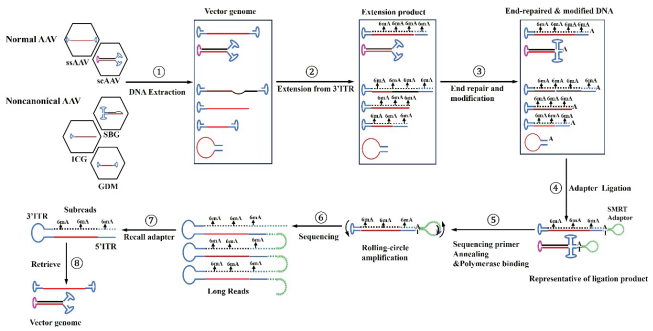
Previous work has demonstrated that both P5- and ITR-associated nucleic acid contaminants from plasmids used to produce recombinant adeno-associated virus (rAAV) vectors can be expressed *in vivo*, highlighting a need to further characterize their implications and develop strategies to increase vector purity. These sequences are encapsidated within fully functional virions, resulting in transcription and translation of undesired plasmid-derived sequences. We hypothesized the packaging of contaminant plasmid sequences is related to the P5 promoter structure and Rep gene expression during production, and that by manipulating critical elements of the P5 promoter and modulating Rep expression, we can reduce the encapsidation of these contaminant sequences. To prevent this encapsidation, we mutated the Terminal Resolution Sequence (TRS) loop mimic on the AAV2 P5 promoter. We have previously shown that placing a spacer between the P5 TRS loop and Rep protein Binding Elements (RBE) reduces its encapsidation three fold, however, it is still present at a relatively high quantity. By directly mutating this site we can reduce encapsidation of nearby DNA by an additional ten-fold. This improvement in purity coincides with a modest reduction in overall viral titer. Rep westerns and fluorescent reporter assays highlight changes in promoter activity correlated with changes to vector titer and purity. These assays were used to identify the implications of promoter modifications on promoter function. Modifications which improved purity and had the most detrimental effect on viral titer, had the strongest impact on promoter activity. Specifically, we observed decreased large Rep expression and increased small Rep expression. We then found that additional modifications to compensate for altered promoter activity rescued viral titer, suggesting these assays may be relevant in establishing and optimizing conditions for rAAV production. These modified promoters may prove to be viable alternatives to the canonical AAV2 P5 routinely used in rAAV production. Large scale production, vector characterization and *in vivo* assays with these new AAV promoters are currently underway. In conclusion, rAAV contamination can be reduced by rationally engineering the P5 promoter. Our findings have implications for making safer, purer products for rAAV gene therapy.

468. Thorough Molecular Configuration Analysis of Non-Canonical AAV Genomes in AAV Vector Preparations

Junping Zhang[1], Xiangping Yu[2], Matthew Chrzanowski[2], Jiahe Tian[3], Derek Pouchnik[4], Ping Guo[5], Roland W Herzog[1], Weidong Xiao[1]

[1]Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN,[2]Nikegen LLC, Wynnewood, PA,[3]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY,[4]School of Molecular Biosciences, Washington State University, Pullman, WA,[5]Lewis Katz School of Medicine, Temple University, Philadelphia, PA

The unique palindromic inverted terminal repeats (ITRs) and single-stranded nature of adeno-associated virus (AAV) DNA are major hurdles to current sequencing technologies. Due to these characteristics, sequencing non-canonical AAV genomes present in AAV vector preparations remains challenging. To address this limitation, we developed Thorough Molecule Configuration Analysis of non-canonical AAV genomes (TMCA-AAVseq). TMCA-AAVseq takes advantage of the documented AAV packaging mechanism, in which encapsidation initiates from its 3'-ITR, for AAV sequencing library construction. Any AAV genome with a 3'-ITR is converted to a template suitable to adapter addition by Bst DNA polymerase mediated extension reaction. This extension reaction helps fixing ITR heterogeneity in AAV population and allows efficient adapter addition to even noncanonical AAV genomes. Additionally, during the second strand extension, dATP is replaced with N6-methyladenine (6mA), this can efficiently distinguish from the new synthesized strand and original sequence. A flowchart of the library preparation and subsequent sequencing by TMCA-AAVseq is depicted in Figure 1. The resulting library maintains the original AAV genome configurations without introducing undesired changes. Subsequently, long-read sequencing can be performed by the Pacific Biosciences (PacBio) single molecule, real-time (SMRT) sequencing technology platform. Finally, through comprehensive data analysis, we can recover canonical, non-canonical AAV DNA, and non-AAV vector DNA sequences, along with their molecular configurations. The method is a robust tool for profiling thorough AAV-population genome. TMCA-AAVSeq can be further expanded and applied to all parvoviruses and their derivative vectors.



469. Engineering of AAV Replication Protein

Jixin Liu[1,2], Conradin Baumgartl[1,2], Dirk Grimm[1,2,3]

[1]BioQuant, Heidelberg University, Heidelberg, Germany,[2]Department of Infectious Diseases/Virology, Section Viral Vector Technologies, Medical Faculty, Heidelberg University, Heidelberg, Germany,[3]German Center for Infection Research (DZIF) and German Center for Cardiovascular Research (DZHK), Heidelberg University, Heidelberg, Germany

Adeno-associated virus (AAV) has become the most popular viral vector for therapeutic gene transfer in humans. However, numerous questions surrounding the biology of the wild-type virus persist, restricting the streamlining and upscaling of AAV vector manufacturing. A particularly enigmatic step is the replication and packaging of the recombinant AAV DNA genome, in which the four pleiotropic Rep(lication) proteins play pivotal roles that are only partially understood. Previous research including work from us showed that the Rep proteins of the most studied AAV serotype 2 (AAV2) are compatible with the inverted terminal repeats (ITRs) of several other serotypes. Hence, they can support the replication and encapsidation of an embedded genome, with the notable exception of the AAV5 ITRs that differ significantly in sequence and structure. Nonetheless, it is possible that wild-type Rep proteins, while adapted evolutionarily for the processing of wild-type viral genomes, may only function suboptimally during replication and/or packaging of recombinant AAV DNA. Moreover, they might also perform subpar in combination with synthetic ITRs that have been engineered or evolved independently, and that may ultimately replace the wild-type AAV2 ITRs which are used in most of the current vectors. Likewise, with the growing number and diversity of AAV capsid variants that were designed or enriched for certain properties, the likelihood concurrently increases that wild-type Rep proteins will no longer mediate an optimal capsid-genome interaction and DNA packaging. Altogether, these considerations have motivated us to adapt our previously established pipeline for DNA family shuffling of AAV capsid proteins to the Rep proteins as well, with the aims to: 1) improve the production yield of wild-type and novel AAV capsids, 2) reduce the proportion of empty capsids during the production process, 3) enhance the replication and/or packaging of recombinant genomes based on different AAV ITRs, and 4) decode the mechanisms, sequences and structures that underlie Rep-capsid and Rep-ITR interactions. To this end, we have shuffled *rep* genes from a wide variety of naturally occurring AAV isolates, resulting in complex libraries for subsequent interrogation through iterative *in vitro* screening for the aforementioned enhanced properties, using a collection of AAV genomes carrying wild-type or synthetic ITRs as templates for viral DNA replication and packaging. The enrichment of shuffled Rep variants with desired properties is comprehensively monitored at high quality and quantity using next-generation sequencing pipelines, comprising PacBio and Nanopore sequencing, which we have adapted and optimized in parallel for this purpose.The results of this work are expected to benefit our understanding of natural AAV biology including genome replication and packaging, while also enabling the improvement of the components and processes required for large-scale AAV manufacturing.

470. Advancing Recombinant AAV Manufacturing: TESSA® Platform for Scalable and Efficient Gene Therapy Production

Weiheng Su[1], Chloe Fustinoni[1], Marek Kucej[1], Pablo Monje[1], Cristina Araujo-Cuevas[1], Michael White[1], Louise Montgomery[1], Daniel Chow[1], Matthew Peckett[1], Qian Liu[1], Yi-Hsin Fan[2], Yu-Pin Su[2], Bibek Ghosh[2], Man-shiow Jiang[2], Ryan Cawood[1], Weimin Valenti[2]

[1]OXGENE, Oxford, United Kingdom,[2]WuXi Advanced Therapies, Philadelphia, PA

Recombinant adeno-associated virus (rAAV) vectors hold significant promise for gene therapy; however, challenges persist in achieving efficient and scalable production. This study investigates the 'Tetracycline-Enabled Self-Silencing Adenovirus' (TESSA®) platform, offering a robust solution for rAAV manufacturing devoid of adenovirus contamination. TESSA® provides a versatile approach to rAAV production, utilizing either the TESSA® Duo, which involves two vectors for AAV rep/cap and the rAAV transfer genome, or the TESSA® Pro method, which employs co-infection with existing rAAVs for propagation. These approaches empower researchers to produce cost-effective, scalable, and rapid rAAV vectors. The TESSA® Duo method, requiring the construction of two TESSA® vectors, proves highly effective, yielding over 30 times more rAAV compared to the triple transfection process across various serotypes, including AAV1-9 & rh10, with productivities exceeding 1E+6 vector genome copies (GC) per cell. Scaling up production in 50 L and 200 L bioreactors using TESSA® achieves impressive yields of >7E+11 GC per mL of cell culture and >1E+17 GC of Drug Substances for rAAV6 and rAAV2. In contrast, the TESSA® Pro approach streamlines rAAV propagation by eliminating the need to encode the rAAV transfer genome within a second TESSA® vector. Co-infection with HEK293 cells using rAAV seed stocks and TESSA® RepCap enables >5,000-fold amplification of input rAAV materials, yielding up to 4E+11 - 2E+12 vector genome copies (GC) per mL of cell culture. TESSA® Pro proves particularly efficient for propagating AAV serotypes that exhibit high-transduction efficiency in HEK293 cells, including AAV2 and AAV6, enabling high rAAV productivity from a relatively low AAV vector input of 50-250 GC per cell. Critical observations indicate that the TESSA® Pro process exhibits high efficiency in rAAV genome packaging, generating up to 90% full capsids before purification. This process is presumed to expedite the replication and packaging of the single-stranded rAAV genome post-nuclear entry, bypassing the conventional requirement for rescuing the AAV ITR embedded within the double-stranded DNA structure associated with other rAAV manufacturing systems. To ensure the production of clinically viable rAAV gene therapy drugs

and facilitate product approval, the TESSA® technology integrates in-depth in-house testing capabilities, including biosafety assessments, viral clearance, and product release testing.

471. A Novel Dual-Isotope Labeling Method for Quantification of AAV Tissue Biodistribution and Cellular Internalization Following Systemic Injection

Hongzhi Wang, Ran Li, Shraddha S. Sadekar, Amrita Kamath, Ben-Quan Shen

Preclinical and Translational Pharmacokinetics and Pharmacodynamics (PTPK), Genentech Inc., South San Francisco, CA

Introduction: An understanding of the biodistribution profile of different components of Gene therapy products following in vivo administration is an important element of the nonclinical development program. Current biodistribution studies emphasize vector genome as the surrogate for PK analysis of AAVs. However, the AAV capsid as an essential component involved in cell binding, internalization, and trafficking within the targeted cell cannot be overlooked. Radioisotopes are ideal tracers with high sensitivity for in vivo imaging and have been applied to AAV capsid modification. Limitations remain as the tracer could not provide detailed distribution on tissue level. The quantification method falls short on distinguishing the AAV been transduced into the cell from the vectors remaining in extracellular matrix. Such limitations may lead to inaccurate PK/PD prediction.Methods/Results: We developed a dual isotope labeling strategy combining iodine-125 (non-residualizing) and indium-111 (residualizing) radionuclides to the capsid to obtain the information regarding both capsid distribution and cellular uptake of AAV vectors. Each capsid was covalently conjugated with around ten 125I or four 111In with high radiochemical purity (>98%). Size measurement by DLS and SEC-HPLC verified AAV capsid's structural integrity and physical property remain intact by the conjugation chemistry. In vitro studies demonstrated the isotope conjugation didn't alter the biological activity of the labeled AAV vectors. The labeling method has been successfully applied to most of wild type and engineered AAV capsids. Dual isotope labeled AAV9 and AAV-PHP.eB as tool molecules has been systematically injected to C57BL/6 mice, followed by tissue collection and counting 125I and 111In individually. Based on the mechanism, 125I represented the intact AAV vectors mainly located in extracellular matrix, while 111In reveal the location of both intact and dissociated AAV vectors in both interstitial spaces and cells. The difference of 125I and 111In radioactivity elucidated the cellular uptake of AAV vectors. From the results, AAV capsid biodistribution profile provided by 111In was consistent with previous reports. Similar tissue biodistribution patterns were observed in major organs, where both AAV serotypes showed accumulation at early time point in highly perfused organs such as liver and spleen, and AAV-PHP.eB exhibited a remarkable elevated brain penetration. Analyzing the cellular uptake result derived by 125I/111In together further reveal detail spatial distribution information in collected organs and tissues. While Liver and spleen have highest capsid accumulation (~40% at day 1), the internalized capsid is relatively low (~12% at day 1), indicating AAVs was mainly located in extracellular matrix of high perfused organs at early time point without entering into the cells. qPCR result also shown a better correlation with cellular uptake corrected result than with total capsid distribution. Conclusion: The dual-isotope labeling method not only trace capsid biodistribution but also provides valuable insights into AAV vector spatial distribution in the context of tissue microenvironments. The results demonstrated dual-isotope labeling method as an efficient tool to elucidate a complete AAV distribution profile, thus facilitating lead candidate selection, capsid optimization and providing guidance on the administration route selection and human dose prediction. Utilizing two SPECT isotopes, this labeling strategy holds promise for integration with SPECT/CT imaging, providing a noninvasive imaging method for preclinical pharmacokinetic studies.

472. Single-Nuclei RNA Sequencing Enables High Resolution Quantification of Transgene Expression in the CNS After AAV Delivery

Kyle Ford[1], Diogo Pessoa[1], Yian Khai Lau[1], Brian Fochtman[1], Zoe Zhaostein[1], Mei Zhu[1], Jessica Willen[1], Zikun Wang[1], Ellen P. S. C. Furber[2], Audrey Lee-Gosselin[2], Fares Bassil[2], Tao Yang[1], Edmund C. Schwartz[1], Ben Shykind[1], Anindya Sen[1], Mansuo Shannon[1], Sitharthan Kamalakaran[1]

[1]Prevail Therapeutics, New York, NY,[2]Prevail Therapeutics, Indianapolis, IN

Efficient delivery of genetic cargo to disease relevant cell types is a ubiquitous challenge in gene therapy. Adeno-associated virus vectors (AAVs) have been used to deliver therapeutic cargos to the central nervous system (CNS), but their efficacy is often limited by poor biodistribution or expression in the target cell types. Consequently, there is a pressing need for methods which can quantify AAV transgene expression with cell-type level resolution. Here, we developed a single nuclei RNA-seq (snRNA-seq) methodology for tracking AAV tropism *in vivo.* We isolated nuclei and generated single nuclei transcriptomes using commercially available reagents, enabling flexible processing of flash-frozen tissue samples. To improve transgene detection, we developed a custom transgene amplification protocol. By amplifying the transgene from the 10x cDNA pool, we can detect orders of magnitude more transgene molecules compared to the unamplified control. We modified the standard bioinformatics pipeline to address unique challenges in AAV transgene quantification. Our pipeline retains high quality nuclei by adopting strict quality filtering measures and doublet removal. To avoid bias due to RNA content difference between cell types, we chose to normalize the transgene expression using a set of stably expressed genes across cell types. We also estimated the noise level due to the ambient RNA and adaptively remove background in a cell type-specific manner. Comparing to standard sequencing and analysis protocols, our in-house methodology demonstrated higher sensitivity and allowed more confident quantification of transgene expression rates across cell types and treatment groups, enabling stratification of capsids/promoters by neural specificity. We then used this methodology to profile transgene expression in the mouse CNS, post injection of various serotypes/promoters via intracerebroventricular (ICV), intravenous (IV), or intracisterna magna (ICM) route of administration. By generating 100,966 single-nuclei transcriptomes, our data showed that ICV delivered AAV9 transduced various cell types in the mouse brain, including neurons, microglia, astrocytes, and oligodendrocytes, while an IV delivered engineered capsid transduced various cell types in the spine cord. All

tested promoters drive robust expression in neurons. Expanding the snRNA-seq methodology to non-human primate, we profiled 4,709 nuclei after ICM delivery of AAV9, quantifying the transduction and expression for various cell types in the spinal cord. We compared the differences in cell-type abundances between NHP and mice, showing a reduced neuron/non-neuron ratio in the NHP spinal cord. In conclusion, we established an efficient single-nuclei RNA sequencing method and collected initial mouse and NHP datasets in the CNS post AAV administration. Looking ahead, we are exploring methods to improve capture of rare cell types and to quantify genome editing with cell-type level resolution. We anticipate cell-type resolved assays will be key to evaluating novel capsids, promoters, and regulatory elements for next generation AAV therapies.

473. Optimization of AAV Production Using New Statistical Design of Experiment: Space Filling Designs

Nida Sayed-Zubairy

Boston Institute of Biotechnology, LLC, Southborough, MA

Applications of adeno-associated virus (AAV) in gene therapy has increased significantly over the past two decades due to its remarkable safety profiles and efficiency of gene delivery into various tissues. Demand for the supply of recombinant AAV vectors has also increased to fulfill pre-clinical and clinical needs. Low titer and process scalability remain two key issues in AAV manufacturing. Productivity of AAV vectors is often affected by the AAV serotype, cell culture type, the size of Gene of Interest (GOI), cell density, transfection method, plasmid DNA concentration/ratio and other factors. Based on risk assessment of factors that may impact AAV production, we have evaluated process parameters using a 24-run Space Filling Design generated by the JMP Statistical Software. Space-Filling Designs (SFDs) are a class of experimental designs created with the objective of covering the entire design space as completely as possible; this in turn allows more accurate modelling of complex response surface behavior typically found in bioprocesses. SFDs are very flexible in structure, do not require any prior assumptions about response surface shape and typically require fewer runs for larger numbers of factors than the commonly used response surface designs. The commonly used classical and optimal response surface designs primarily distribute the design points near the boundary of the experimental region such that little or no data is collected in the interior. As a result, the experimenters have no direct knowledge of the kinetic behavior inside the design region. Without data describing the interior kinetic behavior it is not possible to build accurate response surface models. In this study, we will describe the value of SFDs and how to establish critical process parameters (CPP) and operating ranges for optimum and robust AAV production in suspended HEK293 cells in Ambr® 15 Microbioreactor System.

474. Comparison of Common Methods for Cell Transfection and rAAV2 Purification with Commercially Available Options

Mariana Santana Dias, Gabriela S. Almeida, Rafael F. Azevedo, Hilda Petrs-Silva

Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Adeno-associated viral vectors have emerged as a powerful tool for human gene therapy. Nowadays, they represent the most common viral vectors for in vivo gene therapy applications. They are safe, with low toxicity and immunogenicity, and promote stable expression with high transduction efficiency for several tissues, especially due to its diversity of serotypes. Since the first cloning of the AAV genome, the production process of rAAV vectors has been developed and optimized over time. However, it is still laborious, costly, and vector titers need to be improved, especially for clinical applications. Apart from traditional, firstly applied, methods of cell transfection and vector purification for rAAV production, nowadays there are several commercially available options that require less laboratory apparatus. In the process of establishing bench-scale rAAV production in our laboratory in Brazil, we have compared two different methods of cell transfection and three methods of vector purification. Transfection was examined with calcium phosphate and a commercially available transfection kit for rAAV production (Gibco #A51217), which has been adapted from cell suspension to a cell factory. Furthermore, we compared purification with iodixanol gradient followed by anion exchange FPLC with commercial kits (Takara #6232 and Norgen #63250), using calcium phosphate as the transfection method. Each rAAV2 production was carried out in a 4-layers cell factory. Results indicated a higher transfection efficiency of the calcium phosphate method (with the transduced area of 27.5 %) as compared to the commercial option (8.0 % of transduced area) at 24 hours after transfection, which led to higher rAAV titers after a similar purification method and analysis by qPCR (5.97E+07 vs. 2.52E+06 vg/mL). When comparing purification strategies, Norgen kit (2.96E+08 vg/mL) reached the highest particle titer by qPRC analysis, followed by iodixanol gradient + FPLC (5.97E+07 vg/mL) and then the other commercial kit, by Takara (1.26E+07 vg/mL). Overall, among all tested methods, transfection with calcium phosphate and rAAV purification with Norgen kit or iodixanol gradient + FPLC provided higher rAAV2 titers, indicating that the classical non-commercial is still very efficient for rAAV production when compared to expensive commercially available options.

475. Targeting Gene Activation to Specific Cell Populations Using Convolutional Neural Networks

Michael Leone[1], BaDoi Phan[1], Jing He[2], William R. Stauffer[2], Leah C. Byrne[3], Rebecca P. Seal[2], Andreas Robert Pfenning[1]

[1]Computational Biology, Carnegie Mellon University, Pittsburgh, PA,[2]Neurobiology Department, University of Pittsburgh Medical Center, Pittsburgh, PA,[3]University of Pittsburgh, Pittsburgh, PA

Gene therapies have the potential to transform healthcare, but are often hampered by a lack of specificity. These limitations are especially problematic in the central nervous system, where even a single tissue

can be composed of dozens of distinct neuron subtypes with different functions. Although systematic screening of candidate cell type-specific enhancers has yielded useful tools, the low success rate and time consuming nature of the experiments prevents these techniques from being applied more broadly. To improve the specificity of gene activation to a population of interest, we have developed the SNAIL approach (specific nuclear-anchored independent labeling). SNAIL uses an ensemble of convolutional neural networks trained on open chromatin data to prioritize the cell type-specificity of candidate enhancers. We have applied our approach to successfully label a variety of rare or subtle neuron subtypes in the cortex, the striatum, and spinal cord in a combination of rodent and non-human primate experiments. In parallel to the development of computational approaches, we paired massively parallel reporter assays with AAVs to measure the specificity of hundreds of enhancers in vivo in a single experiment. As a proof of concept, we have applied SNAIL to attenuate features of chronic pain without influencing baseline responses to touch.

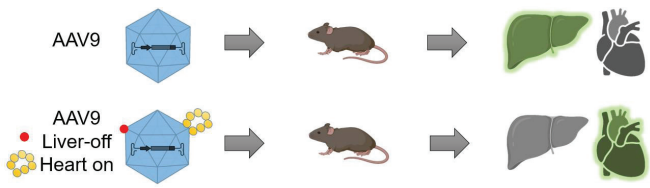
Wednesday Posters: AAV Vectors - Capsid Engineering

476. Liver-Off and Heart-On Tropism with Engineered AAV9 Capsid

Huachun Liu[1,2], Reynette Estelien[1], Julio Sanmiguel[1], Kevin Bersell[3,4,5], Hiroko Wakimoto[4], Christine Seidman[4,5,6], Nerea Zabaleta[1,2]

[1]Schepens Eye Research Institute, Mass Eye & Ear, Boston, MA,[2]Department of Ophthalmology, Harvard Medical School, Boston, MA,[3]Department of Medicine, Brigham and Women's Hospital, Boston, MA,[4]Department of Genetics, Harvard Medical School, Boston, MA,[5]Cardiovascular Division, Brigham and Women's Hospital, Boston, MA,[6]Howard Hughes Medical Institute, Chevy Chase, MD

Adeno-associated viruses (AAVs) are indispensable in gene therapy, providing a safe and precise means to deliver therapeutic genes and treat a variety of genetic disorders. However, when used for heart gene therapy, natural serotype AAV9 has limited effectiveness in transducing cardiac tissues and exhibits substantial off-target transduction in the liver, which would introduce potential liver toxicity during AAV9-based gene therapy. Thus, generating a cardiac tissue-specific variant serotype would tremendously boost the efficiency and safety of AAV9-mediated heart repair. To address this challenge, we engineered the AAV9 capsid by integrating a lab-developed liver-off mutant (GGSS to GAST at VP1 from 266 to 269) with muscle-targeting peptides (MyoAAV4E and 2A) developed by another group. In the variable region VIII of AAV9, the MyoAAV4E and 2A peptides, which include RGD domains, are specifically inserted to improve muscle targeting, with 2A developed in mice and 4E in non-human primates. When delivered systemically, our GAST+2A and GAST+4E variants significantly reduce liver transduction by 0.14- and 0.04-fold, increasing heart-targeting efficiency by 49- and 61-fold at transgene mRNA expression level, respectively. The modified capsid holds great promise for heart gene therapy, allowing for significantly reduced dosage and alleviating liver toxicity.



477. Approaches for Developing an AAV Gene Therapy Candidate: A Hemophilia A Case Study

Hui Wang

Genevoyager, Philadelphia, PA

Currently, commercially available AAV products can cost millions of dollars per patient. Due to high prices and limited production capabilities, only a few hundred patients can receive treatments each year, leaving a considerable number without access to medication. In the pursuit of developing safe, effective, affordable, and accessible AAV gene therapies, Genevoyager offers one-stop services for gene therapy product development. Using Hemophilia A (HA) as a case study, we highlight various approaches: 1) Optimization of liver-specific promoter: We engineered a liver-specific promoter, ATH2, demonstrating higher FVIII activity *in vitro* as compared to traditional HLP promoters (approximately 1.2 and 2.2-fold increase in HepG2 and HUH7). In mouse studies, the hFVIII expressed under ATH2 promoter exhibited approximately 9-fold and 2.5-fold increase at 2 weeks, while displaying approximately 1.7-fold and 1.3-fold increase at 10 weeks, as compared to HLP and TTRm promoters, respectively. 2) FVIII gene modification for improved secretion and activity: We inserted an additional segment between the A2 and A3 domains, resulting in a 2 to 3-fold increase in FVIII secretion and activity levels *in vivo*. 3) Capsid engineering for enhanced yield: We mutated key sites of the AAV capsid, leading to a 2 to 3-fold increase in yield. 4) Optimization of production system: We utilized our proprietary Bac/Sf9 system for AAV production. In comparison to the traditional Sf9 system, there is a significant 4 to 30-fold increase in yield and 2 to 5-fold increase in FVIII activity. By leveraging the exclusive liver-specific promoter, optimized processes, and the innovative Bac/Sf9 system, Genevoyager introduces comprehensive approaches for developing a promising and affordable gene therapy product for Hemophilia A. These highlights also demonstrate our capability to provide one-stop services for the development of gene therapy products.

478. Engineered Enhancer Bolsters AAV Tropism to the Heart

Lauren E. Switala[1], Lin Di[1], Daria Fedyukina[2], Andrei Maiseyeu[1]

[1]Case Western Reserve University, Cleveland, OH,[2]BioHeights, Cleveland, OH

Several adeno-associated viral (AAV) vector strategies for cardiac muscle targeting have been developed to enable disease-modifying treatments for heart failure and genetic cardiomyopathies. However, significant off-target accumulation of intravenously (i.v.)-delivered AAV has led to the high effective dose requirement even for novel cardiac-specific AAV serotypes. We engineered cargo-less polymeric

nanoparticles that drastically improve heart delivery of AAVs when administered prior to AAV i.v. injection. Our lead formulation (referred to as ePL, enhancer polymer) increases selectivity of AAVs to the heart and de-targets them from the liver. Serotypes known to have high (AAVrh.74) and low (AAV1) heart tissue tropisms were tested with and without ePL. We demonstrated up to an order of magnitude increase in heart-to-liver accumulation ratios in ePL-injected C57BL/6 mice. Key advantages of ePL include its excellent safety profile and complementarity to serotype engineering strategies. ePL does not need to be co-formulated with an AAV product, thus simplifying IND CMC requirements. Our study introduces an exciting possibility to direct a variety of AAV vectors to the heart at significantly reduced i.v. doses.

479. Novel Engineered Cardiac-Specific AAV Vectors via Directed Molecular-Evolution in Human-Relevant Model Systems Outperform Current Benchmark Vectors for Clinical Cardiac Gene Therapy

Gregor Habeck[1], Joerg Schweiggert[1], Michael Smuglov[1], Johanna Ferdinand[1], Dorothea Kehr[1], Kristin Cimen[1], Xenia Kramp[1], Hugo A. Katus[1], Marc Johannes Lerchenmueller[1], Matthias Mietsch[2], Lena Erffmeier[2], Rabea Hinkel[2], Patrick Most[1], Martin Busch[1]

[1]AaviGen GmbH, Heidelberg, Germany,[2]German Primate Center, Goettingen, Germany

Introduction and Objective: Recombinant adeno-associated viral (rAAV) vectors currently used in gene therapy (GT) clinical trials for hereditary and acquired heart failure (HF) have a high potential for clinical toxicity due to a lack of cardiac transduction specificity. For example, despite numerous cases of fatal liver failure by rAAV9-based gene therapy medicinal products (GTMPs) in patients due to the predominant hepatic tropism of rAAV9, the capsid continues to be a vector of choice due to its high in vivo myocardial transduction efficiency. Against this background, we have developed an integrative experimental-bioinformatic next-generation Cardiac AAV Platform (next-CAP) to engineer synthetic rAAV capsids that meet the clinical demand of high-performant in vivo cardiac transduction specificity as a foundation for safe and precise cardiac GTMPs against various HF forms. *Approach and Results:* Feeding novel mammalian heart-derived AAV capsid sequences into a genetically-engineering platform that leverages DNA-shuffling and peptide-display methodologies, we generated a highly diverse AAV capsid library with over 200 million variants. This library was subjected to an iterative cross-species in vivo evolution procedure by intravenous injections in mice, farm pigs and non-human primates (NHP) to identify synthetic AAV capsids with optimal in vivo cardiac transduction specificity and efficacy. In order to ensure reliable AAV capsid identification, we employed unique molecular identifier to combine long- and short-read sequencing of AAV genomes to ultimately select a top-100 cardiac-enriched AAV capsid portfolio against a variety of clinically relevant off-target organs, including the liver, central and peripheral nervous system or organs of the immune system (n=3 individual library injections in mice, pigs and NHPs). Subsequent co-injection of this top-100 vector portfolio in mice, pigs and NHPs (n=3 each) with current clinical and pre-clinical vector benchmarks for cardiac GT (e.g., rAAV9, rAAV2i8, rAAVrh74, rAAV2-THGTPAD and myo-AAV) yielded a top-10 portfolio of novel heart-specific AAV capsids that outperformed these benchmarks in terms of tissue-specificity in the human-relevant farm pig and NHP model systems. Our novel cardiac-specific AAV capsids displayed a homogeneous distribution pattern of the left and right ventricle of pigs and NHPs with an up to 20-fold higher heart/liver vector genome copy (vgc) ratio compared to all benchmarks as well as optimal de-targeting of other clinically relevant tissues, such as dorsal root ganglions, brain or spleen in NHPs. In addition to the clinically desirable on- and off-target biodistribution profile, our technical workflow concurrently enriched HEK293 cell high-titer producing cardiac-specific rAAV capsids to eventually enable scalable large-batch manufacturing to meet the demand for rare and common cardiac diseases alike. *Conclusion:* Our versatile next-CAP pipeline enabled the development of novel synthetic cardiac-specific rAAV capsids characterized by a high-performant transduction specificity and efficacy of human-sized pig and NHP hearts, respectively, that clearly outperformed available AAV benchmark vectors yet proposed for cardiac GT. In light of their inherent good producibility, our vector portfolio puts us into the ideal position to effectively assess the best therapeutic hear-specific rAAV-promoter/target systems for subsequent assessment of dose-expression, -efficacy, and -toxicity relationships to move our therapeutic HF GTMP pipeline towards clinical trials.

480. Engineered Cardioskeletal-Directed AAV Capsids That Detarget the Liver

Widler Casy[1], Tiffany Willacy[2], Jen Gifford[2], Jenny Marlowe[1], Cockrell Adam[1]

[1]R&D, Solid Biosciences, Charlestown, MA,[2]Solid Biosciences, Charlestown, MA

Gene therapy remains a promising therapeutic path for treating various monogenic diseases. Currently, AAV-mediated therapeutic treatments for musculoskeletal and cardiac indications require high doses to transduce the large quantities of affected muscle and to achieve widespread therapeutic benefit. However, the use of large vector doses has been associated with liver damage, acute liver failure and even death, as reported in some clinical trials. AAV capsid engineering, aimed at detargeting the liver, offers a solution to address these issues by developing more precise and efficacious vectors. Numerous strategies exist to engineer novel AAV capsids, and our group is continuing to employ an iterative rational design approach, using naturally occurring capsids. Previously, we described these engineered capsids as capable of targeting cardiac and musculoskeletal tissues. Among them, AAV-SLB101 has a 2-4x increase in muscle transduction and ~0.5x decrease in liver tropism in comparison to AAV9 in mice, both DMDmdx and wild type, and non-human primates. This capsid is being used in Solid Biosciences' SGT-003 program for the treatment of DMD that recently had an IND cleared by the US FDA. When designing newly engineered capsids, we followed a stepwise approach to globally detarget tissues, including the brain, liver, and other peripheral organs, while introducing cardiac and musculoskeletal tissue targeting modifications to the AAV capsids. The newly engineered capsids underwent assessment for production, transgene delivery efficiency, and efficacy in vitro and in vivo. Quantitative evaluation included assays for in vivo imaging, protein expression, and vector copy number. Through the combination of liver detargeting and muscle targeting

modifications in wild type capsids the resulting capsids maintained or improved cardiac and musculoskeletal transduction while detargeting the liver. These precisely engineered capsids could lead to gene therapy vectors with high efficiency, efficacy, and safety profiles for treating cardiac and musculoskeletal indications, which require a systemic delivery approach.

481. Novel AAV Capsids with Favorable Pre-Existing Immunity, Biodistribution, and *In Vivo* Potency

Silvia Ramirez, Justin Ishida, Darlene Chen, Kahsay Gebretsadik, Vincent Leonard, Hassibullah Akeefe, Peter Colosi, Sripriya Ravindra Kumar, Pooja Agarwal
BioMarin Pharmaceutical Inc, San Rafael, CA

Adeno-associated viral (AAV) vectors have emerged as effective delivery vectors for gene therapy applications. However, pre-existing immunity and off-target tissue uptake and resulting dose-limiting toxicities including dorsal root ganglia (DRG) and liver toxicity remain critical challenges. According to epidemiological analyses, between 40%-80% of the human population is seropositive for antibodies against common AAV serotypes leading to potential patient exclusion from AAV clinical trials and limiting the number of patients that can potentially benefit from these therapies (Wang et al, 2019). There is also significant interest in identifying capsids with superior in vivo potency, as evidenced by enriched RNA/protein- to-DNA profiles or cell type selectivity (Kondratov et al, 2021). In an effort to find novel AAV capsids with low pre-existing immunity, favorable biodistribution, and strong in vivo potency, we have invested in AAV capsid discovery and engineering efforts and generated a large library of proprietary natural and engineered AAV capsids. Systematic characterization of these capsids for safety, biodistribution, potency, and manufacturability is being evaluated, but early studies reported here have identified several novel capsid clades with favorable pre-existing immunity and distinct tissue tropism profiles. Pre-existing immunity screening was conducted using a pooled human immunoglobulin G (IgG) neutralization assay and identified several novel capsids with up to a 10-fold reduction in pre-existing immunity relative to benchmark capsids with clinical precedence. To determine the in vivo biodistribution of these novel capsids following intravenous (IV) administration, single capsid chemiluminescent screens were conducted in rodents. Interestingly, distinct clade-specific profiles were observed, with some capsids selectively enriched in the liver while others showing liver-detargeting and cardiac or skeletal muscle tropism. Translatability of this biodistribution in larger mammals was explored for a subset of clades through a 9-capsid multiplexed barcoded rAAV distribution study in cynomolgus macaques. Next generation sequencing (NGS) based DNA and RNA barcode analysis of 20+ tissues, collected six-weeks post IV administration, was conducted to inform transduction and transcription profiles of each capsid. This multiplex analysis identified several key differences in biodistribution as well as RNA/DNA profiles across different tissues, suggesting that the capsid's influence may extend beyond delivery, as predicted, to additionally impact potency and/or durability. Follow-up analysis of the same multiplex in rodents yielded similar trends, demonstrating translatability across species. Notably, the NHP multiplex study identified a novel capsid clade with preferential targeting of the heart muscle (38% relative barcode enrichment in the ventricles) and significant liver detargeting (0.6% barcode enrichment). These results were also confirmed in a single capsid NHP study and additional characterization of this novel clade is ongoing. In summary, we report here the identification of novel clades of natural and engineered AAV capsids with low pre-existing immunity and desirable tissue distribution profiles, warranting further investigation of their suitability for gene therapy applications. Manufacturability, stability, and potency evaluation in relevant healthy and disease models has been initiated and will be reported in future meetings.

482. AAV DNA Shuffle Library of GH Loop Variable Regions for Directed Evolution of Cardiotropic Capsids

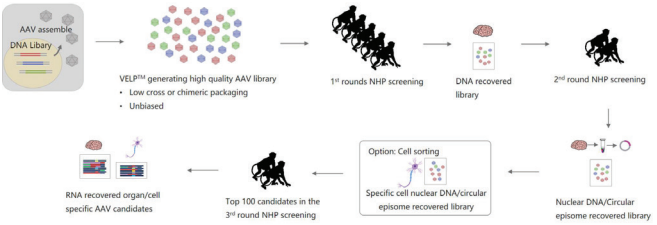
Prasad R. Konkalmatt, Reva Shenwai, Kathy Ivey
Tenaya Therapeutics, South San Francisco, CA

Gene therapies targeting cardiomyocytes (CMs) for treating disorders such as genetic cardiomyopathies require a relatively high vector dose when treated systemically to achieve broad distribution across nearly 100% of the CMs. While AAV9 serotype offers effective transduction of CMs at systemic doses ≥ 3E13 vg/kg body weight, lowering doses may reduce the potential for off-target toxicities and decrease the cost of goods. Therefore, the development of novel AAV capsids with improved tropism to CMs is critical to enable the next generation of therapeutically relevant cardiac gene expression at lower doses with the potential for use in more prevalent forms of heart disease. Strategies involving directed evolution of Adeno-associated virus (AAV) capsid libraries created by random DNA shuffle or rational design to modify receptor binding sites have been employed with limited success. As an alternative approach, we have developed a novel AAV capsid library, the GHVR shuffle library, by randomly shuffling the five variable regions (VRs; VR-IV to -VIII) located between the G and H beta sheets of the capsid protein from the 16 natural AAV serotypes on the AAV9 capsid backbone. We targeted the GH loop since it is present on the exterior of the AAV particle and play a key role in the receptor binding and internalization of the AAV virus particle, and thus drive tropism. This strategy allows us to evaluate a genetically diverse molecular space that is known to determine infectivity, while still limiting the theoretical capsid pool to a manageable size of 1.05E6 capsid variants. To our knowledge, this is the first example of this approach being used for identification of novel capsids with improved tropism for a specific organ or tissue. Using a CM-specific promoter to drive expression following systemic administration, we employed RNA-based recovery of the CM-tropic variants, as determined by next-generation sequencing. The GHVR library was subjected to two rounds of screening, first in mice and then in non-human primates (NHPs), which identified shuffled variants with CM tropism in both species. After the second round of screening in the NHPs the size of the capsids recovered from hearts was reduced to a total of 1.3E4 variants, enriched with the AAV13 at position VR-IV; bat AAV at VR-V; AAV5 at VR-VI; AAV11 at VR-VII; and AAV9 at VR-VIII. Our data indicate tolerance of capsid assembly to VR sequences from the other serotypes at their respective positions in the AAV9 capsid backbone and reveal patterns of VR sequence enrichment from non-AAV9 capsids. Further analysis of these enriched variants is expected to reveal chimeric AAV capsids that can offer enhanced transduction to CMs compared to AAV9.

483. VELP™: An Efficient AAV Capsid Evolution Platform Based on Novel Viral Library Production Strategy

Lei Zhao, Tongxin Liu, Fengkuan Shi, Xingrong Luo
Cure Genetics, SuZhou, China

Introduction: Adeno-associated virus (AAV) is one of the best viral-delivery cargo in many gene therapies because of the different organ/tissue tropism for each wtAAV. However, the specificity of organ/cell targeting efficiency is still required as an ideal delivery vector to reduce the dose-dependent toxicity. Thus, novel AAV serotypes with specific organ/cell tropism plays important roles in development of innovated gene therapies. Directed evolution is a normal way to generate the organ-directed property. However, extensive problems would be existed in directed evolution which are 1) bias, chimeric and cross-package of viral particle library, especially in RNA recovery strategies which requires high amount of library DNA in recombinant AAV packaging; 2) false positive data by DNA recovery strategy which does not correlated to GOI expression; 3) limited RNA recovered data due to the low efficient of tissue specific promoters. Those problems may cause the loss of the valuable candidate since non-significant predominant sequences and the poor population of recovered data for validation. Therefore, we used VELP (Vector Evolution by Library Production) platform to generate unbiased plasmid library and minimize the cross-package in the initial round of selection by reduce and balanced MOI of ITR-Rep-Cap-ITR plasmid during the packaging of natural AAV library. Our plasmid library was then packaged by HEK293T cell with optimized process to generate high transduction activity library. Then we recovered nuclear DNA/circular episomal DNA in the 1st round. The compact and organ specific AAV library will be generated after 1st round followed by the routine RNA recovery strategy for 1-2 rounds selections. Our high qualified AAV starting library with well-established data recovery strategy can generate more reliable candidates with organ/cell-specific tropism, which could be the ideal capsid in the further trials of gene therapies. Results and Discussion: The low redundancy of VELP library was test by NGS after 1st round NHP screening. In liver for instance, our NGS data showed that 7-mer peptide insertion library is 85.6%-91.3%, imply that only a small part of redundant sequences in the library including the other length of peptide insertion (6, 8, 9-mer and any other length of inserted peptide). Moreover, in the 7-mer peptide insertion library, the data without stop-codon is 99.93%-99.96%. The transduction activity after selection of VELP library was monitored by in vitro HEK293T cell assay. The VELP library was generated from 107 random AAV capsid plasmids resulting 19 viral sequences while the conventional packaging generated 88 viral sequences. After VELP packaging, almost all viral sequence represented high transduction activity but very low activity by conventional packaging. The DNA data recovered from cell nuclei are more accurate than the information from tissue DNA while are also larger amount of data recovery than RNA recovered data. This strategy can be good back-up for RNA recovery strategy. Based on our approach, the heart, kidney and muscle targeted screening had been generated in NHPs. Conclusion: The VELP platform generates non-redundant, low cross packaged and high transduction efficiency AAV starting libraries and recovered different information within particular screening cycle. The unique nuclear DNA/circular episomal DNA recovery in VELP platform generate a backup before RNA recovery which might loss lots of candidates. This strategy significantly increases the amount of reliable organ/cell-specific sequences.

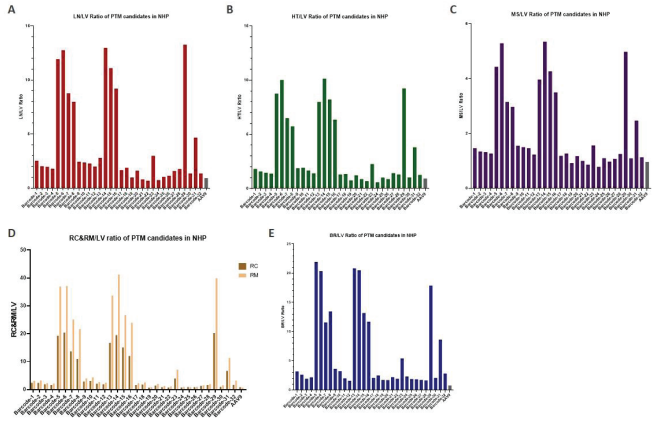


484. Studies of Biodistibution Regulated by the Combinations of Glycosylation Profile on AAV9

Lei Zhao, Tongxin Liu
Cure Genetics, SuZhou, China

Introduction: Adeno-associated virus (AAV) is one of the best viral vector in gene therapy. However, several biological properties such as post-translational modifications (PTMs) of particular AAV serotype still remains unknown. Glycosylation is one the most important PTMs during the protein expression in eukaryotic cell lines. Different cell line such as mammalian HEK293 and insect Sf9, represent different glycan-profiles. For instance, HEK293 cells, which confer mostly complex- and high-mannose-type glycans, while Sf9 cells, which impart mainly paucimannose-type glycans. The different glycans may induce different immunogenicity and biodistributions of protein or assembled nanoparticle. Although previous studies have identified glycosylation site on most of wild-type serotypes (AAV2-AAVrh10) by LC/MS, it is still lack of study of structure-function relationship. Here, we selected 5 N-glycosylation sites (253, 462, 497, 552, 706, based on AAV9 VP1 numbering) and designed the full combination of those site with/without N-glycans based on AAV9 according to the most frequency of detected in AAV2-AAVrh10. The titer during AAV packaging and the biodistribution in vivo (rat and NHP) have been studied based on qPCR and NGS-barcode technology. Some particular site of N-glycans or combinations play important role in AAV production and liver de-targeting. Results and Discussion: The 32 combinations of 5 N-glycosylation sites have been introduced to AAV9. After triple-plasmid packaging by HEK293T cell, the titers were monitored by qPCR. As showed in Fig. A-E, the barcode-1-16 represents comparable titer with wtAAV9 while barcode 17-32 showed significant reduced titer. The different between the two groups is barcode 1-16 have N253 glycosylated motif but barcode 17-32 carried Q253 non-glycosylated amino acid. This result indicated that the N-glycosylation site 253 originally from AAV2 also effect the titer in AAV9. The biodistribution studies of the 32 variants were then preformed in rat and NHP. Barcode 1-16 behaved strong liver de-targeting effect in rat while only barcode 5-8 and 13-16 represented strong liver de-targeting effect in NHP. Interestingly, barcode 5-8 and 13-16 have very high non-liver/liver ratio in NHP indicating that those N-glycan combinations strongly reduce the liver accumulation by particular pattern of PTMs. This potential liver de-targeting property could help the design of AAV capsid engineering to reduce the liver toxicity in systematic administration of AAV vector. Conclusions: Glycosylation might play an important role in AAV capsid potency by

effecting the productivity and biodistribution. Our work generated the full combinations of 5 N-glycosylation sites which reported before to study the biodistribution. To balance the titer and liver de-targeting properties, we find a motif with NXQXX (based on 5 N-glycosylation sites 253, 462, 497, 552, 706) are potentially reduce the liver target effect which might be used in combine with other novel AAV serotypes.



485. Extending ML Toolbox for AAV Design: AAV-RoBERTa

Oleksandr Kondratov[1], Arunava Banerjee[2], Sergei Zolotukhin[1]

[1]College of Medicine, Pediatrics, University of Florida, Gainesville, FL,[2]Department of Computer & Information Science & Engineering, University of Florida, Gainesville, FL

Multiple machine learning approaches have been extensively explored in recent years to predict AAV library fitness: feedforward artificial neural networks (ANN), convolutional neural networks (CNN), variational autoencoders (VAE), recurrent neural networks (RNN) etc. The most remarkable characteristic of the aforementioned models is their original purpose - analysis of plain data, images or video, respectively. Therefore, adapting AAV sequence to these models required techniques such as one-hot encoding and sometimes extra null dimensions to match dedicated data dimensionality of each model. At the same time, AAV shell protein sequences represent a set of amino acid strings with nonlinear interactions arising from complex structural organization. Such protein organization of AAV has conceptual similarities with human languages, presenting an opportunity to leverage robust natural language processing (NLP) approaches. Recent advances in language-to-language translation in the domain of NLP have been based on deep feed forward networks incorporating attention nodes. These networks, known as transformers, have demonstrated great improvements in accuracy. By abandoning the serial sequence to sequence approach as well as the recurrent network, not only does the transformer circumvent the vanishing gradient problem, but it can also learn from orders of magnitude more data due to its inherent parallelism. Possibility to feed to the model plain sequences (text) makes it friendly to users with biological background. In our initial exploration, we compared packaging prediction efficiency of already published feedforward ANN, modified CNN and NLP based approach on the same raw AAV2 mutant dataset. As an NLP model we selected Robustly Optimized BERT Pre-training Approach (RoBERTa) trained from scratch (AAV-RoBERTa) with subsequent fine-tuning on packager/non-packager dataset. As anticipated, the NLP approach showed its superiority over simpler models in accuracy. Further, we generated NGS datasets for five distinct AAV libraries representing four different serotypes with approximately 400M packaged variants. This dataset was used to train a multiserotype version of RoBERTa (aka XLM-AAV). We tested XLM-AAV vs single AAV-RoBERTa models in their perplexity, prediction accuracy and potential of sequence representation. All models demonstrated size dependence in perplexity improvement but for XLM-AAV model this effect was more pronounced.Our future research is directed on investigation of AAV structural relations captured by XLM-AAV and its usage for acceleration of directed evolution.

486. Efficient Transduction of Nasal Epithelial Cells Achieved through the Intranasal Administration of a Newly Developed Adeno-Associated Virus, AAV-GC09

Cenfeng Chu

OBiO Technology (Shanghai) Corp., Ltd., Shanghai, China

Rhinologic diseases pose a significant health concern, leading to systemic symptoms that impact mood, cognition, and sleep. Among these, atopic rhinitis (AR) stands out as one of the most prevalent disorders, affecting 10-20% of the global population. Unfortunately, effective treatments for AR remain elusive. The adeno-associated virus (AAV) has emerged as a highly efficient gene delivery tool, holding therapeutic promise for common diseases like AR. In the realm of respiratory diseases, intranasal administration is a widely adopted drug delivery method. This study devised a 7-peptide insertion AAV library, based on the natural serotype AAV1 at amino acid sites 590-591. Mice were infected through intranasal administration, and multiple *in vivo* screening rounds were conducted. Through sequencing data validation, a novel AAV vector, AAV-GC09, emerged as the top candidate, efficiently infecting nasal epithelial cells. Importantly, intranasal administration in mice demonstrated the vector's specificity and safety by not infecting lung cells. In the clinical landscape, monoclonal antibody AR drugs targeting critical pathways, such as the interleukin-4 receptor alpha antibody, are currently undergoing clinical testing. Leveraging the AAV delivery tool holds the potential for sustained antibody expression, significantly enhancing the overall patient experience. Our findings underscore the substantial potential of AAV-GC09 as a vector for rhinologic gene therapy, offering a promising avenue for addressing the challenges associated with atopic rhinitis and other rhinologic conditions.

487. A Comparative Analysis of Engineered Liver Tropic Capsids Across Different Species: Towards Cross-Species Translation

Joy Wang, Shiliang Hu, June Deng, Yinxing Chen, Nannan Jia, Kirsten Lapoint, Neil Huang, Gregory Preston, Jay Wang, Nupur Kale, Noel Walsh, Lauren M. Drouin, Matthew Chiocco, Matthias Hebben, Mariana Nacht, Jing Liao

Alexion Genomic Medicine, AstraZeneca Rare Disease, Lexington, MA

Rapid advancements in genomic medicine, particularly in viral vector-based delivery systems, underscore the necessity for AAV capsids suitable for translation from rodent models to humans. Therefore, there is a high need for a robust platform to assess the tropism and potency of engineered capsids. In our study, we focused on liver-tropic capsids and conducted a comprehensive analysis across diverse models, including *ex-vivo* primary human hepatocytes, *in vivo* wild-type (WT) mice, different liver humanized mouse models, and non-human primates (NHP), to evaluate the transduction efficiency of different liver-specific capsids designed for genomic medicine applications. In primary human hepatocytes, WT mice, and humanized mouse models, we employed reporter genes GFP and human FIX to evaluate transduction efficiency in different models. Droplet digital PCR measured GFP RNA expression levels, GFP protein was quantified by IHC staining, and ELISA was used to measure human FIX protein levels. In the NHP study, we barcoded different capsids and validated transgene RNA levels quantified by next-generation sequencing (NGS). Consistent results were observed for capsid ranking in two different liver humanized mouse models, FRG and PXB mice, aligning with the outcomes in human primary hepatocytes. Notably, the top capsid from human models also demonstrated excellent transduction efficiency in the WT mouse liver, suggesting its potential use in preclinical, primate, and human studies. Importantly, the lead capsid identified in the WT mouse study, humanized mice, and primary human hepatocytes remained the top performer in the NHP study, the most relevant model to humans for liver-tropic capsids. The results of this comparative analysis not only deepen our understanding of capsid behavior but also lay the groundwork for a standardized screening platform for capsids, streamlining preclinical testing across diverse species. The identification of a common capsid with tropism and transduction capabilities across various species represents a significant advance in the field of genomic medicine.

488. A Rationally Designed AAV Capsid Variant of sL65 Can Achieve Efficient Cross-Species Liver Transduction

Shiliang Hu, Joy Wang, Yinxing Chen, Nannan Jia, June Deng, Kirsten Lapoint, Megan Ericson, Jay Wang, Shreya Amarwani, Noel Walsh, William Lee, Lauren Drouin, Gregory Preston, Matthias Hebben, Mariana Nacht, Jing Liao

Alexion Genomic Medicine-AstraZenea Rare Disease, Lexington, MA

sL65 is the first human liver tropic AAV capsid developed using LogicBio's sAAVy platform. sL65 is a synthetic capsid through DNA shuffling of cap proteins from 12 natural serotypes, demonstrating superior non-human primate (NHP) liver tropism compared to well-known liver tropic capsids like AAV8 and LK03. However, sL65 exhibits lower mouse liver tropism than AAV8. We addressed this by engineering sL65 to ensure consistent transduction across species, enhancing preclinical data reliability for both mouse and NHP livers. For the AAV capsid engineering, we employed a rational design strategy, modifying the variable region I (VR-I) of sL65. GFP served as the transgene reporter in WT mice studies, while hFIX was the reporter gene in cynomolgus macaque (NHP) experiments. Quantification of cell entry and transgene expression for new sL65 variants was performed using ddPCR and NGS sequencing. Of the 6 sL65 variants produced, our new capsids demonstrated excellent AAV manufacturability. Some sL65 variants exhibited increased mouse liver tropism compared to the original sL65 capsid and natural serotype AAV8. Notably, new variant sL65-V6 showed about 2 and 10-fold transgene delivery compared to AAV8 and sL65, respectively, in mouse liver. In NHP studies, sL65-V6 showed about 2 and 6-fold transgene liver delivery compared to sL65 and LK03, respectively. Addressing the variability in transduction capabilities among different AAV capsids in mice and NHPs, our engineered sL65-V6 variant demonstrated efficient transgene expression across mice, NHPs, and human primary hepatocytes. In summary, considering manufacturability, high transduction efficiency in NHP livers, and cross-species capability, our new AAV capsid, sL65-V6 emerges as a strong preclinical and clinical candidate for liver targeted gene therapies.

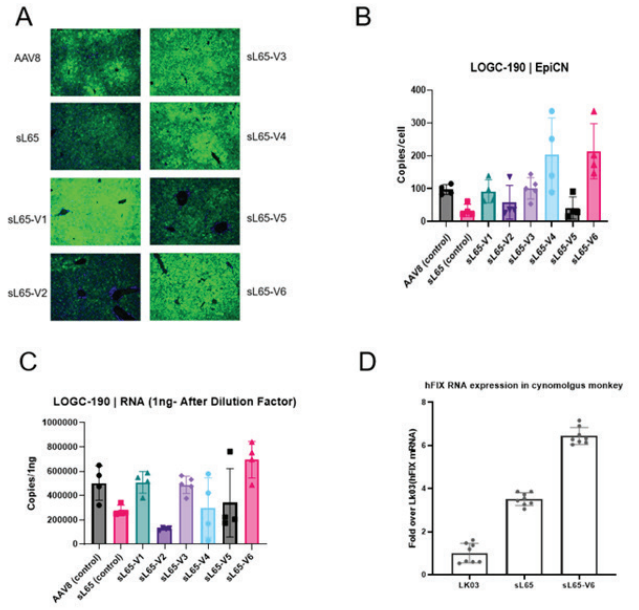


Fig1. A, WT Mouse liver transgene expression by different capsids. B, episomal copies number to check AAV capsid variants entry to WT mouse hepatocyte. C, transgene mRNA expression by different capsid in WT mice. D transgene mRNA expression comparison by different capsids in cynomolgus macaque.

489. Identification of AAV2-13 Hypervariable Regions Mediating Liver Transduction and Detargeting in Rodents and Non-Human Primates

Justine Nozi[1,2], Novella Tedesco[1,2], Christian Leborgne[1,2], Bérangère Bertin[1,2], Louis Bergantz[1,2], Ante Mihaljevic[1,2], Coralie Pecquet[1,2], Theoni-Maria Michalopolou[1,2], Nathalie Daniele[1], Laetitia Van Wittenberghe[1], David A. Gross[1,2], Tiziana La Bella[1,2], Giuseppe Ronzitti[1,2]

[1]GENETHON, Evry, France,[2]Université Paris-Saclay, Univ Evry, Inserm, Genethon, Integrare Research Unit UMR_S951, Evry, France

Adeno-associated viral (AAV) vectors are widely used in gene replacement strategies in preclinical and clinical contexts. Along the years gene therapy with AAV vectors showed efficacy for the targeting of different organs and sustained expression over time (Mendell JR,

2017; Nathwani AC, 2014; D'Antiga L, 2023). The excellent safety profile and the proven efficacy resulted in the multiplication of clinical trials to address unmet medical needs that eventually led to achieve marketing authorization for some of the AAV-based products. Despite these enormous successes, limitations in their use still exist, and a better understanding of the mechanisms mediating liver targeting and detargeting is required. In this work, we conducted a shuffling study of the hypervariable regions (HVRs) of AAV8 with homologous regions from AAV2-13 capsids isolated from human liver (La Bella T, 2020) to pinpoint regions involved in liver tropism. HVRs were shuffled in AAV8, a capsid with a robust liver tropism, by substituting them individually or as combinations. Shuffled capsids were combined with a ubiquitous or a hepato-specific promoter to evaluate the *in vivo* and *in vitro* transduction efficiency in mouse. Our results highlighted the HVR5 as a region of the AAV capsid able to improve the specificity of liver targeting. Noteworthy, HVR5 shuffled capsids combined with ubiquitous promoter led to a modest improvement in liver transduction. On the contrary, when combined with the hepatic promoter, shuffled capsids outperformed AAV8 transduction efficiency, suggesting that modifications of the HVR5 may increase the selectivity of AAV vectors for hepatocytes. We then tried to combine other HVRs with the HVR5 to evaluate whether a synergic effect was observed. The combination of HVRs 1, 7, 8 and 10 with HVR5 led to a dramatic decrease of liver targeting suggesting that the simultaneous replacement of multiple HVRs of AAV8 with the corresponding regions of wild type AAV impaired the liver tropism regardless of the promoter used. On the contrary, individual HVRs 1, 5, 7, 8 and 10 substitutions did not independently impact liver transduction. Validation of the association of certain HVR regions with liver targeting and detargeting was performed in non-human primates. Importantly, we found that shuffled AAV capsids containing regions that mediated liver detargeting in mice had a similar profile in primates pointing to an inter-species conservation of the detargeting characteristics associated to these regions. To conclude, in this study, rational shuffling of regions derived from wild-type AAV2-13 capsids isolated in human livers into homologous regions of the AAV8 capsid, allowed to identify determinants of liver transduction and detargeting across species. This knowledge is fundamental to develop AAV capsids for diseases where concomitant muscle and liver gene transfer maybe required to reduce the impact of the immune response by achieving sufficient transgene expression to induce liver-mediated peripheral tolerance without liver overloading (Sellier P, 2023). When validated in appropriate preclinical models and with clinically relevant transgenes, the use of capsids derived from shuffling of HVRs may thus provide safer AAV vectors tailored to the specificities of the disease.

490. A Novel AAV9 Mutant with a Remarkably Short Circulation Half Life Leads to Exclusive Liver Transduction Following Systemic Administration

Pratheppa Rajagopal[1], Teruyuki Nishi[2], Kei Adachi[1], Jessica Dillon[1], Anusha Sairavi[1], Hiroyuki Nakai[1]

[1]Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR,[2]KANEKA Corporation, Takasago, Japan

Introduction: AAV capsid engineering has emerged as a promising strategy to overcome challenges. To explore novel phenotypes of AAV capsids attractive for clinical translation, we adopted an NXT/S scanning mutagenesis approach to introduce the NXT/S motif into AAV9 capsid protein. **Methods and Results:** We introduced the NXT/S motif into AAV9 capsid in a systematic manner to create potentially N-glycosylated amino acid residues at non-native site. Total 258 AAV9 mutants were created and their biological phenotypes were determined in vitro and in mice using the AAV Barcode-Seq technology. Briefly, the mutant vectors and the wild-type AAV9 vector were packaged in HEK293 cells using the barcoded double-stranded (ds) AAV-U6-VBC vector genome as the transgene. The dsAAV-U6-VBC vector genomes harbor a human U6 small nuclear (sn) RNA promoter-driven non-functional noncoding RNA expression cassette containing a pair of 12 nucleotide-long viral DNA barcodes (VBCs). From AAV Barcode-Seq analysis using tissue culture cells and samples from mice treated with this library, we identified an AAV9NXTmutx that can transduce HepG2 cells at least an order of magnitude better than AAV9 and show exclusive liver transduction with a modest enhancement compared to AAV9 and minimal off-target effects in mice. For further evaluation, we injected C57BL/6J mice intravenously with ssAAV-CAG-nlsGFP-VBC vector packaged with the wild-type AAV9 or the AAV9NXTmutx mutant capsid. Three weeks post injection, the protein quantification (ELISA) and histopathological analysis of the liver and non-hepatic organs revealed the preferred liver tropism of the AAVNXTmutx as opposed to the systemic transduction with AAV9. To understand potential mechanisms of liver exclusivity of this mutant, we determined pharmacokinetic profile in the blood following administration and computationally determined capsid surface electrostatic charges by Adaptive Poisson-Boltzmann Solver (APBS). The pharmacokinetic study revealed an interesting characteristic of the mutant being cleared extremely rapidly, cleared within minutes, as opposed to AAV9 showing prolonged persistence in the circulation. APBS analysis highlighted the presence of a newly created basic amino acid patch on the surface of the AAV9 capsid that is not present in the wild-type AAV9. To assess the effect of this NXT mutant on other serotypes, we engrafted a similar mutation on other AAV serotypes to evaluate enhanced liver transduction. The AAVxNXT library containing AAV3, 5, 7, 8, 9, 10, DJ, KP1, KP3, Anc80, LK03, NP59, rh10 and its mutant was packaged with different barcodes. The in vivo analysis of AAVxNXT mutant library exhibited a slightly elevated liver transduction in AAV10, 8, 9, and rh10 in the mutant group, whereas the mutant in AAV DJ, KP1, KP3, Anc80 had a deteriorative effect. Considering the difference in efficacy of AAV transduction between animal models and humans, the chimeric xenograft mouse model with human hepatocytes was used to further understand the mutant behavior. The humanized chimera mice were administered with AAVxNXTmutant library, and the human hepatocytes were subjected to NGS analysis, revealing that the mutation had the similar effect as in C57BL/6J mice. In addition to the minimum beneficial properties in AAV8, 10, and 9 serotypes, a deteriorative effect was observed for other serotypes. **Conclusion:** Since the enhanced liver transduction and restricted biodistribution may lead to the usage of a lower vector dose which in turn minimizes the dose-dependent host immune responses, this mutant may represent a novel attractive candidate for liver-directed gene therapy with minimum off-target effects.

491. BI-GT101 Enhances Adeno-Associated Virus Uptake and Overall Transduction Efficiency in a Serotype-Independent Manner

Olaniyi Olarewaju, Franziska Held, Christian Schoen, Udo Maier, Francois du Plessis

AAV Gene Therapy Group, Research Beyond Borders, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riß, Germany

The need for high AAV doses to achieve clinical efficacy remains a key challenge to AAV utility in the clinic. High vector dose increases cost and has been majorly implicated in the activation of immune responses directed against the AAV capsid, as well as the therapeutic payload. Hence, numerous efforts are currently directed at improving AAV transduction efficiency in order to overcome these challenges. In this study, we demonstrate the potency and efficiency of BI-GT101, a small peptide, which, upon pre-incubation with AAVs, effectively enhances transduction efficiency by two- to four-fold in multiple cell lines of epithelial and fibroblastic origins *in vitro*. Additionally, BI-GT101 outperforms other commonly used cell penetrating peptides such as TAT, BIP2 and ARF which have been previously used to enhance AAV transduction. Moreover, we show similar enhancement of transduction for multiple AAV serotypes, suggesting that the mechanism of action for BI-GT101 is serotype-independent. Mechanistically, BI-GT101 increased vector DNA-uptake by two-fold, resulting in a corresponding increase in the uncoated vector genomes. Our data reveals BI-GT101 primarily influences cellular uptake of AAV particles in a HSPG-dependent manner, as we observed no differences in the vector genome uncoating efficiencies between WT-AAV and BI-GT101-supplemented-AAVs. Next, BI-GT101 was genetically incorporated in the GH2/GH3 loop of the VP1 of a transduction-incompetent AAV2 (HSPG-motif ablated AAV2), this led to the restoration of the transduction efficiency of the modified AAV2 displaying BI-GT101 to levels comparable or approximately 2-fold higher than WT-AAV2 in HEK293T cells and HT1080 cells, respectively. Therefore, showing that BI-GT101 facilitates HSPG receptor binding and subsequent uptake of the modified AAVs. In conclusion, we demonstrate that combining/supplementing AAVs with BI-GT101 robustly enhances AAV transduction efficiency, thereby opening new opportunities to reduce vector doses by a factor of two to four. This could be significant in gene therapy applications in compartmentalized environments such as the knee, eye, CNS, and in the inner ear.

492. A Novel AAV2 Mutant SonuAAV™ Expressing mGjb2 Can Improve Hearing in Adult Gjb2-Deficient Mice by Cochlear Perilymph Administration

Yoshinori Tanaka[1], Kazusaku Kamiya[2,3], Toshiyuki Miura[1], Sachiko Okamoto[1], Tatsuji Enoki[1]

[1]Takara Bio Inc., Kusatsu, Shiga, Japan,[2]Department of Otorhinolaryngology, Juntendo University Faculty of Medicine, Tokyo, Japan,[3]Gap Junction Therapeutics, Inc., Tokyo, Japan

Hearing loss is one of the most common sensory disorders. Current therapies for hearing loss involves the use of hearing devices to amplify sound, but this treatment still has limitations in terms of hearing sensitivity. Recently, AAV gene therapy has emerged as a promising strategy for the treatment of genetic diseases and has demonstrated clinical efficacy in several organs, such as retina, central nervous system, and muscle. Mutations in the gap junction protein beta 2 (GJB2), which encodes Connexin 26 (CX26), have been reported as a major cause of inherited hearing loss. Several studies targeting the GJB2 gene using AAV vectors have shown successful hearing correction in neonatal rodent model. However, there are currently no effective AAV vectors for inner ear gene delivery to rescue the hearing in adult mice. To date, peptide library screening approach by inserting short randomized peptide exposed on the surface of viral particles, has been established as a AAV capsid engineering method for tissue-specific transduction. In this study, we generated a random peptide library using AAV2 and inoculated it into cochlear explant culture of neonatal mice. After several days, the supporting cells identified as Myo7a negative cells, were isolated from the explants and then AAV genomic DNA was extracted from these cells. AAV capsid gene was recovered through PCR amplification followed by re-cloning to create a next generation AAV library. Second and third round of directed evolution were performed using adult mice instead of neonatal explants. The next generation of the AAV random peptide library was injected into the cochlear perilymph of adult mice. After 4 weeks post-injection, CX26 positive cells were isolated from the cochlear tissue. Finally, several candidates were selected as gene transfer vectors into inner ear cells after the third round of directed evolution. To evaluate the transduction efficiency mediated the selected peptide insertions, we generated seven AAV2 mutant candidates harboring the AcGFP gene under the control of the CMV promoter and then inoculated these AAV vectors into neonatal mouse cochlear explants. As a result, two AAV2 candidates (AAV-TB4 and TB5) showed significantly high AcGFP expression in both hair cells (HCs) and supporting cells (SCs) of cochlear explants. The AcGFP expression of AAV-TB5 (designated SonuAAV™) was approximately 95-fold higher than that of AAV2 and nearly 80% of both HCs and SCs were transduced. We further examined whether SonuAAV expressing mouse CX26 could improve hearing in conditional CX26 knockout mice. The SonuAAV-CX26 vector was injected into the perilymph through the lateral semicircular canal of inner ear in 4-weeks-old deaf mice, and then the auditory function was monitored by auditory brainstem response (ABR) every two weeks. After 6 weeks, successful hearing improvement was observed in the SonuAAV-CX26 vector administrated mice. Further confirmation is still required for histological identification of Gjb2 protein expression and gap junction formation between the SCs by SonuAAV gene transfer in non-human primate models and human cells. However, our data demonstrates the significant potential of SonuAAV as an efficient gene delivery vector into inner ear cells and its effectiveness in improving hearing impairment.

493. Enhancing Gene Delivery in Gene Therapy: The Role of Adhesive Phenolic Molecules in AAV Assembly

Yu Ri Nam[1], Zheng Rong Lau[2], Jeong Pil Han[3], Su Cheong Yeom[3], Jae-Hyung Jang[2,4], Haeshin Lee[1,4]

[1]Korea Advanced Institute of Science and Technology (KAIST), Daejeon, Korea, Republic of,[2]Yonsei University, Seoul, Korea, Republic of,[3]Seoul National University, Pyeong Chang-gun, Korea, Republic of,[4]GluGene Therapeutics Inc., Seoul, Korea, Republic of

Introduction The adeno-associated virus (AAV) has emerged as a remarkably effective tool in human gene therapy, owing to its notable safety record and non-replicative nature. This has resulted in the FDA and EMA approving a range of AAV-based gene therapies, highlighting the vast potential of gene therapy in developing novel treatments for genetic disorders. Despite its considerable promise, AAV's potential is tempered by its limited payload capacity, approximately 4.7 kb, making the delivery of larger genes for diseases such as Duchenne Muscular Dystrophy (DMD), Cystic Fibrosis, and Hemophilia A a significant challenge. Researchers have explored various solutions, including trans-splicing, overlapping, and hybrid dual AAV vectors, yet the simultaneous delivery of dual AAV vectors remains a challenge. To address this, in this study we propose a supramolecular AAV assembly using adhesive phenolic molecules. This innovative technique enables the assembly of viral capsid proteins for the targeted delivery of multiple genes to specific cells or tissues. **Methods** We prepared suprastructured AAV using adhesive phenolic molecules, mixing them with AAVs in volumetric ratios of either 1:1 or 1:9, based on the solubility of the molecules. To characterize these assembled particles, we utilized ultraviolet-visible (UV-Vis) spectroscopy, measuring at a wavelength of 600 nm (A600) to estimate aggregation. Additionally, the size and morphology of the particles were analyzed using atomic force microscopy (AFM) and transmission electron microscopy (TEM). To assess cellular uptake efficiency, human embryo kidney (HEK) 293T cells were employed; following a 48-hour transfection, the efficiency was quantified using fluorescence-activated cell sorting (FACS), and was visualized through fluorescence microscopy. Furthermore, for an in vivo gene delivery, intranasal injections of supra-structured AAVs carrying a cystic fibrosis gene into mice was administered. Post two weeks of injection, we performed ELISA analysis to evaluate the efficacy in gene delivery. **Results** In our experiments, the suprastructured AAVs showed a notable increase in turbidity at A600, correlating with the concentration of the phenolic molecule. Size analysis via AFM revealed a significant height increase in the suprastructured AAVs, ranging between 100 to 250 nm, compared to the approximate 22 nm height of single AAVs. TEM images further confirmed this aggregation, showcasing particles clustered by the adhesive molecules. In *in vitro* analyses, the suprastructured AAVs enhanced the co-transduction efficiency of dual AAV vectors, showing an improvement of 76-80% over treatments with non-assembled AAVs. This indicates a substantial increase in the effectiveness of gene delivery. Moreover, *in vivo* experiments yielded promising results, with a noticeable increase in the concentration of GFP in subjects treated with phenolic molecule-assembled AAVs compared to those treated with non-assembled vectors. **Conclusion** Our study showcases a breakthrough in gene therapy with the development of a supramolecular AAV assembly technique using adhesive phenolic molecules, effectively overcoming AAV's payload limitations and demonstrating enhanced gene delivery in both in vitro and in vivo models, thereby offering promising avenues for multiple therapeutic gene delivery.

494. Rationally Designed a Novel AAV Capsid for Intra-Articular Gene Delivery

Shuang Luo[1], Qing Xie[1,2], Qingwei Li[1], Qiang Zheng[1,2]
[1]Chengdu Origen Biotechnology Co., Ltd, Chengdu, China,[2]Sichuan Provincial Key Laboratory of Innovative Biomedicine, Chengdu, China

Arthritis is a musculoskeletal inflammation that affects various joints in the body. It is often accompanied by excruciating pain, impacting daily lives, and seriously affecting patients' physical and mental health. Current treatments include the use of nonsteroidal anti-inflammatory drugs. Systemic administration often leads to considerable side effects, while local administration requires patients to visit the hospital regularly for procedures, causing inconvenience. Gene therapy, which possesses a single administration with long-term effectiveness, has been explored to address the treatment of this disease. In the present study, an adeno-associated virus (AAV) mediated gene therapy was developed and evaluated in a collagen-induced arthritis (CIA) mouse and New Zealand rabbit model. Briefly, the AAV vector was intra-articular injected into the knee joint of the animals, and the transduction efficiency, types of transduced cells in the joint, off-target transduction in other tissues and duration of AAV expression in the joint were evaluated thereafter. Our vector consists of an engineered AAV capsid as a delivery vehicle and a coding sequence for anti-TNF (Enbrel) as the transgene. The results showed that the expression of the target protein in the joint of the New Zealand rabbit lasted for over 9 months after the injection with 1E11 vg AAVs for each knee. The tissue tropism of our engineered AAV was evaluated in CIA 57BL/6 mice's joints as well. In this experiment, 5E10 vg AAVs were administered to each knee of the mice. It showed our AAV vector mainly transduced A-type synoviocytes, DC cells and T cells, but not transduce B-type synoviocytes and chondrocytes after intra-articular injection of the mice. These results demonstrate that our vector has a high transduction efficiency in diverse joint cells and long-term expression after intra-articular administration.

495. Developing a Potent Gene Therapy Candidate for Facioscapulohumeral Muscular Dystrophy (FSHD) Through High Throughput AAV Capsid and Cargo Engineering

Saurav Seshadri, Kyle Brown, Shayan Tabebordbar, Edward Marsh, Jana Jenquin, Janelle Stricker, Brian Ferguson, Mark Fielden, Sharif Tabebordbar
Kate Therapeutics, San Diego, CA

Facioscapulohumeral muscular dystrophy (FSHD) is an autosomal-dominant genetic disorder affecting an estimated 1 in 8,000 individuals. Patients with FSHD typically exhibit progressive weakness of muscles in the face, shoulders, upper arms, legs, and abdomen; approximately 20% of patients will eventually require the use of a wheelchair, and the majority experience pain and fatigue, often debilitating. De-repression of the transcription factor *DUX4* has been identified as the pathogenetic mechanism in FSHD. Insufficient epigenetic silencing of the *DUX4* gene in patient muscles results in aberrant expression of DUX4, which is toxic to mature myofibers. Therefore, knockdown of the *DUX4* transcript has been advanced as a therapeutic strategy for FSHD and has shown efficacy in preclinical models. Here, we demonstrate efficacy of a novel MyoAAV-mediated RNAi therapy

for FSHD both *in vitro* (in human patient-derived myotubes) and *in vivo* (in the ACTA1-MCM;FLExDUX4 mouse model, a bi-transgenic line with inducible expression of DUX4 in skeletal muscles, which reproduces disease-relevant biochemical, histological, and functional phenotypes of FSHD). First, we conducted a tiling screen to identify the most potent DUX4-targeting artificial miRNA sequences, using an engineered HEK293 cell line stably expressing a tagged mutant DUX4 transgene. Top hits from this screen were validated in FSHD patient-derived myotubes. These cells contain a disease-causing contraction in the D4Z4 region of chromosome 4q that is responsible for epigenetic silencing of *DUX4*, and upon differentiation, show expression of *DUX4* and its transcriptionally regulated target genes. Candidate miRNA sequences were ranked based on the magnitude of DUX4 and target gene knockdown achieved in these cells, and selected sequences were carried forward for additional characterization. We performed small RNAseq in human myotubes to assess processing efficiency of the miRNA-containing transcript. To ensure that our candidate miRNA sequences were specific for DUX4 and did not cause knockdown of unintended transcripts, we applied an unbiased, empirical approach using RNAseq in human primary myotubes. We selected our lead miRNA candidate based on optimal potency and processing, and minimal off-target effects. In separate experiments, we evolved liver de-targeted, muscle-tropic MyoAAV capsid variants in non-human primates. We packaged our lead miRNA sequence in our top liver de-targeted, muscle-tropic MyoAAV and tested the resulting candidate *in vitro* and *in vivo*. We confirmed potent knockdown of DUX4 and target genes, efficient processing, and minimal off-target effects at a relevant dose in human myotubes. To assess *in vivo* efficacy, we used ACTA1-MCM;FLExDUX4 mice. We dosed these mice with our candidate or vehicle, and induced DUX4 expression in all mice by injecting tamoxifen. Treatment with MyoAAV delivering DUX4 miRNA protected mice from severe impairment in treadmill time to exhaustion, prevented expression of markers of histopathology, and blocked elevations in DUX4 and target genes, all of which were observed in vehicle-treated mice. Taken together, these data support the further development and potential of our gene therapy candidate for the treatment of FSHD.

496. Rationally Engineered AAV Variants for Efficient Gene Delivery to Muscle

Philip C. Sin[1], Hanyang Hu[2], Zengmin Du[3], Dandan Li[2], Shihao Wu[3], Huaqing Xue[3], Wei Jiang[2], Xia Wu[2], Jane Zheng[3], Xiao Xiao[3]

[1]Belief BioMed Limited, Hong Kong, Hong Kong,[2]Research, Belief BioMed Limited, Shanghai, China,[3]Belief BioMed Limited, Shanghai, China

Background: Recombinant adeno-associated virus (rAAVs) vectors are among the most promising for *in vivo* gene therapy. AAV-mediated systemic gene delivery into muscle has been a major challenge for muscle disorders gene therapy, with capillary blood vessels posing the principle barrier and limiting vector dissemination. To achieve exceptional therapeutic efficacy, AAV-mediated muscular disease gene therapy often requires injection of a large dose of virus (>1E+14 vg/kg). Serious adverse events and toxicity have been observed in some high dose systemically delivered AAV gene therapy clinical trials, highlighting the need for more efficient AAV vectors to achieve efficacy at lower doses. **Method:** We utilize integrin overexpression cell and adenovirus helper to replicative screen the engineered AAVs with rationally designed short peptide insertion. The screening process was repeated a total of 5 times. In the very last round of selection, the engineered AAV capsid DNA were extracted and put through next generation sequencing. We identified several top clones as integrin targeted engineered AAV vector candidates. To evaluate rationally engineered AAV variants in muscles of rodents or non-human primate, we generated myotropic AAV candidates delivering reporter gene under the control of CB promoter and then injected these vectors intravenously. After 3 weeks, we analyzed reporter gene expression in whole-body, whole-tissue and sliced sample, and compared with AAV9 or myoAAV4. **Result:** Significant tdTomato expression mediated by three AAV9 mutations were detected in whole-body muscles and heart and the expression level and coverage were much higher than wild-type AAV9. Moreover, AAV9.P7, a non RGD motif peptide chimera, was efficient in myotropic transduction in common with myoAAV4A. We next mutated several sites of AAV9.P7, known to mediate receptor interactions and affect tropism, to reduce liver transduction. AAV9.P7.4, with a specific single site mutation, showed desired liver de-targeting and maintained efficient muscle transduction. In addition, we investigated whether we could transfer the peptide of AAV9.P7 to natural serotype, usually AAV5, due to its low seroprevalence. Fortunately, AAV5.P7, which carried the same inserted peptide of AAV9.P7, was the same in muscle infectivity as AAV9 but stronger than its parent AAV5. Interestingly, AAV5.P7 gained muscular tropism, and were no longer liver targeting, maybe due to peptide transplant destroyed sialic acid binding site. Novel capsids validated in rodents do not always translate to NHP. After validating the AAV9.P7 capsids in rodents, we therefore decided to assess its transduction potential in rhesus macaque, a common pre-clinical research model for AAV gene therapy. Compared with the parent AAV9, systemic delivery of AAV9.P7 packaged with CB-LacZ efficiently transduced cardiac and skeletal muscle in 2~3 years old rhesus macaque. **Discussion:** We demonstrate the rational design of AAV9 variants displaying integrin targeted peptides on the viral capsid and the identification of three myotropic AAV9 variants through cell based high-throughput method. Moreover, AAV9.P7, a non RGD motif peptide modified AAV9 variant, showed myotropic efficiency in systemic gene delivery in both rodents and NHP. We rationally designed AAV9.P7 and gained several mutations, including AAV9.P7.4, that showed high myotropic efficiency with liver de-targeted. The integrin targeting peptide rationally translated from AAV9.P7 to low seroprevalence serotype AAV5, enabled serotype switching to AAV5.P7 for broad application in clinic. Our results suggest that AAV9.P7, AAV9.P7.4 and AAV5.P7 are useful gene therapy delivery tool for muscular diseases.

497. Development of a Computational Pipeline to Improve Engineered Capsid Identification from Defined and Random AAV9 Peptide Insert Libraries

Rosemary L. Meggersee, Zhe Zhang, Daniel Lefever, Jenny A. Greig, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

The large peptide insert libraries used to engineer AAV9 capsids for tissue transduction generate enormous amounts of data. Depending upon the target tissue, it is often difficult to identify candidates with

genuine transduction advantages. Bioinformatic analysis tools have helped to alleviate this issue, enabling the identification of several engineered capsids with greater tissue specificity. However, it has recently become apparent that these standard bioinformatic analysis methods have limitations. Multiple groups have found that their best capsid is not part of an obvious motif family and may not even be their top ranked capsid. To address this drawback, we have developed several analysis tools and "hit picking" methods for a defined library isolated from multiple nonhuman primate (NHP) tissues. The methods we employed included unsupervised principal component analysis to confirm that different tissues had distinctive profiles of peptide abundance. We applied k-means clustering to identify subgroups of peptides with identical patterns of relative abundance with respect to input vector across multiple tissues. We summarized the frequency of peptide positions by subgroups and compared peptides of the same subgroup with each other to evaluate their sequence similarity. This analysis pipeline enabled us to identify previously unseen and unappreciated patterns and associations that informed the selection of several candidates for follow-up studies. In vivo characterization of these identified capsids in mice and NHPs demonstrated that these capsids outperformed AAV9 in their respective tissue targets. In mice, we observed transduction activity of between 40 and 500 times that of AAV9 depending on the tissue type; increases of 10 to 20 times over AAV9 were observed in NHPs. We leveraged this knowledge to fine-tune our bioinformatics pipeline for a large, random insert library on tissues that are known to be difficult to transduce and to thus identify potential next-generation capsids.

498. Identifying Muscle Stem Cell Tropic AAV Variants through Directed Evolution

Harry M. Chen[1], Michael R. Emami[2], Courtney Young[2], Kevin Liu[1], Braeden Freitas[1], Emily Skuratovsky[1], Thomas A. Rando[3], Oluwatayo Ikotun[1], April D. Pyle[1], Melissa J. Spencer[1]

[1]UCLA, Los Angeles, CA,[2]MyoGene Bio, San Diego, CA,[3]Stanford, Palo Alto, CA

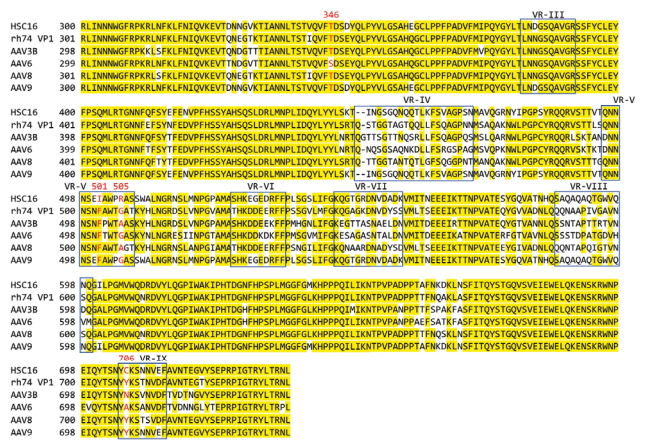
Duchenne Muscular Dystrophy (DMD) is a rare genetic disease caused by frameshifting mutations in DMD, resulting in a lack of dystrophin protein production. While AAV-microdystrophin gene replacement therapy was recently given accelerated FDA approval for DMD, the therapeutic efficacy appears to reduce with time, likely due to turnover of skeletal muscle and replacement by muscle stem cells. Redosing AAV is not an option; however, a strategy to retain durable dystrophin is to target Muscle Stem Cells (MuSCs) with CRISPR/Cas9 to correct genomic DNA in the proliferating cell population. We have previously identified 8 MuSC targeting and 10 Skeletal Muscle (SkMu) targeting AAVs through directed evolution of a 7 amino acid sequence, inserted between amino acids 589A and 590Q in the surface region VIII (VR-VIII) of AAV9. Individual validation of these variants through systemic administration in mice has revealed that they significantly outperform wtAAV9 in MuSC targeting while also retaining their SkMu tropism. In addition, in vitro studies in primary human myoblasts (HSMMs) have demonstrated a similar enhanced tropism compared to wtAAV9. These data have been confirmed through flow cytometry alongside qPCR of viral genomes. While further characterization must be done, these new variants provide a potential new platform for achieving long-term dystrophin restoration and durability when AAV is used to deliver CRISPR components in DMD and other myogenic diseases that involve muscle degeneration and regeneration.

499. Rational Design of AAV-rh74, AAV3B, and AAV8 with Limited Liver Targeting

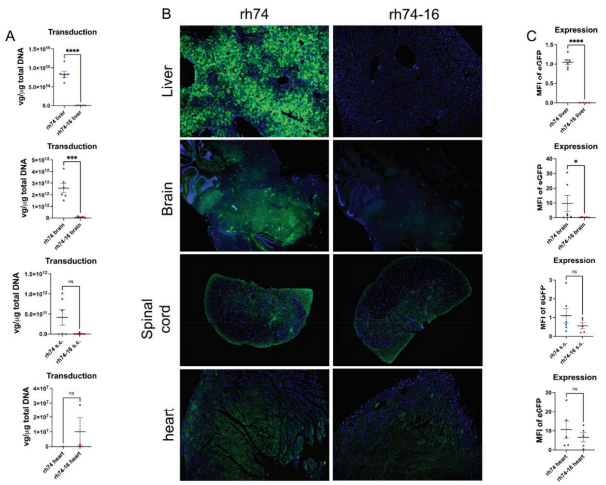
Christopher Chan, Kathryn K. Harris, Sergei Zolotukhin, Geoffrey D. Keeler

Department of Pediatrics, University of Florida, Gainesville, FL

Introduction Recombinant adeno-associated viruses (rAAVs) are one of the most widely used vectors for gene therapy, with several adeno-associated virus vector-based gene therapies currently FDA-approved for the treatment of diseases such as Duchenne muscular dystrophy and hemophilia B. rAAVs are engineered to lack viral genes and are particularly suited for use in gene therapy because of their low immunogenicity, ability to transduce many different cell types, and persistent transgene expression after initial treatment administration. However, most AAV serotypes transduce the liver at a high rate when administered peripherally, thus increasing the necessary dose for the treatment of other tissues. High dosage of AAV has been associated with adverse side effects in recent clinical trials. To minimize this risk to patients, there is a need to produce rAAV vectors that effectively transduce target tissues at lower doses. De-targeting rAAV vectors from the liver would prevent rAAV from being removed from the bloodstream before reaching target tissues. One naturally occurring clade F AAV serotype, AAV hematopoietic stem cell 16 (AAVHSC16), has been found to show reduced liver transduction when administered peripherally compared to other closely related AAV serotypes. Three residues on the capsid surface of AAVHSC16 are associated with reduced galactose binding. We hypothesized that point mutagenesis of these surface residues in AAV serotypes from other clades would be associated with reduced transduction of liver tissue. **Methods** Four non-clade F, clinically relevant AAV serotypes were identified who differed from AAVHSC16 at the residues associated with reduced galactose binding: AAV3B, AAV6, AAV8, and AAVrh74. These serotypes were mutated at those three residues, and self-complementary rAAV-GFP vectors were produced for both the wild-type and mutant capsids. We assayed the wild-type and mutant vectors either *in vitro* in HuH7 cells or *in vivo* using a murine model to compare transduction and transgene expression.



Results While wildtype AAV3B transduced over 80% of HuH7 cells *in vitro* as assayed by FACS, the mutant capsid had effectively no transduction of HuH7 cells. AAVrh74 and AAV8 had significant reductions in transduction efficiency of the liver in their mutants, while AAV6 had overall low liver transduction in both the wildtype and the mutant. Our data indicates that mutagenesis results in decreased transduction and transgene expression in liver tissue.



Conclusion Mutagenesis at the residues associated with reduced galactose binding in AAVHSC16 resulted in decreased transduction and transgene expression in liver tissue by AAV serotypes from other clades. This method may be a means to de-target additional AAV vectors from the liver, which could result in the development of AAV therapies with more limited adverse side effects.

500. Structure Guided Design of the AAV9 Capsid Evading Antibodies Derived from Zolgensma Patients

Jon Zachary[1], Austin Nelson[1], Jane Hsi[1], Lindsay Potts[1], Paul Chipman[1], Mohammad Ghanem[2], Ian E. Alexander[3], Grant J. Logan[4], Juha Huiskonen[2], Robert McKenna[1], Mario Mietzsch[1]

[1]University of Florida, Gainesville, FL,[2]University of Helsinki, Helsinki, Finland,[3]Sydney Children's Hospitals Network, Westmead, Australia,[4]Children's Medical Research Institute, Westmead, Australia

A major challenge for wide-spread utilization of AAV vectors in gene therapy applications is the presence of pre-existing neutralizing antibodies (NAbs) in a large portion of the human population. These antibodies originate from prior exposure to naturally circulating AAVs and primarily target the capsid leading to vector inactivation and loss of treatment efficacy. As a result, patients having these antibodies or exceeding defined antibody titer thresholds are usually excluded from receiving these therapeutics. A strategy to circumvent NAbs is the design of novel AAV capsids capable of evading pre-existing antibodies. However, this capsid-engineering approach requires knowledge of the capsid-antibody interactions. Previously, we have mapped the overall binding sites of 21 monoclonal antibodies (mAbs) derived from three Zolgensma® patients to the AAV9 capsid by cryo-EM at a resolution range of 5-10 Å. For a detailed understanding of the interactions, high resolution cryo-EM data was collected for each of the capsid-antibody complexes. 3D-reconstruction of the data with imposed icosahedral symmetry resulted in cryo-EM maps for the different AAV9-Fab complexes at resolutions ranging from 1.88 to 3.27 Å. The capsids in all maps displayed high-resolution features, which allowed the building of reliable models. In contrast, the antibody portions of the complexes, with the exception of two, showed "blurred" densities preventing effective model building and characterization of the binding interface. These blurred antibodies were bound at or near an icosahedral symmetry axis. At these binding locations, the bound antibodies do not conform to icosahedral symmetry of the capsid and as a result appeared distorted in the cryo-EM maps. Localized reconstructions of these regions resolved the bound antibodies and allowed the unbiased modelling of the binding interface. Furthermore, these structures demonstrated induced structural changes of the capsid upon binding by the antibody. Subsequently, key amino acid residues were identified and new AAV9 variants produced via site-directed mutagenesis PCR. Ultimately, we produced a capsid variant with six amino acid substitutions relative to wtAAV9 that was capable of fully escaping 18 of the 21 mAbs at high antibody titers, while also displaying improved transduction efficiency and unchanged yields using standard AAV production methods. This study demonstrates that high resolution antibody binding mapping coupled with structure guided rational design is a powerful tool for the design of new AAV capsid variants that can expand the patient cohort treatable with AAV vectors to include patients that were previously excluded due to their pre-existing antibodies.

501. NAVIGATE: A Directed Evolution Platform for Engineering Novel Ocular and Muscle AAV Capsids in Non-Human Primates

Elad Firnberg[1], Sigmund K. S. Tejada[1], Samantha Yost[1], April R. Giles[1], Timur Gaynutdinov[1], Victor Chen[1], Steven Foltz[1], Kirk Elliot[1], Benjamin P. Heithoff[1], Jared B. Smith[1], Jessica Gumerson[1], Brendan Lilley[1], Subha Karumuthil-Melethil[1], Heonhwa Choi[2], Jason T. Kaelber[2], Olivier F. Danos[1], Andrew C. Mercer[1], Ye Liu[1]

[1]REGENXBIO Inc., Rockville, MD,[2]Institute for Quantitative Biomedicine, Rutgers, The State University of New Jersey, Piscataway, NJ

Most AAV vectors currently used in the clinic feature capsids from naturally occurring serotypes, but could be further optimized for specific indications and routes of administration. Directed evolution has proven itself as an effective method for engineering improved properties into proteins, included AAV capsids. In this work, we developed a new capsid discovery engine termed NAVIGATE (**N**ovel **A**AV **V**ector **I**ntelligent **G**uided **A**daption **T**hrough **E**volution), based on mRNA-expression selection in target tissues. We designed a library construct in which the AAV *cap* gene is expressed under a CMV promoter with an AAV *rep* intron to allow for library packaging and ubiquitous expression in all tissues of interest. Diversity was generated by insertion of 7-mer randomized peptides in the AAV VR-IV or VR-VIII surface-exposed loops. We optimized the plasmid library construction and plasmid transfection process to generate AAV libraries with a high diversity of up to 10^8 variants with minimal cross-packaging, mosaicism, and wildtype capsid sequence contamination. Library composition was evaluated on the AAV genome level by

next-generation sequencing (NGS) and on the protein level by LC-MS analysis of VP proteins. We constructed libraries for multiple AAV scaffolds including AAV3B, AAV5, AAV8, and AAV9. In addition, we incorporated a 496-NNN/AAA-498 mutation in AAV9 to impart a liver-detargeting phenotype. Libraries were administered to non-human primates (NHP) by two different routes of administration and tissues were collected after three weeks for mRNA extraction and NGS. Second round libraries were generated based on a subset of 2000-4000 peptides and selection repeated in NHPs. In the third round, top capsids were packaged individually with a barcoded CAG.TdTomato transgene and administered as a pool. Finally, top performing capsids were evaluated *in vivo* by single vector administration. Cryo-EM was used to elucidate the structure of evolved capsids and infer structure-function relationships. NAVIGATE campaigns were conducted for suprachoroidal space (SCS) delivery to ocular tissues and intravenous (IV) delivery to muscle. The SCS campaign identified cross-species compatible capsids with >200-fold improvement in mRNA expression in NHP RPE/choroid compared to AAV8 and >100-fold improvement in secreted transgene product level in mini-pig aqueous humor. The IV muscle campaign identified cross-species compatible capsids with 7-fold lower liver mRNA expression and 2-fold improved skeletal muscle and heart mRNA expression in NHP compared to AAV9. Liver-detargeting and muscle expression was reproducible in both C57Bl6/J and *mdx* mice with a CAG.TdTomato and Spc5-12.microdystrophin transgene, respectively. As such, the NAVIGATE platform is a powerful AAV capsid discovery engine that can evolve capsids with improved properties in primates for potential use in future gene therapies.

502. Engineered AAV Capsids for Efficient Muscle Transduction in NHP

Shuyuan Chen, Zhenhua Wu, Xiaodong Pang, Shaoyong Li, Zhongwan Li, Runyuan Zhang, Guanlin Li, Xing Lan, Yuling Jiang, Yanyan Zheng, Changpeng Wan, Guo-jie Ye

Exegenesis Bio Inc., Lower Gwynedd, PA

Systematic administration of muscle tropic Adeno-associated virus (AAV) vectors containing therapeutic payloads represents a promising treatment for muscular dystrophy. Currently, very high doses of AAV are generally administrated intravenously due to relative low transduction efficiency of AAV capsids in the muscles. The administration of high dose AAVs has been reported to elicit significant adverse effects, particularly hepatic impairment. We aimed to engineer AAV capsids with enhanced muscle transduction and liver de-targeting profile. By using our AAVarta® platform, an AI-aided AAV capsid evolution discovery technology, peptide insertion capsid libraries based on AAV9 were generated and screened in non-human primates (NHP). The screening process relies on functional mRNA recovery in the muscles by using an in-house developed potent synthetic muscle promoter. Three weeks after intravenous administration of AAV libraries in the NHP, the muscle tissues were harvested for total RNA isolation. The capsid-specific primers were used for reverse transcription and PCR. The purified PCR products were subjected to Next-generation sequencing (NGS). The top capsid candidates were selected by AAVarta® enrichment ranking analysis and individually produced as recombinant AAVs for validation. In mice, one variant AVT919 showed significant improvements in cardiac and skeletal muscles over MyoAAV2A, a benchmark myotropic AAV capsid. In Cynomolgus macaque, AVT919 delivered via intravenous injection also demonstrated significantly enhanced transgene expression over AAV9 in the cardiac and skeletal muscles, while the transduction in the liver is comparable to AAV9. To reduce the ability of AVT919 targeting the liver, two focused libraries containing point mutations in different loops were generated. After screening in NHPs, certain AVT919 variants showed complete liver de-targeting while maintaining efficient muscle transduction as indicated by NGS data. Top candidates were selected and packaged with reporter gene for multiplex validation studies. By analyzing transgene expression level and genome copies in the tissues, several variants demonstrated efficient muscle transduction and liver de-targeting in NHP. In summary, these engineered myotropic and liver de-targeted AAV capsids could provide efficient and safer delivery platforms for muscle-targeting gene therapies.

503. Generating Human-Evolved, Cell-Type-Specific AAV Capsids for Targeted Gene Delivery

Alba Guijarro Belmar, Panayiota Demosthenous, Riccardo Privolizzi, Marta Zinicola, Hanna Luniak, Brandon Roberts, Devan Parmar, Calvin Chad Smith, Miranda Mathews, Andrew Murray

Sania Therapeutics, London, United Kingdom

Targeted delivery of therapeutic genes remains a defining challenge in gene therapy. The ideal viral delivery system would target the cell or tissue type of interest with minimal tropism for other cell types or organ systems. Highly directed tropism would also allow the vector to be administered at a low dose and enable local delivery of the gene therapy, limiting toxicity and associated immune responses. Adeno-associated viral (AAV) vectors are safe and versatile and have recently emerged as the preeminent delivery vehicle for gene therapies. The main advantage of AAVs is that their tropism can be directed towards a particular cell type by recombinant engineering of the capsid through directed evolution. This process has resulted in numerous novel and effective gene therapy vectors. However, the major drawback of directed evolution is the highly species-specific properties of the new vector, where the tropism of a particular vector can vary from one animal species to the next. As directed evolution strategies typically occur in animal models, this severely hinders the translation of novel AAV vectors into human patients in the clinic. Sania's approach to AAV vector development aims to generate novel, targeted vectors that can be successfully and rapidly translated into the clinic. Our proprietary platform, R-Scan, utilises a combination of human induced pluripotent stem cell (IPSC) derived cell types and microfluidics to perform directed AAV capsid evolution in an in vitro human system. The first clinical application for R-Scan is in the neurological space, specifically in neuro-muscular disorders such as spasticity. Spasticity causes severe and painful muscle contractions in patients due to hyperexcitable spinal motor neurons and we have used R-Scan to evolve AAV vectors that can target motor neurons directly via intramuscular injection. We first recreated this spinal-motor neural circuit using a combination of IPSC-derived motor neurons and myocytes in our R-Scan platform. To ensure we capture the phenotypic diversity of the human population we use a pooled line of IPSCs from multiple donors. For capsid screening Sania has generated 16 different

proprietary capsid libraries based on six parental serotypes and three diversification strategies - 1) random mutagenesis across the full length of the genome or to the external loops of the capsid, 2) insertions of random peptide sequences in specific locations of the capsids and, 3) insertion of rationally designed motifs known to target our cell type of interest. So far, R-Scan has generated multiple AAV vectors that show superior transduction of both human motor neurons in vitro and human neuromuscular organoids. Capsids generated from the R-Scan platform also show efficient transduction of mouse motor neurons following intramuscular injection, demonstrating translatability from our in vitro system to whole animal. Biodistribution analysis shows that intramuscular injection of our motor neuron targeted AAVs prevents vector spread and transduction in off target tissues. Additionally, we have shown that our novel capsids can be manufactured to similar titers as wild type vectors. We are further developing R-Scan so we can generate human-evolved vectors for multiple neuronal circuits and cell types. Ultimately, our goal is to utilise R-Scan to deliver targeted, safe and enduring AAV gene therapies for disorders of high unmet need.

Wednesday Posters: AAV Vectors - Non-Human Primates and Large Animal Models

504. CAP-002: Systemic AAV Gene Therapy with Next Generation Capsids for Treatment of STXBP1 Encephalopathy

Allison Knoll[1], Alberto J. Lopez[1], Troy Sandberg[1], Brandon Wheeler[1], Kenna Whitley[1], Lubov S. Grigoryeva[1], Pedram Davoodian[1], Matthew Frias[1], Mark Chin[1], Celeste Stephany[1], David Goertsen[1], Evan Walsh[1], Pasha Tchourilov[1], Hari Acharya[1], Paul Denis[1], Wu Chen[2,3], Joo Hyun Kim[2,3], Alysee Michaels[2,3], Armando Rivera[2,3], Yusuf Sham[2,3], Catalina Elwonger[2,3], Jose Paulo Carneiro[2,3], Mingshan Xue[2,3,4], Swati Tole[1], Nick Goeden[1], Nicholas Flytzanis[1], Susan Catalano[1]

[1]Capsida Biotherapeutics, Inc., Thousand Oaks, CA,[2]Department of Neuroscience, Baylor College of Medicine, Houston, TX,[3]The Cain Foundation Laboratories, Jan and Dan Duncan Neurological Research Institute at Texas Children's Hospital, Houston, TX,[4]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX

Syntaxin-binding protein 1 (STXBP1) encephalopathy is a rare, devastating neurodevelopment disorder and epilepsy affecting approximately 1:30,000 newborns and caused by *de novo* heterozygous mutations in the STXBP1 gene, which regulates neuronal communication through presynaptic vesicle and neurotransmitter and neuropeptide release. Genetic mutations that impair STXBP1 function alter neuronal excitability and cause a variety of clinical features with no approved therapies today: treatment-resistant epilepsy, severe intellectual disability, speech and language impairments, movement impairments, behavioral disruptions, and early mortality caused by sudden unexpected death in epilepsy. We performed dose-ranging pharmacology studies in a Stxbp1 haploinsufficiency mouse model that closely recapitulates the phenotypes of human disease. These studies allow for a detailed interrogation of phenotypic correction across the behavioral domains of epilepsy and cognitive and motor impairment. Using three different doses that achieve varying levels of STXBP1 supplementation throughout the brain, behavioral results support that dose-dependent reversal of disease phenotype can be achieved across all three domains, with long-lasting correction out to a year post-intervention. These results demonstrate that AAV gene therapy has the potential to be disease-modifying and provide a long-lasting solution for the treatment of STXBP1 encephalopathy but requires the biodistribution and supplementation of STXBP1 protein in neurons widely across the brain. Using our high-throughput, non-human primate (NHP)-based AAV engineering platform, novel AAVs have been engineered to cross the blood-brain barrier and efficiently deliver therapeutic cargo at breakthrough levels throughout the brain after intravenous delivery. The CAP-002 candidate consists of one such engineered capsid packaging the human STXBP1 gene, that can achieve brain-wide distribution in up to 70% of neurons in the NHP, depending on brain area, at low to moderate doses, and supplement STXBP1 protein at comparable levels that reversed disease phenotype in the mouse model. This breakthrough profile of brain transduction, coupled with significant de-targeting of the liver compared to AAV9, allows CAP-002 to be delivered at lower doses than historically used with systemically delivered gene therapies, providing a greater therapeutic window for mitigating potential toxicities and pathology associated with high-dose intervention. This well-tolerated efficacy profile is expected to provide a disease-modifying solution and achieve meaningful prospect of direct benefit for patients suffering from STXBP1 encephalopathy.

505. Assessment and Comparison of Safety of AAV9 Bicistronic HexA/HexB Vector Following Cerebrospinal Fluid Administration in Nonhuman Primate and Ovine Models

Nathan K. Yingling[1], Pamela St. Louis[1], Hector Ribeiro Benatti[1], Erin F. Hall[1], Jillian Gallagher[1], Abby McElroy[1], Meghan Blackwood[1], Luz Yvette Lopez Diaz[1], Elizabeth Parsley[2], Sarah Nath[1], Deborah J. Fernau[1], Toloo Taghian[1], Robert King[1], Miguel Sena-Esteves[1], Allison M. Keeler-Klunk[1], Heather L. Gray-Edwards[1]

[1]UMass Chan Medical School, Worcester, MA,[2]Tufts Cummings Veterinary School, North Grafton, MA

GM2-gangliosidoses - Tay-Sachs disease (TSD) and Sandhoff disease (SD) are neurodegenerative disorders due to mutations in the genes that encode the α (*HEXA*) & β subunits (*HEXB*) of the enzyme Hexosaminidase A. TSD and SD disease are clinically similar and manifest in infantile, juvenile, and late-onset forms. While infantile and juvenile patients face early mortality, late-onset patients can have a normal lifespan but are wheelchair bound. An ongoing clinical trial for infantile TSD and SD utilizing two monocistronic AAVrh8 vectors encoding HEXA & HEXB has shown positive effects on disease progression and increases in CSF HEXA expression in TSD patients. However, to circumvent the need for two vectors and bi-thalamic injections, a bicistronic AAV9 vector encoding both HEXA & HEXB cDNA (AAV9Bicis_HexA/HexB) was developed. Here, we evaluated

the safety of the AAV9Bicis_HexA/HexB vector in wild-type nonhuman primates (NHPs) and sheep.NHPs and sheep were randomly assigned to an AAV9Bicis_HexA/HexB treated (NHP: n=2; sheep: n=2) or a PBS control cohort (NHP: n=1; Sheep: n=1). AAV or PBS was administered through combined bilateral intracerebroventricular injection, cisterna magna, and lumbar intrathecal injections, with treated animals receiving a total of 2.38E14 vg (2.9 x the proposed patient dose). NHPs were immunosuppressed (prednisolone 1mg/kg), for 1 month starting at the time of injection followed by a taper. Animals received a baseline evaluation and were then monitored for 90 or 180 days by physical & neurological examinations, MRI, sensory nerve conduction velocities (NCVs), urine, CSF, and blood analyses. At the endpoint, animals were terminated, and tissues were collected for analysis. Postoperatively, the PBS NHP developed seizures which responded to Levetiracetam. All subsequent NHPs received Levetiracetam without incident. No other neurological signs were noted in this study. NCVs showed no differences between time points. Weight changes, calculated as a percent change from baseline, showed an initial drop which was regained by day 180 in NHPs. All sheep demonstrated expected weight gain. Elevated LFTs were noted in all animals during the 1st week, returning to normal range thereafter. CBC, chemistry, and urinalysis were otherwise unremarkable. Analyses of CSF revealed no detectable protein in NHPs, with pending cytospin microscopic analysis. At 90 days histopathologic evaluation showed mild nerve fiber degeneration in the cervical and thoracic dorsal root ganglion consistent with AAV administration. Histopathology for the 180-day NHPs and sheep is pending. Neutralizing antibodies were detected in all NHPs and one of the AAV treated NHPs had a positive ELISPOT to AAV9 at day 30. No positive ELISPOTs to the transgene were noted. Sheep NAb and ELISPOTS are underway.These findings highlight the safety profile of the bicistronic AAV9 HexA/HexB vector for the treatment of GM2-gangliosidoses. We also demonstrate the potential for sheep as a surrogate to NHPs to highlight toxicities associated with CNS-directed AAV gene transfer. Forthcoming studies will include analysis of MRI data, HexA enzyme activity, and AAV vector biodistribution.

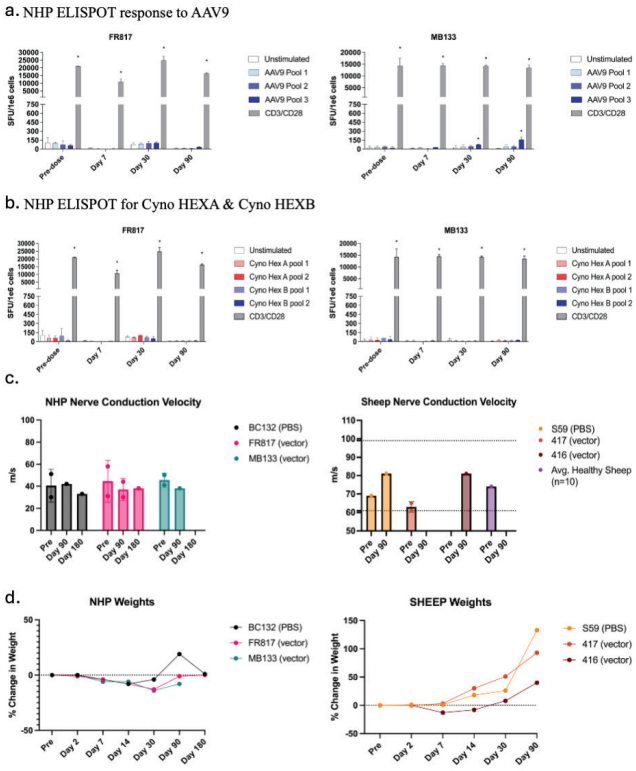


Figure 1. (a) ELISPOT response to AAV9 pools in treated NHPs (* indicates significant response). (b) ELISPOT response to Cyno HEXA & Cyno HEXB in treated NHPs. (c) NCV testing results comparison between models. (d) Fluctuations in weight as percent change from baseline, with a comparison between animal models.

506. Distinct Immune Signature of AAV9 Related Toxicity Following Intra-CSF Dosing of Cynomolgus Monkeys: Key Roles for IP-10, IP-9, MIG, IL-12 and IL-17

Ghiabe Henri Guibinga[1], Kelley Penraat[2], Xiaoguang Li[1], Justin Le[1], Jonathan Flynn[1], Sophie Lemire[3], Tony Del Rio[1], Keith Mansfield[2], Eric Johnson[2], Eloise Hudry[2], Emily Meseck[4], Fatih Ozsolak[1]

[1]Novartis, Biomedical Research, Biologics Research Center (BRC), San Diego, CA,[2]Novartis, Biomedical Research, Pre-clinical Safety (PCS), Cambridge, MA,[3]Novartis, Biomedical Research Disease of Aging and Regenerative Medicine, Data Science, Basel, Switzerland,[4]Novartis, East Hanover, NJ

Cells in the peripheral nervous system (PNS) and central nervous system (CNS) are the main intended targets of AAV9-mediated gene transfer, particularly when administered through intrathecal lumbar puncture (IT/LP). This method allows the AAV vector carrying the therapeutic gene to directly access the cerebrospinal fluid (CSF) cavity, potentially providing disease-modifying benefits for various neurological disorders. However, these benefits must be considered alongside the recognized toxicities associated with AAV9 IT delivery, such as immune cell infiltration and neuronal degeneration, which have been observed in non-human primates (NHP) in areas like the dorsal root ganglia, brain, and spinal cord. In our study, we utilized proteomic and multiplex-cytokine panels to analyze immune-related injury markers in CSF and serum samples from NHP following

intrathecal delivery of AAV9. Our findings identify several cytokines and chemokines, including IL-12, interferon gamma induced protein 10 and 9 (IP-10 and IP-9) as well as monokine induced by gamma interferon (MIG), as potential key mediators of injury in the CNS and PNS after AAV9 intrathecal administration. The data demonstrate elevated levels of IP-10, IP-9, and MIG chemokines in the serum and CSF of NHP four weeks after AAV9-GFP intrathecal dosing. These changes in cytokines/chemokines may correspond to inflammatory and neurodegenerative events as evaluated through histopathology analyses. NHP treated with "empty" or transcriptionally inert AAV9-derived capsids did not show similar elevated levels of these pro-inflammatory chemokines, nor did they display apparent injury events in the CNS or PNS. Furthermore, the inclusion of miRNA-182 or/and miR-183 target sequences in AAV9-GFP encoding cargo to mitigate dorsal root ganglia (DRG) toxicity resulted in a reduction of GFP expression in cervical, thoracic, lumbar, and sacral DRG neurons. This reduction was accompanied by a lower incidence/severity of microscopic neuronal degeneration and immune cell infiltration. Specifically, animals treated with AAV9-GFP containing the double miR-182 and miR-183 elements exhibited the greatest reduction in GFP expression and the lowest incidence of DRG toxicity. However, in these animals, there was no simultaneous decrease in IL-12, IP-10, IP-9, and MIG levels in either the serum or CSF. Instead, a decrease in IL-17, a pro-inflammatory cytokine secreted by activated DRG satellite cells and known to be implicated in DRG-related neuropathic injury, was observed in the CSF and serum. Our data emphasize two distinct immune signatures following AAV9 intrathecal dosing in NHP: 1. The first immune signature is characterized by elevated levels of IL-12, IP-10, IP-9, and MIG, indicating broad immune-related events in the PNS and CNS that involves both innate and adaptive immunity and may extend beyond DRG toxicity. 2. The second immune signature is marked by changes in CSF and serum levels of the neuropathic-specific cytokine IL-17, which is present at the time of injury and may potentially be more closely associated with the incidence of DRG toxicity events. Therefore, if this is confirmed IL-17 could potentially serve as a more suitable marker for specifically tracking DRG toxicity in nonclinical studies.

507. Newly Identified Dorsal Root Ganglion-Enriched miRNAs Fail to Suppress AAV Transgene Expression *In Vivo*

Michael S. Fleming, Emily Crosier, Daniel M. Dubreuil, MiAngela Ramos, Shih-Ching Chou, Swathi Ayloo, Brenda Richards, Amy Richards, Shelley Nass, Bindu Nambiar, Denise Woodcock, Catherine O'Riordan, Martin Goulet, Christian Mueller, Shyam Ramachandran

Genomic Medicine Unit, Sanofi, Waltham, MA

Dorsal root ganglia (DRGs) sit at the interface of the central nervous system (CNS) and peripheral blood circulation. Due to their high vascularization by fenestrated capillaries and contact with subarachnoid cerebral spinal fluid (CSF), DRGs receive high exposure to AAV administered systemically or intra-CSF, often leading to significant transduction of DRG neurons. Recent reports have described subclinical histopathological findings in non-human primate (NHP), pig, dog, rat, and mouse DRGs following AAV administration. One mechanism hypothesized to underlie DRG toxicity is high levels of transgene expression in DRG neurons following AAV transduction. One approach to limit transgene expression in a tissue-selective pattern takes advantage of endogenous miRNA-mediated RNA knockdown and tissue-selective miRNA expression. Inclusion of miRNA target sequences in the UTR of an exogenous transgene can lead to the degradation of transgene transcript specifically in tissues endogenously expressing that miRNA. A recent study utilized DRG-enriched expression of miR183 to inhibit transgene expression in DRG neurons transduced by AAV, resulting in the prevention of histopathological findings in DRG. In this study, we aimed to replicate miR183-mediated detargeting of AAV expression while testing the DRG detargeting capacity of additional DRG-enriched miRNAs. We first identified DRG-enriched miRNAs from the literature and by small RNA sequencing of human and NHP DRGs, CNS tissues, and peripheral tissues. We identified seven miRNAs with enriched expression in human and/or NHP DRG relative to brain. We confirmed *in vitro* that the inclusion of the targets of these miRNAs, or miR183, in transgene UTR inhibited expression upon co-transfection with the matching miRNA mimic. We then administered AAV.GMU001 encoding a reporter gene and miRNA target sequences, either alone or in combination, to NHPs via an intra-CSF route of administration. We found no decrease in transgene expression in DRGs for transgenes containing targets of miR183 or the newly identified DRG-enriched miRNAs relative to an unmodified transgene. In a parallel study with a different reporter gene, we also found that inclusion of miR183 target sequences had no effect on transgene expression in NHP DRG. From these results, we conclude that miRNA-mediated detargeting of expression from primate DRGs may be context-specific and cannot be easily translated across different capsids, doses and/or transgenes.

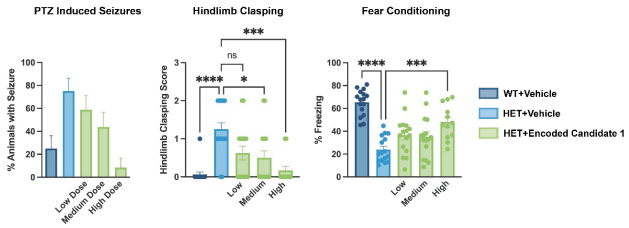
508. Advancing Gene Therapy for STXBP1-Related Disorders Through Targeted Vector Engineering: Efficacy and Safety Results in Mice and Non-Human Primates

Rangoli Aeran, Annie Tanenhaus, Sheila Sears, Nathan Moerke, Adam Miller, Camille Artur, Yosr Bouhlal, Peter Bove, Alex Diaz de Arce, Saki Shimizu, Jason Le, Keith Place, Dixon Hoffelt, Jennifer Su, Ming Chen, Brooke Babineau, John McLaughlin, Myat Soe, Winnie Lin, Dhruv Bole, Kristen Valentine, Liz Hallam, Serena Liu, Puja Dhanota, Steven A. Tan, Ben Zhao, Raghu Hosur, Suresh Poda, Archana Belle, Stephanie Tagliatela

Encoded Therapeutics, South San Francisco, CA

Rationale and Methods: *De novo* heterozygous variants in the neuronal *STXBP1* gene cause severe, early-onset developmental and epileptic encephalopathy. Adeno-associated virus (AAV)-based gene replacement therapy offers the potential for a transformative, disease-modifying approach for *STXBP1*-related disorders. In proof-of-concept experiments, bilateral intracerebroventricular (ICV) injection of an AAV9-vectorized *STXBP1* transgene under the control of a ubiquitous (EF1a) promoter led to robust, dose-dependent transgene expression and rescue of key behavioral phenotypes in *STXBP1* haploinsufficient mice (*STXBP1*$^{+/-}$), supporting the viability of this approach. However, ubiquitous overexpression of the neuronal-selective *STXBP1* gene,

controlled by EF1a, led to off-target expression in non-neuronal tissues and DRG toxicity in non-human primates (NHPs). To address this limitation, we engineered potent, cell-selective promoters and DRG de-targeting elements to mitigate unintended effects. Through targeted vector engineering, we developed i. novel promoter cassettes to drive potent and selective *STXBP1* expression across excitatory and inhibitory neuronal cells, and ii. a novel 3'UTR regulatory element to de-target expression in the DRG. **Results:** In *STXBP1*$^{+/-}$ mice, optimized promoter candidates achieved robust neuronal expression and phenotypic rescue with minimal off-target expression. The addition of a DRG de-targeting regulatory element in the 3'UTR significantly reduced DRG transgene expression, while maintaining neuronal expression and behavioral rescue. In NHPs receiving a unilateral ICV injection, widespread vector biodistribution and consistent CNS transgene expression were observed. These vectors were well-tolerated for over 50 days with no adverse events post-administration. Furthermore, the 3'UTR DRG de-targeting element reduced DRG expression by 10-fold, rescuing DRG toxicity with no adverse histopathologic effects or nerve conduction alterations. **Conclusion:** Together, our preclinical studies demonstrate robust efficacy for these engineered gene replacement candidates in mice, with conserved expression and tolerability in NHPs. These data highlight the value of vector engineering and support continued development of a best-in-class gene therapy for *STXBP1*-related disorders. **Figure 1:** Dose-dependent rescue of tonic/clonic seizures, abnormal hindlimb clasping, and fear conditioning phenotypes in *STXBP1*$^{+/-}$ mice using a cell-selective promoter cassette to drive *STXBP1* expression.



509. Endoplasmic Reticulum Stress Induction at the Transcriptional Levels in the Liver of Non-Human Primates Dosed with Toxic Doses of AAV9PHP.B-CBh-SMN1

Pedram Moeini Gavgani[1], Elisabet Guruceaga Martinez[2], Thomas A. Lanz[3], Larry O. Whiteley[4], Gloria González-Aseguinolaza[1], Tomas Aragón[1], Carmen Unzu[1]

[1]DNA and RNA Medicine Division, CIMA, University of Navarra, Pamplona, Spain,[2]Bioinformatics Platform, CIMA, University of Navarra, Pamplona, Spain,[3]Pfizer Inc., Drug Safety Research & Development, Groton, CT,[4]Pfizer Inc., Drug Safety Research & Development, Cambridge, Cambridge, MA

Introduction: Adeno-associated viral vector (AAV)-mediated gene therapy has emerged as a leading *in vivo* gene delivery platform, particularly for treating rare diseases like spinal muscular atrophy (SMA). Despite its success, reports of serious adverse events and liver toxicity associated with the administration of high-doses of AAV (>1×10^{14} vg/kg) in both preclinical and clinical studies have raised concerns in the field. Liver toxicity manifests as elevated liver enzymes, liver failure and even death, and understanding the underlying mechanisms of hepatotoxicity has become crucial. Recently, recombinant AAVs have been shown to induce endoplasmic reticulum (ER) stress by overwhelming ER protein folding capacity and to promote the activation of the unfolded protein response (UPR). The UPR is a homeostatic mechanism that surveys the quality of protein folding within the ER to maintain normal proteostasis within the cell. However, in the event of unresolved stress the UPR can also promote apoptotic cell death. Since the UPR drives a well-established transcriptional response to cope with stress, the goal of this study is to analyze the transcriptomic signature of ER stress in RNA obtained from the liver of non-human primates (NHP) receiving increasing doses of AAV9PHP.B-CBh-SMN1, an investigational gene therapy for SMA, that caused severe hepatotoxicity. **Methods:** Principal component analysis (PCA), gene set enrichment analysis (GSEA) and the evaluation of the expression of UPR/ISR-specific genes were performed on RNA sequencing data from the livers of NHPs (males and females) and rats (males) receiving different doses of AAV9PHP.B-CBh-SMN1. 2×10^{13}, 5×10^{13} and 1×10^{14} vg/kg of AAV9PHP.B-CBh-SMN1 and sacrificed 4 days after dosing (Palazzi et al. 2021). **Results:** In NHPs, PCA analysis showed that RNAseq datasets clustering was driven by two principal parameters, dose and sex. In primate samples GSEA revealed the enrichment of genes related to cytotoxicity and genotoxicity as well as the loss of enrichment in genes related to liver function after high dose AAV administration. In contrast, in rats GSEA showed a clear enrichment of genes related to the IFN response and immune response, but not in toxicity-related genes, regardless of the AAV dose. Analysis of the expression of transcripts modulated by the integrated stress response (ISR) and either of the three independent UPR mechanisms revealed upregulation of the ISR related genes (PERK, GADD34, ATF4 and CHOP markers) and downregulation of BLOS1, CES1, CES2 and SCARA2 mRNA levels, indicating IRE1-dependent decay (RIDD) activation, in both male and female NHPs in a dose dependent manner. In contrast to NHPs, no activation of the ISR and IRE1 (RIDD) arms was observed in treated rats. **Conclusions:** Our gene expression analysis on liver samples of NHPs and rats revealed a dose-dependent activation of ISR/UPR in NHPs by rAAV9-PHP.B-CBh-SMN1 administration and suggests an important role for these cell intrinsic mechanisms in AAV-mediated hepatotoxicity in NHPs. We postulate that modulation of specific ISR/UPR mechanisms might provide an effective strategy to prevent hepatotoxicity resulting from high dose AAV therapies.

510. Expression and Distribution of AAV9 and a Novel AAV2 Variant Following Intrathecal and Subpial Administration in African Green Monkeys

Michael R. Weed[1], Addison Gralen[2], Ivette M. Sandoval[2], Kimicia Isaac[1], Ernell Nisbett[1], John Elsworth[1], Matthew S. Lawrence[3], Martin Marsala[4], Fredric P. Manfredsson[5]

[1]Virscio, Inc., New Haven, CT,[2]Barrow Neurological Institute, Phoenix, AZ,[3]Virscio Inc, New Haven, CT,[4]Department of Anesthesiology, University of California-San Diego, La Jolla, CA,[5]Barrow Neurologic Institute, Phoenix, AZ

Clinical efficacy of CNS-targeting AAV gene therapies has been limited by relatively narrow biodistribution and inefficient transduction of neurons after targeted CNS administration and intra-CSF AAV vector delivery. In the latter, transduction is often limited, with sporadic distribution of infected cells in various anatomical locations, but with a majority of transduction occurring in perivascular cells and in cells in close apposition to the choroid plexus. The current studies evaluated biodistribution and neuronal transduction of AAV9 and a novel AAV2 mutant, with modifications of the AAV2 capsid resulting in increased distribution and infectivity. AAV9 or AAV2 mutant vectors expressing green fluorescent protein (GFP) were administered to adult male (N=9) and female (N=9) animals into the lumbar intrathecal (L-IT), cisterna magna (CM) or C3-C4 subpial spaces (N=3 per vector/site). L-IT and CM administrations consisted of a dose of 5E12 vector genomes (vg) in 500 µL. Subpial administrations consisted of a dose of 1.5E11 vg in 300 µL. No adverse events were noted during the in-life portion of this study. Animals were euthanized 21 days post administration with the brains fixed intact for immunohistochemistry. Peripheral tissue samples were collected for vector biodistribution and transgene quantification. Postmortem evaluation of transduction shows that either capsid efficiently transduced brain parenchyma and cells in the spinal cord; however, efficacy was strongly dependent on delivery method, with IT and CM producing greater transduction of the parenchyma, and subpial delivery resulted in vastly improved transduction of the spinal cord.

511. High-Resolution Quantitative Analysis of Multiple AAV Capsids in Rodent and Primate Models Using Multiplexed Reporter Protein Tagging Platform

Matthew A. Child[1], Alexandra Tsolias[2], Charlotte Chung[1], Rupinderjit Kaur[1], Nilesh Pande[1], Amy Bruce[1], Chiso Egbuchulam[1], Jeff Thompson[1], Alexis Bloedel[1], Kyle Grant[1], Jeyashree Natasan[1], Ruohong Zhou[1], Dhiral Shah[1], Netsanet Gebremedhin[1], Mathieu E. Nonnenmacher[1]

[1]Voyager Therapeutics, Cambridge, MA,[2]Voyager Therapeutics, Inc., Lexington, MA

Technologies for directed evolution of Adeno-Associated Virus (AAV) capsid libraries have dramatically improved in the last decade, and the generalization of high-throughput functional *in vivo* screens (iTransduce, M-CREATE, TRACER™, TRADE, BRAVE, DELIVER, etc.) is generating vast number of capsids with promising characteristics. This abundance of capsid variants can generate a bottleneck at the characterization stage, especially in non-human primate models. For each capsid variant to move forward as a clinical candidate or an experimental tool, it is of paramount importance to acquire high-quality quantitative and qualitative data, such as viral genome quantification, transgene mRNA expression, percentage of transduced cells, cell type specificity and regional distribution. Existing platforms combining RNA barcoding and single cell RNA-seq have been validated with up to 30 capsid variants in a single animal, but they are limited by low spatial resolution and the lack of protein expression data. Here we designed a medium-throughput platform for simultaneous analysis of up to 10 capsid variants in a single animal while providing transgene DNA quantitation, transgene mRNA quantitation and transgene protein immunohistochemical detection. This was accomplished by 1) engineering a high-sensitivity reporter transgene scaffold to allow RNA and protein detection at low dose and 2) optimizing a series of C-terminally fused peptide tags that can be detected with distinct tag-specific antibodies. The Multi-Tag platform was first optimized in a mouse model using the neurotrophic 9P31 capsid and showed highly consistent detection in the brain at a well-tolerated dose. We then further demonstrated the validity of the platform in a 10-capsid pilot marmoset study and in cynomolgus macaque studies where we routinely tested 5 capsids per animal. Finally, we successfully demonstrated the compatibility of the multi-tag system with spatial transcriptomics in a mouse pilot experiment.

512. Efficient Intravenous Transduction of Schwann Cells in Neonatal Mouse

Yasemin Özgür Günes[1], Catherine Le Stunff[1], Andoni Echaniz-Laguna[2], Romina Aron-Badin[3], Pierre-François Bougnères[4]

[1]INSERM, Le Kremlin-Bicêtre, France,[2]Department of Neurology, APHP, Bicêtre Hospital, Le Kremlin-Bicêtre, France,[3]CEA, CNRS, Université Paris-Saclay, MIRCen, Laboratoire des Maladies Neurodégénératives, Fontenay aux Roses, France,[4]Therapy Design Consulting, Vincennes, France

We report a successful biodistribution study in peripheral nerves using intravenously administered rAAV9-MAG-GFP vector during the neonatal period (P10). Previous gene therapy attempts have used intra-thecal injections of AAV9 vectors *carrying Jet, Mpz, or CbH promoters* (Bailey 2018, Kagiava 2021, Bailey 2020 respectively). We confirmed the efficacy of this route of administration in 4 adult wild type mice and 1 adult *Macaca fascicularis* injected with rAAV9.MAG.eGFP (2E13 vg/kg) intrathecally. In this experiment, transgene expression was observed in >75% of Schwann cells in lumbar roots and in proximal regions of a peripheral nerve. Here, we generated a gene therapy mediated by a rAAV9 vector and the MAG promoter to deliver genes in mouse models of peripheral neuropathies. Four wild type mice were injected at the neonatal period at postnatal day 1 (P1) (n=2) and day 10 (P10) (n=2) with rAAV9.MAG.eGFP (5E13 vg/kg) through intravenous injections. We chose rodents of this age because the blood nerve barrier (BNB) is not functional before 13 days of life but that its function is established by 16 days. We observed transgene expression in >75% of Schwann cells in lumbar roots and in proximal parts of a peripheral nerve. This observation indicates that Schwann cells can be targeted via an intravenous route in the first few days of life.

513. Development and Preclinical Testing of an AAV-Based Gene Therapy Product for Parkinson's Disease

Teresa Torre-Muruzábal[1], Jose L. Lanciego[2], Anne Michel[3], Filip De Vin[1], Sofie De Munter[1], Tine Brouns[1], Duy Tien Ta[1], Caroline Eykens[3], Irina Thiry[1], Sofie Molenberghs[1], Kristel Vuerinckx[1], Kevin D'Ostilio[3], Maria del Carmen Romero Pita[3], Marcia Weber[3], Veronick Benoy[1], Michael Linden[3], Julie Tordo[3], Els Henckaerts[1]

[1]Trellis Research Group, KU Leuven, Leuven, Belgium,[2]Area de Neurociencias, Centro de Investigación Médica Aplicada (CIMA) and Centro de Investigación en Red de Enfermedades Neurodegenerativas (CIBERNED), Pamplona, Spain,[3]UCB Pharma S.A., Leuven, Belgium

Parkinson's Disease (PD) is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the appearance of intracellular α-synuclein inclusions. Besides motor symptoms, patients could also suffer from non-motor complications such as anxiety, depression and dementia and, currently available therapies are largely symptomatic. Aiming for a treatment that offers more than symptom control, we developed a gene therapy approach based on adeno-associated virus (AAV)-mediated overexpression of the lysosomal enzyme β-glucocerebrosidase (GCase). Mutations in *GBA1*, the gene which encodes GCase, are among the most common genetic risk factors for the development of PD. To select the most suitable construct, we created different ITR-containing transgene cassettes with promoters of variable strengths. After *in vitro* selection of the constructs, we produced AAV vectors and tested them *in vivo*. To achieve targeted delivery to the pathological brain areas, we used AAV-TT, a neurotropic capsid which has been shown to undergo efficacious retrograde transport in the brains of both small and large animal models. To deliver the therapeutic AAV, we performed intraparenchymal stereotactic injections in wild type rats as well as nonhuman primates (NHP). We assessed vector biodistribution and promoter strength in both models by analysing GCase expression and enzymatic activity in the target tissues. Leveraging the capability of retrograde transport of AAV-TT, administration of our selected construct in rat striatum induced an increase of GCase expression of 52% in the SN. Similarly, successful delivery to the SNpc in the NHP brain was achieved by administering bolus injections at 4 different sites in the caudate nucleus and putamen, which led to a significant increase of GCase activity in the SNpc compared to the contralateral hemisphere. Additionally, we tested the capability of our vector to travel retrogradely to the SNpc in NHP under conditions of MPTP-induced neurodegeneration, mimicking the level of neuronal damage present in patients at the time of treatment. Even with a severe dopaminergic lesion in the SNpc, expression of the GCase protein in the SNpc increased significantly after administration in the caudate nucleus and putamen. Finally, the AAV-based GBA1 therapeutic vector demonstrated potency and pharmacological efficacy in a rodent model of Gcase deficiency, and additionally led to neuroprotection and α-synuclein clearance in an NHP model of synucleopathy. In summary, these preclinical data demonstrate the early feasibility of an AAV-mediated GCase overexpression approach to treat PD.

514. Evaluation of a Novel AAV Capsid with Widespread CNS and Peripheral Biodistribution in Non-Human Primates

Jie Bu, Jessica Hogestyn, Swathi Ayloo, Jae Ryu, Ben Chou, Jeffery Ardinger, Miangela Ramos, Charles Miller, Lydia Blatnik, Yuxia Luo, Amy Richards, Catherine O'Riordan, Martin Goulet, Christian Mueller, Shyam Ramachandran

Genomic Medicine Unit, Sanofi, Waltham, MA

Lysosomal storage disorders (LSDs) are characterized by lysosomal dysfunction caused by the accumulation of toxic metabolites, leading to debilitating neurodegenerative symptoms. The current standard of care for many LSDs is enzyme replacement therapy (ERT), where recombinant enzyme is replaced via regular intravenous infusions. Gene therapy, the delivery of nucleic acids encoding a functional copy of the defective gene, is particularly suited for the treatment of LSDs, as they are typically well-characterized monogenic disorders. Furthermore, gene therapy has the benefit of being a one-time treatment, without the peak-to-trough fluctuations of ERT. Adeno-associated viruses (AAV) have emerged as the preferred delivery vector for neurological gene therapy due to their favorable safety profile and longevity of transgene expression in post-mitotic cells. However, clinical translation of AAV-based gene therapies has been limited due to the inability of naturally occurring AAVs to broadly transduce the human brain. We report here the development of a novel virus, AAV.GMU1, with improved transgene expression in non-human primate central nervous system (CNS) compared to the CNS-tropic AAVrh10. Importantly, AAV.GMU1 yields higher transgene expression compared to AAVrh10 at lower vector exposure in tissues, a finding with profound safety and dose-selection implications. Additionally, we evaluated AAV.GMU1 biodistribution by different administration routes relevant to the treatment of LSDs: intracerebroventricular (ICV), intra-cisterna magna (ICM) and intravenous. Both ICV and ICM infusions of AAV.GMU1 led to widespread transduction and robust transgene expression in the CNS, with ICV demonstrating enhanced exposure in subcortical brain regions. Safety findings were favorable for ICM, while bilateral ICV administration led to increased histopathological findings in the brain. Intravenous administration of AAV.GMU1 resulted in widespread systemic exposure and robust transgene expression in liver and different muscle groups. In summary, we present AAV.GMU1 for the treatment of LSDs requiring widespread therapeutic gene delivery throughout the CNS and peripheral organs.

515. Biodistribution Assessment across Eight Non-Human Primate Studies of AAV9 Uncovers Tissue Specific Variation in Virus Transcriptional Productivity

Muhammad Shahrukh, Tony Del Rio, Fatih Ozsolak

Novartis Pharma AG, San Diego, CA

Adeno-associated virus (AAV) based gene therapies have garnered significant attention and investment in recent years due to their commercial success in tackling retinal disease, spinal muscular atrophy, and hemophilia. These AAV vectors provide a means for effective delivery of therapeutic genetic material prompting disease

correction. When evaluating the safety and efficacy of recombinant AAV vectors, the biodistribution profile plays a critical role in novel therapy development. Herein, we performed a biodistribution metadata analysis of eight separate preclinical studies in which cynomolgus macaques (*Macaca fascicularis*) were dosed with versions of self-complementary or single-stranded AAV9 capsids equipped with chicken ß-actin or cytomegalovirus promoters expressing fluorescent reporter proteins. These studies cover a broad range of administration routes including intravenous (IV), intracisternal magna (ICM), and intrathecal lumbar puncture (IT) infusion. We provide unique insights into the biodistribution gradient that results from intrathecal infusion of self-complementary AAV9 in adult and juvenile cynomolgus macaques Through the metadata analysis of AAV9 biodistribution, distinct trends emerge that showcase variable transcriptional productivity of viral DNA, predominantly in the liver. To understand the observed difference in transcriptional productivity in liver tissues, we performed epigenetic profiling of tissue-localized viral DNA. These findings shed some light on plausible strategies to consider in preclinical development of AAV9 mediated gene therapies.

516. Non-Human Primate Evaluation of an Engineered AAV Capsid for Retinal Cell-Specific and Biofactory-Based Ocular Gene Therapies

Heikki Turunen, Amanda Miles, Barbara Diaz-Rohrer, Sarah Hilton, Sam Chen, Katie Maryak, Sam Sinai, Kathy S. Lin, Jamie Kwasnieski, Adrian Veres, Eric Kelsic

Dyno Therapeutics, Watertown, MA

Ocular diseases including glaucoma, wet age-related macular degeneration (AMD), geographic atrophy (GA) and inherited retinal diseases (IRDs) may be treated using new innovative gene delivery capsids that have safer profiles and are more effective at lower doses. To design novel AAVs with these properties, we iteratively train generative AI using in vivo data from cynomolgus monkey eyes injected with multiplexed AAV capsid libraries. Here we describe the characterization of a novel capsid discovered through this approach, which exhibits improved retinal cell transduction when compared to AAV2 and external engineered capsids, with favorable retinal cell-specificity and low ocular inflammation. To evaluate retinal transduction capability, cynomolgus monkey eyes were injected intravitreally (IVT) with a capsid library, or a single capsid. We measured transduction through bulk NGS, quantification of immunofluorescent histological images and expression of a secreted protein in ocular fluids from a biofactory approach. Bulk NGS measurements from variant-associated barcodes extracted from the retina indicate an 80x improvement over AAV2. Quantification of images indicate the novel capsid consistently outperformed a commonly referenced external engineered capsid, with a 2.6x increase in retinal transduction when co-injected into the same eye, each at ~1e11vg. Similarly a 2.5-3x increase in expression of the secreted protein in vitreous humor levels was seen when delivered by IVT injection compared to the external engineered capsid (~2-3e11vg). This improved biofactory-based expression directly demonstrates the novel capsid's high potential as a vector for delivering gene therapies to treat wet AMD, among other ocular therapies employing secreted payloads. Furthermore, no inflammation was observed when this capsid was delivered alone by IVT with a secreted protein reporter gene. Next, we evaluated the cell-specific transduction of the novel capsid by IVT delivery. We did this through single-nucleus RNA sequencing (snRNA-seq) and cell-specific immunostaining of histological sections. snRNAseq confirmed transduction of all major NHP retinal cell types, including ganglion cells, bipolar cells and rods. Cell-specific immunostaining also demonstrated ganglion cells were most highly transduced in the macula, whereas bipolar cells and rods were highly transduced in the peripheral and central retina. By snRNA-seq, this capsid showed 2-3x higher transduction across these cell types compared to the external engineered capsid, closely matching histology quantification results. These results demonstrate the potential of this novel capsid as a field-leading capsid for delivery of ocular gene therapies. Potential improvement in applications like biofactory-based gene therapy approaches for wet AMD or GA and higher cell specific targeting of cells including photoreceptors and bipolar cells for treatments of IRDs and optogenetic-based therapies are highlighted. With continued validation of capsids exhibiting high transduction or unique retinal cell specificity, we will continue to expand the applicability of AAV therapies to unique ocular disease profiles.

517. Swine Reporter Models for Somatic Cell Genome Editing

Jarryd M. Campbell[1], Rebecca Milliken[1], Sierra Long[1], Hinsoukpo Dagan[1], Dennis Webster[1], Derek Korpela[1], Daniel F. Carlson[2]

[1]Recombinetics, Inc., Eagan, MN,[2]Recombinetics, Eagan, MN

Delivery of gene editing reagents to the appropriate tissues and cells at an effective dose is a significant challenge for *in vivo* gene editing therapies. Rodent reporter models activated by recombinases or gene editing reagents have been indispensable for development of new delivery modalities. However, their small size and anatomy do not accurately mimic the effectiveness of delivery to large mammals. As part of the NIH Somatic Cell Genome Editing (SCGE) consortium, the Recombinetics Swine Reporter Models are swine models designed to track gene editing in any tissue or cell in the body. It does this by harboring a genetic reporter in every cell that can only be activated by gene editors like CRISPR or Base Editor or by Cre recombinase. Once activated, the reporter will permanently make fluorescent markers that can be used to track gene delivery in any tissue or cell in as soon as one week or in months to years even with non-viral vectors. The first Swine Reporter Model (SRM-1) harbors a genetic reporter that is based on the rodent Ai9 allele. The Ai9 allele has been modified to allow for activation by a SpCas9, SaCas9, AsCas12a, or Cre. The reporter is driven by a highly active and ubiquitous CAG promoter and was positioned at a safe harbor site by gene editing to avoid genotoxicity caused by the integration. After removal of a 3xStop cassette by CRISPR or Cre the cell will begin producing a fluorescent gene (tdTomato) and a sodium iodide symporter gene (NIS) that can be tracked by fluorescent activated cell sorting (FACS) or immunohistochemistry (IHC). The NIS gene also allows for non-invasive imaging of cell activation after injection with a radioactive iodide tracer using sensitive imagine modalities like single-proton emission computed tomography (SPECT) or positron emission tomography (PET). We intravenously

injected AAV9-Cre ($7.3x10^{12}$ GC/kg) into 3-week old SRM-1 piglet and collected 41 tissues after three weeks for analysis of AAV9 viral distribution and reporter activation measured by DNA recombination and tissue immunohistochemistry. AA9 injected in a male piglet was found to have widespread tropism with most accumulation in liver, muscle, adrenal glands, and testicular tissue. A subset of tissues was analyzed by immunohistochemistry, co-staining for tdTomato and NIS, to visualize the reporter activation. The resulting images showed clear and robust reporter activation can be visualized at the tissue and cellular level. The level of activation was highest in skeletal muscle, liver, and testicular tissue, followed by heart muscle, spleen, and no activation in the brain tissue. Overall, this experiment demonstrates the utility of the reporter for tissue- and cell-specific delivery of AAV. We next plan to deliver CRISPR editing tools by packaging mRNA with LNPs and delivering to the brain and liver to the reporter to further validate model utility. The second reporter animal, SRM-2, can be activated by adenine base editor and will also undergo *in vivo* validation studies. We anticipate these animal lines will be useful for both discovery studies of new delivery modalities and late-stage IND-enabling studies such as dose-escalation, pharmacokinetics, and pharmacodynamics analyses to streamline preclinical evaluation for gene editing-based therapies.

518. Determining the Maternal Safety of In-Utero AAV Gene Therapy (IUGT) Using a Sheep Model

Oluwaseun O. Babatunde[1], Martin Rodriguez[1], Sarah Wachtman[1], Jovanna Perez[1], Jennifer Wilson[1], Walaa Mohamed[1], William Vaughan[1], Anthony Atala[1], Christopher Doering[2], H. Trent Spencer[2], Christopher D. Porada[1], M Graça D. Almeida-Porada[1]

[1]Fetal Research and Therapy Program, Wake Forest Institute for Regenerative Medicine, Winston Salem, NC,[2]Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta and Dept. of Pediatrics, Emory University, Atlanta, GA

In utero gene therapy (IUGT) harbors the promise of curing a wide range of monogenic disorders and can preempt disease-induced organ damage that could occur during gestation, thus allowing for the birth of a healthy child. Still, ethical issues surrounding fetal therapies can never be overlooked, especially concerning the primary interests of the woman's well-being. Here, using sheep as a model, we investigated whether the pregnant ewes were affected by IUGT using AAV5-PGK-eGFP (n=4), AAV6-PGK-eGFP (n=3), and excipient alone (n=2). Here, using sheep as a model, we investigated whether the pregnant ewes were affected by IUGT when sheep fetuses at 60-65gd received PGK-eGFP-AAV5 or PGK-eGFP-AAV6 at a dose of $3x10^{13}$ vg/kg, or excipient as a control. All the injected fetuses were confirmed to be transduced by the respective vectors. Blood samples were collected from the pregnant ewes, prior to, immediately after, four days, and fourteen days post-AAV IUGT. Blood samples were analyzed by digital PCR (dPCR) for the AAV-eGFP transgene and the AAV-ITRs to determine whether circulating AAV DNA was present in the maternal plasma, and to assess whether circulating maternal blood cells had been transduced (eGFP and ITRs copies/cell).dPCR performed on plasma and hepatic tissue-derived DNA demonstrated AAV5- or AAV6-derived ITRs or eGFP were never present, at any time point, in the circulation or in the hepatic parenchyma of any of the ewes. In addition, ELISAs to detect anti-AAV5 IgG and anti-AAV6 IgG were performed on maternal plasma to determine if an immune response had developed against the AAV vectors. No anti-AAV5 or anti-AAV6 antibodies were detected in plasma samples collected from any of the ewes, either prior to IUGT or fourteen days post-fetal injection of AAV5-PGK-eGFP and AAV6-PGK-eGFP, confirming that after IUGT, AAV5 and AAV6 vectors are confined to the fetal environment and are absent in maternal circulation/tissues. In addition, we investigated whether delivery of AAV vectors to the fetus resulted in placental or cord tissue transduction by performing dPCR for the eGFP transgene and immunohistochemistry (IHC) for the eGFP protein. The copies/cell of AAV5-eGFP transgene in the placenta(s) and umbilical cord(s) were 0.5±0.3 and 0.5±0.4, respectively; and AAV6-eGFP transgene copies/cell in the same tissues were 0.00005±0.0007 and 0.06±0.04, respectively. However, despite the presence of copies/cell in placenta(s) and cord tissue(s), IHC revealed the absence of eGFP protein expression in all tissue(s) analyzed. Therefore, in the sheep model, IUGT using AAV5 or AAV6 vectors does not pose a risk for the pregnant ewes, and the placenta might serve as a selective barrier, preventing the passage of AAV vectors injected in the fetus to the ewe's circulation/tissues.

519. Defining *In Vivo* Biodistribution and Transduction Efficiency of AAV5 and AAV6 Vectors for *In Utero* Gene Therapy Using a Fetal Sheep Model

Oluwaseun O. Babatunde[1], Martin Rodriguez[1], Sarah Wachtman[1], Jovanna Perez[1], Jennifer Wilson[1], Walaa Mohamed[1], William Vaughan[1], Anthony Atala[1], Christopher Doering[2], H. Trent Spencer[2], Christopher D. Porada[1], M Graça D. Almeida-Porada[1]

[1]Fetal Research and Therapy Program, Wake Forest Institute for Regenerative Medicine, Winston Salem, NC,[2]Aflac Cancer and Blood Disorders Center, Children's Healthcare of Atlanta and Dept. of Pediatrics, Emory University, Atlanta, GA

AAV gene therapy (GT) for hemophilia A (HA) was poised to fulfill the promise of a safe, affordable, correction after a single treatment. Yet, clinical trials (CT) have underscored the risk of hepatoxicity and genotoxicity in adult HA patients receiving GT. Also, pre-existing AAV immunity prevents CT eligibility, and it is unknown whether GT will avoid induction of FVIII inhibitors. We hypothesize that performing in utero AAV-based GT (IUGT) will enable correction prior to birth circumventing both AAV- and FVIII-related immune hurdles. Here we used fetal sheep to define in-vivo biodistribution and transduction efficiency of AAV5 and AAV6 vectors encoding eGFP, under the control of the constitutively active PGK promoter, after IUGT. Sheep fetuses at 60-65gd (equivalent of 16-18gw in humans) received PGK-eGFP-AAV5 (n=6), PGK eGFP-AAV6 (n=8) at a dose of $3x10^{13}$ vg/kg or excipient (n=4). Fetal tissues were analyzed at 14 days post IUGT by digital PCR (dPCR) to determine eGFP and ITRs copies/cell. PGK-AAV5-eGFP transgene copies/cell in the brain, lung, duodenum, spleen, thymus, reproductive organs, kidney, heart, liver and bone marrow were 0.7±0.4; 1.7±0.7; 9.9± 3.0; 18.2±7.9; 3.7±2.4; 10.4±5.9; 3.5±1.3; 3.5±1.5; 2.4±0.6 and 0.13±0.006 respectively, and PGK-AAV6-eGFP transgene copies/cell in the same organs were 0±0.00; 0.013± 0.01; 0.05±0.02; 0.014±0.007; 0.1±0.06; 0.06±0.032;

0.03±0.02; 0.03±0.005; 0.07±0.02 and 0.08±0.04 respectively. RT-dPCR for eGFP mRNA on liver, lung, and spleen of AAV5 animals were 40.4±27.9; 30.5±12.9; 64±27.8 per 10,000 copies of RPL13A and AAV6 44± 23.6; 2.8±2.4; 6±3.5 all per 10,000 copies of the housekeeping gene RPL13A. Immunohistochemistry (IHC) was performed for the detection of the eGFP protein and ELISA to quantify the of amount eGFP expressed in the liver and lungs of the different animals. Animals that received AAV5 and AAV6 had 96.5±45.9 and 20.5±14.4 pg/ml of eGFP protein in the lungs and 121±45.6 and 338±131.8 pg/ml in the liver respectively. In summary, both AAV5 than AAV6 transduce the majority of the organs during the prenatal period with AAV5 exhibiting higher transduction efficiency than AAV6 except for the bone marrow. Nevertheless, despite higher copies/cell of AAV5 in the liver than AAV6, the two AAV serotypes exhibited similar copies of eGFP mRNA transcripts with AAV6 translating into more GFP protein than AAV5. However, the eGFP transcripts were translated into more protein in the livers transduced with AAV6 than AAV5, underscoring the need for further studies to investigate the mechanism(s) by which the AAV capsid can influence transgene expression independent of vector copy number in the target cell.

Wednesday Posters: AAV Vectors - Product Development Manufacturing and Approval Considerations

520. Breaking Boundaries in AAV Production: Exploiting High Salt Concentrations for Enhanced Process Efficiency

Dmitry Zabezhinsky

Merck Life Science KGaA, Darmstadt, Germany

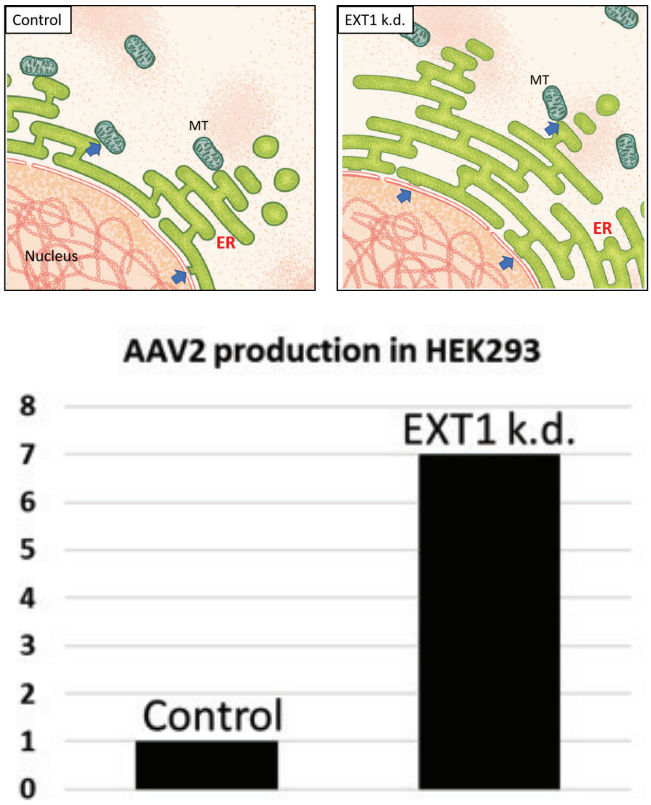
Enhancing adeno-associated virus (AAV) production is of utmost importance in the field of gene therapy. In this study, we have confirmed previous approaches to boost AAV production utilizing higher salt concentrations during the lysis process. Our research yielded impressive results, demonstrating that elevated salt levels, such as 500mM, effectively enhance AAV process intensification by eliminating adhesive DNA from the AAV capsids and reducing aggregation. However, it is worth noting that high salt concentrations exceeding 200mM hamper the activity of standard endonucleases, which are virtually inactive at high salt environment. To overcome this challenge, we have engineered a cutting-edge enzyme called the Benzonase® Salt Tolerant Endonuclease, specifically designed to function optimally in high salt conditions ranging from 500-1000mM. This remarkable enzyme efficiently digests DNA fragments shorter than 10 base pairs and remains fully compatible with commonly used detergents. By incorporating 500mM salt in the lysis buffer, we have achieved remarkable outcomes. Our experimental results indicate a substantial improvement in AAV titers by 20-30% (evaluated using ELISA) and a remarkable 10-fold enhancement in viral transduction capability. These findings provide valuable insights to intensify AAV production, solving one of the key bottlenecks of the gene therapy industry: AAV production yield. The design of this new Salt Tolerant IPEC GMP endonuclease also contributes to meet the highest patient safety standards.

521. Enhancing Viral Vector Production Through Genetic Engineering of Host Cells

Maximilien Lopes, Jean-Claude Twizere

Viral Interactomes Networks, University of Liege, Liege, Belgium

Our engineered Extra-HEK293-AAV cell line boosts viral vectors production by two to sevenfold. Viral vectors stand out as widely used and effective delivery agents of gene therapies. However, one of the main challenges confronting their manufacturing lies in the scaling up of production and addressing the relatively low productivity of host cells, leading to high production costs. In practice, large dosages are required for patient treatment and these viral vectors are packaged in mammalian cells such as human embryonic kidney (HEK293). In the pursuit of enhancing the quantity and quality of gene therapy products, ongoing efforts focus on refining upstream and downstream processes, including culture medium composition, transfection agents, and purification techniques. As a complementary strategy, our focus is directed to the optimization of host cells intracellular machinery required for viral vector production. Specifically, our approach involves genetic engineering of HEK293 cells, designed to yield higher quantities of viral vectors at an industrial scale. We previously demonstrated the regulatory role of the Exostosin 1 (EXT1) glycosyltransferase enzyme in shaping the morphology and dynamics of the Endoplasmic Reticulum (ER). Through the targeted reduction of EXT1 gene expression using shRNAs, multiple pathways are impacted, influencing ER network and associated cellular processes. These observations led us to consider potential applications of depletion of EXT1 in enhancing the efficiency of biomanufacturing. The subsequent work resulted in an improved capacity of the cells to generate recombinant viral vectors, with a production multiplied by a factor of 2 to 7. Following our findings, validation studies were conducted comparing the production capabilities of wild-type cells against the modified cells at the laboratory scale. Subsequently, we adapted our solution from adhesion culture to suspension culture, using serum-free media, a critical step for widespread industrial adoption. This transition was succeeded by a scaling-up phase, incorporating the use of fixed bed bioreactors for adhesion culture and stirred tanks for suspension culture. Recognizing the significance of accurate cell characterization in the pharmaceutical industry and considering the abundance of HEK293 derivatives cells, we took a proactive approach by creating a comprehensive identity card for our cells. This was achieved through a multi-omics study, encompassing the whole genome sequence using NGS PacBio HIFI, transcriptomes analysis using Illumina, and targeted metabolomics. The whole genome sequence facilitates the precise identification of this cell line, when used in highly controlled clinical settings. The transcriptomics and metabolomics data will contribute to the study of the impacted pathways following the EXT1 knockdown, revealing the regulatory mechanisms that underscore the enhanced productivity. Future studies aim to investigate complementary gene interplays and refine the optimization of this HEK293 cell line to produce recombinant biomolecules.



522. Fit from Development to Manufacturing - A Successful rAAV9 Scalability Study in a Suspension Process Using HEK293

Matthias Boscher, Johanna Wagner, Sinisa Petrik, Jared Babic, Beatriz Larena Carnio, Dila Bitnel, Sirri Ndonwi, Regina Staffler, Johanna Weizenegger, Eleni Tsiaousi, Christos Mantzoros, Tobias Kloetzler, Marina Magerl, Gora Matthias, Anita Heinlein, Henze Sandra, Christine Zach, Sebastian Ritter, Andreas Schulze, Alexandra Schobert, Melanie Langhauser, Karl Heller, Markus Hoerer, Ahmed Youssef

Ascend Gene and Cell Therapies GmbH, Planegg, Germany

Design of experiment (DoE) approaches performed in robust scale-down devices such as small-scale bioreactors for upstream process development allow product specific optimization of modular platform processes at speed and with reasonable development costs. However, scaling up the process to larger bioreactors can be challenging. To stay in a single provider's system for all scales can be a good starting point, as most of them already deliver a homologue vessel height to diameter ratio as well as respective impeller, baffles, and sparger designs. But as often in sensitive biological systems, this is not enough. Several process specific and defining conditions like mixing time, power input, impeller tip speed or oxygen transfer rate (OTR) cannot be kept constant together throughout all scales. Also, factors independent of the bioreactor but dependent on scale, such as process parameters and volumes of the cell seed train, must be considered, as they can impact the cell's performance. The aim of this study is to demonstrate scalability of AAV productivity and consistency of product quality across all different bioreactor scales of Ascend's AAV manufacturing platform offering. Many critical quality attributes related to product impurities including full/empty ratio, residual plasmid and host cell DNA (HCD) packaging are strongly determined by the design and choice of the biological starting materials and the upstream process. Therefore, we consider it essential to scale-up processes with the target of consistent quality and productivity across all scales to be able to support our clients across a product's life cycle. This in turn necessitates robust, fit for purpose analytics allowing in-depth product characterization. Here we present AAV batch data from 250 mL to 200 L bioreactor size using several proprietary analytical methods to assess both quantity and quality of the material generated. In summary, we demonstrate a successful scale-up of the upstream process of our modular AAV manufacturing platform using our split two plasmid platform, from bench-scale to 200 L production scale meeting our stringent development target of consistent quality at expected volumetric yield increase. Our commitment to our clients is to support development of their products through all stages, from early preclinical and tox studies to Phase 3 and commercial manufacturing with a de-risked CMC regulatory pathway.

523. Enhanced Transfection Complex Performance for AAV Production

James J. Ludtke[1], Nicolas Rossi[1], Yuyuan Wang[1], Oksana Hart[1], Neha Andhole[1], Austin Stork[1], Jennifer Swanson[1], Becky Reese[1], Laura Juckem[2]

[1]Mirus Bio LLC, Madison, WI,[2]Mirus Bio, Madison, WI

Adeno-associated virus (AAV) has emerged as a leading platform for gene delivery for the treatment of a variety of human diseases. AAV manufacturing is an expensive and relatively complicated multi-step process, which often includes transient transfection, harvest, downstream processing, quantitation and quality assessment. The transient transfection step of AAV manufacturing is arguably the most critical step for efficient AAV production, and is dependent on a robust and reliable transient transfection protocol. Transitioning to large-scale bioreactors is required to meet the increasing demand for AAV gene therapies. The scale-up process of generating transfection complexes presents several technical challenges involving the combining and mixing of large volumes of liquids, a complex formation incubation period, followed by transfer to the bioreactor. Transfection complexes can have a relatively short time window for optimal transfection efficiency, compromising flexibility and convenience within the upstream workflow. Optimal transfection complex formation times (TCFTs) vary from as short as 10 minutes to up to 60 minutes, depending on the transfection reagent and transfection complex specifics (complex formation buffer, DNA concentration, reagent-to-DNA ratio, etc). Mirus Bio has developed tools that can extend the optimal TCFT. Transfection complexes formed with the modified formulation and/or workflow have optimal TCFTs from between 30 minutes to greater than 2 hours. This increased window allows manufacturers to more consistently achieve the optimal time for transfection complex formation reducing the risk of compromised performance and/or failed runs. Data will be presented showing the particle size of the transfection complex via dynamic light scattering and AAV genome and capsid titers. Stabilization will be assessed with

varying transfection formulations, ranging from multi-component lipid and polymer formulations to single component polymeric compounds. The effects of AAV serotype and gene of interest will also be evaluated. The data presented in this poster illustrates extended TCFTs while at the same time generating maximum AAV titer and percent full capsids. This will shift the paradigm for reproducible and large-scale production of AAV by transient transfection.

524. Comparability Study Between Ion-Exchange Monolith and Affinity Resin for Purification of AAV8

Janja Merkelj Koren, Rok Žigon, Andrej Mihevc, Marko Šnajder, Maja Leskovec, Aleš Štrancar

Sartorius BIA Separations, Ajdovščina, Slovenia

The main objective of every downstream process of Adeno-associated virus (AAV) is to achieve high recovery while delivering the purest, most potent product possible. In manufacturing setting capture step is either affinity or cation exchange chromatography, which both concentrate the product and remove impurities. Following capture, the eluate is generally further processed on anion exchange monolith to enrich full capsids and further reduce impurities. In this poster, a side-by-side comparison of capture step on CIMmultus SO3 column and commercially available affinity resin, followed by polishing on CIMmultus QA will be presented. Results will show process and step recoveries, measured by two orthogonal analytical methods -dPCR and PATfix. Comparison will include impurity reduction, with final full AAV purity by PATfix and mass photometry, residual hcDNA, pDNA, host cell protein and endotoxin data. Product column capacity will be presented for both process strategies. Finally, processing time will be calculated and discussed.

525. Unveiling L4-22K and -33K as Novel Adenovirus Helpers for Improved AAV Manufacturing

Michael Gapinske[1], Eduardo Aguilar[1], Brandon S. Razooky[1], Andy Tran[1], Kailey Myers[1], Kenny Shimada[1], Christos Galanis[1], Alexandra Cole[1], Min Zong[1], Raquel Vega[1], Gilad Doron[2], Bryan St. Germain[2], Jason Carte[1], John F. Kerwin[2]

[1]Resilience, San Diego, CA,[2]Resilience, Waltham, MA

Adeno-associated virus (AAV) gene therapy has emerged as a promising therapeutic avenue for a myriad of genetic disorders, leveraging the inherent ability of AAV vectors to deliver and express therapeutic genes in target cells. Producing AAV requires the expression of a multitude of proteins and RNAs, categorized into 4 classes; Capsid (Cap), Replicase (Rep), the transgene of interest (GOI), and Helper. For the last 25 years, the established dogma has maintained that the minimally sufficient system for maximal AAV productivity using Adenovirus Helpers only requires the proteins E2A DBP, E4orf6, E1A, and E1B-55K as well as the viral-associated RNA (VARNA). Here, we present an additional Adenovirus gene, L4-22K/33K, as a necessary component for maximal AAV productivity. We utilized a comprehensive screening approach, dissecting a canonical Adenoviral helper plasmid to determine the L4-22K and -33K protein products are required for high-titer AAV production. These findings facilitated the development of a novel Tetracycline-inducible Helper system with a 100-fold improvement in AAV production over a system lacking the L4 gene, improving our ability to generate a stable cell line for the production of AAV.

526. The Potential of Global Regulatory Harmonization in Gene and Cell Therapy

Claire Marasco, Ashley Marie Shay, Christopher J. Shilling

Regulatory Affairs, Forge Biologics, Inc., Grove City, OH

Global regulatory convergence has the potential to enable faster and more widespread patient access to gene therapy products, especially for rare disease indications. Even so, with 10 in vivo gene therapy approvals in at least 11 global regions over 7 years, the progress toward convergence is unclear. Success in this area has been demonstrated by Project Orbis. Project Orbis, conducted through FDA's Oncology Center of Excellence, has facilitated the parallel review and 205 total approvals of 75 unique oncological drugs in the US, Australia, Canada, UK, Singapore, Israel, Brazil, and Switzerland. With these successes in mind, Project Orbis has been discussed at major industry conferences as a potential model for a gene therapy global regulatory framework. Regulatory frameworks that may be leveraged exist today, including parallel scientific advice, equivalent designation pathways and conditional marketing authorizations. These frameworks will be discussed in this presentation. Though commercial in vivo gene therapy has arrived in at least 11 regions, these approvals are concentrated in the US, EU, and UK. That said, the global industry continues to make important steps toward convergence in the gene therapy regulatory environment. This presentation will share the case studies of industry groups creating "playbook" approaches to gene therapy development that have the potential to expand to a global scope. Further, the presentation will discuss the actions that regulators and standards-coordinating bodies are taking to promote convergence, like ATMP cluster teleconferences, the IPRP, APEC, and the WHO gene therapy regulatory framework. Recently, Dr. Peter Marks from US FDA announced the CoGenT Pilot to promote global collaboration in the regulation of gene therapy products. This is an exciting basis on which harmonization could flourish in this industry, especially as it draws many parallels to Project Orbis, which will be discussed in this presentation. With robust discourse and careful alignment on expectations and priorities in the evaluation of gene therapy products, there is an opportunity to create the scale of success seen with Project Orbis for gene therapies, enabling access to life-changing medicines for patients across the globe.

527. From Benchtop to Cleanroom; Designing an Optimized Platform Purification Process for rAAV Production to Meet the Growing Demand

Blake Gursky, Corben Davis, Smita Menon, Keerthana Subramanian, Ganesh Krishnamoorthy, Frank Agbogbo, David Dismuke

Forge Biologics, Grove City, OH

Recombinant adeno-associated virus (rAAV) vectors have garnered significant attention from the gene therapy community, with their proven clinical success in addressing debilitating chronic diseases

and conditions. However, a major challenge lies in developing an optimized, cost-efficient, and scalable purification process for all rAAV serotypes, including novel capsid designs, to meet the growing global demand for these therapies. Capital investment, time to perform development, high vector requirements, and manufacturing expertise all pose potential obstacles for gene therapy developers. At Forge Biologics, we introduce a tailored downstream platform process designed to support expedited therapeutic rAAV pipeline programs, delivering high yield and high product quality. In our platform process, crude lysate from the optimized upstream process is clarified using systematically tested depth filters. Following clarification, the rAAV vector is captured using a predetermined resin-buffer combination, based upon our experience with multiple serotypes. Depending on the outcomes, a more customized affinity resin process with specific buffer conditions is incorporated into the process for high recovery and product quality. Subsequent enrichment of the vector material involves separating full capsids from empty capsids through either cesium chloride ultracentrifugation or anion exchange chromatography. The method of enrichment is determined by program needs and timeline. The final step involves taking the full rAAV capsids through the final formulation process and filling the drug product utilizing state of the art fill/finish processes. This process yields a highly pure rAAV suitable for early preclinical applications, toxicological studies, or clinical use. At the start of a typical project, process parameters are determined at a 1L scale and then scaled up to larger batch sizes, reaching up to 1000L, depending on the specific client's needs. The success of each purification step is verified through stage-appropriate analytical testing, including GOI titer by ddPCR, residual plasmid DNA by ddPCR, product purity by SDS-PAGE or capillary electrophoresis, genome integrity through next-generation sequencing, empty:full particle ratio by analytical ultracentrifugation, potency through qualified cell-based assays, residual host cell DNA, residual host cell protein, endotoxin, and other relevant tests. This presentation will cover key experimental highlights in the development of Forge's platform purification process.

528. Development of a Scalable Upstream Process for Suspension HEK 293 rAAV Production in Single-Use Bioreactors for Efficient Manufacturing of Gene Therapy Products

Danielle Sexton[1], Jessie Chua[2], Steven Wesel[1], Frank Agbogbo[3], David J. Dismuke[4]

[1]ForgeBiologics, Grove City, OH,[2]Process Development, ForgeBiologics, Grove City, OH,[3]Forge Biologics, Grove City, OH,[4]Forge Biologics, Columbus, OH

The need to supply large patient populations with gene therapy medicines produced using recombinant adeno-associated virus (rAAV) vectors continues to increase with scientific advancement, positive clinical data, and commercial approvals. A pivotal challenge has involved the bottleneck associated with vector yield from suspension bioreactors and establishing a scalable production process. Forge Biologics has developed a platform process based on the transient transfection of Ignition™ HEK293 cells in single-use bioreactors, which has allowed us to help our clients navigate from benchtop to clinic more quickly than traditional development pathways. Our targeted process development approach leverages the benefits of a platform while also allowing clients the ability to optimize critical parameters to ensure overall process robustness. To further enhance yields and productivity, especially for lower-producing serotypes and problematic gene of interest (GOI) vectors, additional examination of growth and purification processes can often lead to substantial improvements in vector yield. This presentation will provide examples of process optimization studies at the plasmid design, transfection, and harvest steps. Product-specific enhancements can include transfection DoE studies, capsid design and screening, and lysis and harvest optimization. The transfection process can often be enhanced by evaluating plasmid DNA (pDNA)/reagent complexes for entry into cells or by changing the ratios of three plasmids traditionally used in rAAV productions. The data presented explores the optimization of transfection parameters, including plasmid ratios, DNA/cell, PEI/DNA ratios, transfection reagent, and feeds. Capsid design and screening can establish effective rAAV rep/cap plasmids, which are critical for yield and quality in rAAV production. Data will be presented on how the manufacturability of rAAV can be improved through optimization of the rep/cap design. Some rAAV capsids, such as rAAV2 and rAAV6, bind to the cell mass, which should be considered for lysis, harvest, and clarification optimization. The data presented demonstrates that harvest timing and lysis conditions can improve the release of the rAAV from the cell debris for improved vector recovery. Beyond these product-specific enhancements, Forge Biologics systematically characterizes the platform across multiple scales. This comprehensive approach enables efficient progression through process development and facilitates the adjustment of manufacturing processes to achieve optimal yield and reduced variability. Data including cell growth and productivity impacts of process improvements will be shared.

529. Characterization of Engineered AAV Capsids from Different HEK293 Cell Culture Fractions, Crude Lysate versus Cell Pellet Material

Heidy Morales, Hari Acharya

Capsida Biotherapeutics, Inc., Newbury Park, CA

Recombinant adeno-associated virus (rAAV) has emerged as a promising delivery vehicle for gene therapies. Building on the capabilities of wildtype AAV serotypes, engineered capsids with improved tissue tropism and reduced toxicity are being developed. Engineered capsids create opportunities to address unmet medical needs for both rare and common diseases across all ages. As the therapeutic space expands, so does the need for higher yielding production processes with consistent product profiles. The main production platform for rAAV uses triple transient transfection of HEK293 cells. Typically, cells are expanded to a target density, transfected with plasmids, incubated, and lysed to harvest the product. Crude lysate includes product from both the supernatant and cell pellet fractions. How does the distribution of product and attribute profile compare between these fractions? Here, we characterize the material from the different cell culture fractions (crude lysate (CL), cell pellet lysate (CPL), supernatant (SN)) and assess product distribution. We also compare material taken through an affinity chromatography capture step or an iodixanol ultracentrifugation step to understand the impact on attribute profiles. The respective downstream trains

offer different benefits for high-throughput screening work. We find the product is not evenly distributed between the fractions and the attribute profile varies (e.g, residual DNA, host cell protein, %fulls). Information generated from characterizing the different fractions can be used to adjust incubation durations, harvest approaches, and downstream processing.

530. Alternative Plasmid Designs Including Two Plasmid Transfection Systems for Improved Production and Packaging of Engineered AAV Capsids

Lysa-Anne Volpe, Jenna Rodden, Heidy Morales, Garrett Garrido, Hari Acharya

Capsida Biotherapeutics Inc., Thousand Oaks, CA

Recombinant adeno-associated virus (rAAV) vectors are one of the most promising delivery systems for gene therapy. Building on the success of wild-type AAV serotypes, engineered AAV capsids for in vivo gene delivery are being developed to improve target tissue tropism, increase transduction efficiency, and reduce toxicity. These novel capsids create opportunities to address unmet medical needs for both rare and common diseases across all ages. The increasing use of engineered capsids in clinical trials has highlighted the need for scalable, higher-producing, cost-effective processes to meet the material and quality demands for commercial manufacturing. To date, triple transient transfection with plasmids is the most common and widely used platform for rAAV generation due to its ease, flexibility, and speed for sequence modification. Although effective, the three-plasmid manufacturing system is complex to optimize, requires higher costs of critical raw materials, and can lead to lot-to-lot variability. Here, we report the development of a two-plasmid transfection system and compare production and packaging performance to our triple transient transfection process. Various plasmid configurations were explored to identify design benefits and functional components to group together. To assess plasmid design impacts, various process conditions were evaluated, including performance in HEK293 cell lines, plasmid backbone selection markers, plasmid molar ratio, DNA input, and transfection reagent optimization. Evaluation of the transfection systems were performed using a HEK293 suspension production process at small scale and purified rAAV particles were measured for encapsidated residual plasmid and host cell DNA levels. Preliminary results from these studies display improved viral production, higher percentage of full particles and decrease in packaged residual DNA impurities.

531. Membrane Chromatography for Preparation of AAV at the Manufacturing Scale

Julio Miguel Huato, Aydin Kavara, Mark Schofield, Kurt Boenning

Cytiva, Westborough, MA

The recent FDA approval of several adeno-associated virus (AAV)-based gene therapies is driving demand for AAV production. One of the biggest manufacturing challenges is removing "empty" capsids, which do not contain the gene of interest. Anion exchange chromatography has emerged as a solution for scalable empty and full capsid separation. Here we develop a process for baseline separation of empty and full AAV capsids using Mustang Q anion exchange membrane chromatography. This process development approach utilizes AAV serotypes 5, 8, and 9 and traverses initial screening using Mustang Q XT 0.86 Acrodisc unit up to manufacturing-scale processes with Mustang Q XT 140. Process development of a two-step elution was performed via response surface DoE, exploring conductivity and the length of the first elution step. Empty and full capsid percentages were quantitated with ELISA and ddPCR assays as well as mass photometry. The results from response surfaces were used to construct statistical models of the process operating space. These models provide optimal conditions for recovery and purity, both of which can exceed 80%. Model predictions were then validated at small scale prior to scale-up. We present the results from our scale-up purification and show that purity and yield are consistent with the results obtained from the response surface model. To demonstrate that Mustang Q membrane chromatography can be a robust manufacturing technique, we tested three different feedstreams of AAV5 on three unique lots of Mustang Q. The feedstreams varied by cell line (HEK and SF9) and cell culture technique (adherent and suspension). Drastic differences in the separation conditions and operating space were identified. Here we show that despite the differences in feedstreams, reproducibility and robustness are high across membrane lots. We demonstrate the ability to cycle Mustang Q membrane nine times to achieve reproducible, back-to-back separations.

532. IsoTag™ AAV as a Versatile, Scalable Method for Screening and Producing AAV at Multiple Scales and Volumes

Kimberly Anderson, Jennifer Haley, Jarrett Dobbins, Chris Ocker, Sophia Petraki, Michael Dzuricky, Kelli Luginbuhl

Isolere Bio, Durham, NC

Adeno-associated virus (AAV) is a prevalent vector used within the gene therapy space due to its safety profiles and tissue tropism. With the popularity of this vector, there is a need for efficient purification of potential AAV candidates at every scale, from small-scale laboratory screening to larger scale manufacturing. IsoTag™ AAV is an affinity-based purification reagent that utilizes liquid-liquid phase separation and can be used at all scales of the development process through manufacturing. IsoTag™ AAV reagent binds and sequesters AAV capsids into droplets, which can be washed to remove contaminants, and subsequently eluted from the droplets for collection of the purified product. The IsoTag-AAV reagent can be utilized for separation at a small scale using low speed centrifugation or utilized at a larger scale in a tangential-flow filtration (TFF) format. Using low speed centrifugation, volumes of 0.1mL to 1L can be processed in 2 hours, independent of starting titers, with >90% capture and up to 80% recovery. The low-speed centrifugation method can serve as a rapid high-throughput method, showing compatibility for multiple serotypes, including serotypes 2, 6, 8, 9, and engineered capsids, and it provides a streamlined purification process for more efficient screening. For volumes starting at 0.5L and above, TFF can be used with commercially available 0.22 to 0.65 micron pore filters. Using TFF, AAV9 was purified using TFF, demonstrating a capture efficacy of >90% and an overall recovery of 70-80% using a neutral pH elution

buffer. Volumes of up to 8L have been successfully purified in under 4 hours. Overall, IsoTag™ AAV provides a versatile solution that can be used to streamline screening of multiple AAV candidates, as well as providing a platform for accelerated process development and scaling of AAV purification for manufacturing.

533. Adeno-Associated Virus (AAV) Production: A Collaborative Approach to Accelerate Process Optimisation

Paul Young, Gareth Berry, Spyros Gerontas, Taelor Patterson

Process Science, Pharmaron Gene Therapy, Liverpool, United Kingdom

Pharmaron believes that forming effective partnerships are critical to advance the chemistry, manufacturing and controls (CMC) development of viral vectors to bring these life changing medicines to patients. By combining expertise and knowledge with collaborative working with industrial and academic partners, Pharmaron has developed a scalable and robust multi-serotype AAV manufacturing platform. Effective partnering with upstream experts has enhanced their transfection system for large scale manufacture of AAV viral vectors in suspension cell culture and driven a significant increase in viral titre and infectious particles. Further, through collaborations on purification science, Pharmaron has developed a toolbox approach to purify different AAV serotypes and products. Through this development they have carefully balanced the purity of AAV vectors, the separation of genome containing (full) from genome-free (empty) AAV capsids and improved process recovery using Pharmaron's state-of-the-art AAV platform analytics. In this presentation they will show how working collaboratively with strategic industrial partners is key to continuously improving Pharmaron's processes and advancing gene therapy innovation into commercial solutions.

534. An Alternate Approach to AAV Purification

Paul Young, Taelor Patterson, Gareth Berry, Spyros Gerontas

Process Science, Pharmaron Gene Therapy, Liverpool, United Kingdom

Pharmaron Biologics, as a leading cell and gene therapy CDMO, have successfully collaborated with BIA Separations (now part of Sartorius) to develop and establish a platform purification process to efficiently, effectively and reproducibly purify AAV gene therapies across multiple products and serotypes which overcomes the challenges of traditional methods of AAV purification. This CIMmultus monolith-based platform can effectively clear impurities and allow for empty: full separation of AAV capsids to produce highly pure, monomeric virus containing the therapeutic gene of interest. This approach, in combination with out multi-serotype upstream production platform, allows Pharmaron to efficiently scale up to a 500L bioreactor in their cGMP manufacturing facility, with the ability to reproducibly generate upwards of 90% genome containing capsids and a reduction in downstream processing time of over 60% in a fully scalable manner from bench to large scale production, benefiting both our customers and their patients through the manufacture of effective gene therapies.

535. Implementing Virus Filtration to Ensure Pathogen Safety of Cell and Gene Therapy Products

Daniel Strauss

Asahi Kasei Bioprocess America, Glenview, IL

Ensuring pathogen safety is critical in the manufacture of biological products to reliably provide safe medicines to patients. Production of cell and gene therapy (CGT) products provides new challenges for the assurance of pathogen safety compared to traditional recombinant products due to raw materials which can pose higher risk of virus contamination and downstream purification steps which provide limited capability to remove or inactivate potential virus contaminants. One of the most effective and robust unit operations for removal of viral contaminants is virus filtration. Unfortunately, the very size-based mechanism which leads to its high degree of virus removal also makes virus filtration difficult to implement for many CGT manufacturing processes. Here, we performed virus spiking studies to evaluate removal of a model virus contaminant from a parvovirus-containing feed utilizing a 35nm pore size filter. The data show robust virus removal and excellent product yields even when challenged with various filtration fluxes and with interruptions in the process. Additionally, case studies will be shown demonstrating the applicability of virus filtration in AAV manufacturing process. Finally, we will discuss strategies and feasibility for the incorporation of filtration as an upstream barrier for protection of other gene therapy and cell therapy products as well. Overall, the increased risks associated with new therapeutic modalities call for implementation of the robust protection capability that only virus filtration can provide.

536. Impact Assessment of ITR Instability Observed in AAV Plasmids on Vector Productivity and Potency

Xingcheng Chen[1], Rashi Batra[1], Lukas Morgan[1], Wen Jin[2], Catherine Moroski-Erkul[3], Vignesh Chinnasamy[4], Siyang Xia[5], Tal Kramer[2], Chia Chu[1], Francesca Vitelli[1]

[1]Viral Vector Process Development, Intellia Therapeutics, Cambridge, MA,[2]Gene Editing Platform, Intellia Therapeutics, Cambridge, MA,[3]Exploratory Pharmacology & Toxicology, Intellia Therapeutics, Cambridge, MA,[4]Analytical Development, Intellia Therapeutics, Cambridge, MA,[5]Computational Sciences, Intellia Therapeutics, Cambridge, MA

Recombinant adeno-associated virus (rAAV) vectors have emerged as effective *in vivo* gene delivery vehicles to treat monogenic diseases. Manufacture of rAAV is more complex than traditional biologics given the limited process understanding and corresponding impact on vector potency. Triple plasmid transient transfection in HEK293 cells is a proven method with approved products on the market. AAV Inverted Terminal Repeats (ITRs), a cis-acting element on the plasmid with multiple essential viral functions, are prone to base pair deletions due to high GC content and strong secondary structures. Recombination and deletion of ITRs in AAV vectors may affect vector function given its known interaction with viral replication proteins and its role as origin of replication. Though we have been successful in minimizing ITR instability events through screening of E. coli strains, short base pair deletions in plasmids can still occur at various levels. Using PacBio sequencing here, we present a case study in which low levels of ITR

instability events in plasmid did not propagate into the genome of purified AAV and high viral titers were still achieved. Furthermore, functional assessment of the produced vector using an *in vivo* model confirmed no impact on vector potency.

537. Design of Experiments (DoE) to Optimize Capture and Recovery of rAAV from Clarified Cell Culture Using Aqueous Two-Phase Extraction

Daniel Crosby, Vijesh Kumar, Ohnmar Khanal
Spark Therapeutics, Philadelphia, PA

The current method for large-scale downstream capture of recombinant adeno-associated viral vectors (rAAVs) using affinity chromatography incurs a high cost and potential alteration of the capsid following a low pH elution, which can impact capsid efficacy. In this work, we used aqueous two-phase extraction (ATPE) to capture rAAVs from clarified cell culture harvest selectively. ATPE utilizes a polymer, typically polyethylene glycol (PEG), and a salt at a high concentration to drive liquid-liquid phase separation. ATPE can recover viral capsids from cell lysate in the interfacial region of the two-phase system, allowing for purification and concentration of the capsids. Particularly for the rAAVs that do not show sufficient affinity to the few commercial capture resins, ATPE is a promising alternative capture step. Compared to the steep cost of AAV capture resins, ATPE uses inexpensive components. However, optimizing ATPE is tedious because several parameters need to be optimized simultaneously. To overcome this challenge, we used a DoE approach to screen multiple conditions to select the optimal rAAV recovery. Using a full factorial DoE, various concentrations of salt and PEG were directly mixed with cell lysate containing rAAVs. Once the two phases were formed, the AAV-rich interface was extracted, and its rAAV concentration was determined using analytical affinity chromatography. The regression model built with the DoE data allowed us to select the PEG and salt concentrations for optimal recovery and purity. The model provided the entire design space for AVV recovery in various conditions. and predicted that lower molecular weight PEG gives higher recovery, which is consistent with the previous studies[1]. The optimal conditions were validated, and ATPE proved a potential alternative to costly affinity resins in capturing AVV from cell culture harvest. 1. Fu XQ, Leong HY, Wang GA, et al. Enrichment and separation of recombinant adeno-associated virus particles by aqueous two-phase extraction. *Biochem Eng J*. 2023;200. doi:10.1016/j.bej.2023.109115

538. Advancing Safety in Bioprocess Purification with Saltonase

Joanna Niska-Blakie[1], Felix Küttner[2], Hanna Kubiak[1], Katarzyna Anczykowska[1], Domenica Martorana[2], Peter Porschewski[2]
[1]QIAGEN, Gdansk, Poland,[2]QIAGEN GmbH, Hilden, Germany

In the dynamic landscape of biologics development, stringent requirements for final product purity, manufacturing consistency, and overall safety demand a thorough removal of plasmid DNA and host cell nucleic acid impurities. The regulatory standards established by the US FDA and other global regulatory agencies emphasize the importance of the purification step, recommending quantifiable limits for host cell DNA at 10 ng/dose, with DNA fragments smaller than 200 bp. This underscores the primary challenge in achieving efficient host nucleic acid removal, which pertains to the robust structure of chromatin, intricately composed of negatively charged DNA and positively charged histone proteins. The disruption of this compact structure is crucial for eliminating impurities. In response to this challenge, our efforts have been directed towards the development of a novel salt-active endonuclease, Saltonase. Engineered to elevate safety standards in bioprocess purification, Saltonase's distinctive strength lies in its ability to sustain activity in both high and physiological salt concentrations among a broad spectrum of pH. Additionally, it exhibits high thermal stability and digestion robustness. These remarkable traits not only facilitate the destabilization of chromatin for enhanced endonuclease access but also ensure the enzyme's efficacy and robustness across diverse workflows. By leveraging these safety advantages, bioprocesses gain an augmented capability to meet regulatory standards, ensuring the utmost safety in the production of viral vectors, exosomes, and other biologics pivotal in gene therapies and targeted nucleic acid delivery. This establishes Saltonase a steadfast and secure choice for bioprocess purification.

539. Towards an AAV Platform Production Media and Process for the Gene and Cell Therapy Community

Nelson Ndahiro[1], Justin Sargunas[1], Junneng Wen[1], Michael Betenbaugh[1], David McNally[2], William Brakewood[1]
[1]Johns Hopkins University, Baltimore, MD,[2]MassBiologics, Fall River, MA

Introduction: This study aims to illuminate the HEK-mediated adeno-associated virus (AAV) production process by developing an open-sourced production platform incorporating a defined serum-free medium, transfection process, and AAV content analytics. Initial attempts using an open-source CHO-medium resulted in cell aggregation, slow growth, and an inability to transfect and produce AAV. Therefore, we endeavored to create an HEK-friendly medium and process conducive to healthy cell growth, transfection, and vector production. Nutrient consumption rates were also studied to elucidate metabolic activity associated with AAV production. **Methods:** To optimize the AAV production process, medium design experiments including medium knockouts and spiking studies were conducted, focusing on identifying inhibitors of cell growth and transfection. Then, metabolic consumption rates were studied to discern differences between AAV-producing cells, non-producing PEI-transfected cells, and un-transfected cells. Additionally, AAV production characteristics for different serotypes were examined. **Results:** The study identified distinct nutrient consumption rates between un-transfected cells, AAV-producing cells, and non-producing PEI-transfected cells. Through medium design experiments, transfection and AAV production was realized in the open-source medium. Nutrient consumption in AAV producers were also examined for different serotypes. **Conclusion:** By developing an HEK-friendly medium where all nutrients are known, and refining the AAV production process, this study will serve to develop a valuable resource for advancing the production of adeno-associated viruses in the field of biomanufacturing. Indeed, this study

will contribute to the establishment of an AAV production platform for the scientific community, offering insights into the optimization of the complex bioproduction process for this crucial class of medicines.

540. Robust Adeno-Associated Virus Aggregate Analysis with Reduced Sample Consumption on Biozen™ dSEC-7 Size Exclusion Column

Tran Pham
Phenomenex, Torrance, CA

Size Exclusion Chromatography (SEC) is widely accepted to separate adeno-associated virus (AAV) monomer from its aggregates but analysis with current SEC columns require high volume sample consumption and lack robustness. Herein, Biozen™ dSEC-7, a chromatographic (SEC) column, is described and tested for various AAV serotype aggregates. Its 700 Å pore size, inert particle chemistry, and applicable particle size(s) are found to enable highly reproducible baseline separation and recoveries of the AAV monomer and its aggregates, even amongst batches of columns, within 10 minutes. The percentage relative standard deviations of the peak area, retention time, and % monomer were all < 1 % with as little as 0.5 µL of sample. This performance enables faster and more accurate determination of AAV aggregates, a critical quality attribute (CQA) in drug substances and drug products, without compromising data quality.

541. The Evolution of AAV Production: Scaling Through Nature Instead of Brute-Force

Lucia Fernandez, Dan Brownell, Matt Ercolino, Jade Bose, Jonathan Morgan, James Huynh, Shangzhen Zhou
VintaBio, Philadelphia, PA

The Cell and Gene Therapy industry witnessed its re-birth and rapid growth in just over one decade. This rapid expansion posed a conundrum to manufacturing engineers: how to scale vector production. While the initial wave of success was largely achieved with vectors generated in adherent production systems, the natural state of the widely used HEK293 cells, the field witnessed a broader shift to suspension-based process. This shift was prompted by the lack of readily available and reliable bioreactors for adherent cells. Despite the great success of suspension-based processes in biological manufacturing, the transition to suspension-based process for viral vector faces many challenges. A number of experiments indicate reduced production efficiency in suspension systems leading to increased production lot size. Furthermore, it was reported that suspension processes generate increased impurities in the crude harvest than their adherent counterparts. The increased production size and impurity burden created significant stress to the downstream purification process. Given the recent advancements in adherent bioreactor technology, particularly the development of fixed-bed bioreactors, the scalability concerns of adherent systems have been greatly diminished. There is potential for production engineers to re-examine the initial production success achieved through simple, cost-effective adherent-based processes, and scale this success to large commercial production to solve some of the industry's biggest bottlenecks. Our goal in this study is to comprehensively examine the scalability and in-process characteristics of our adherent process through the following aims: 1) Evaluate the scalability, repeatability, and process robustness of adherent vs. suspension processes. 2) Investigate material consumption, including plasmids and transfection reagents on viral production in both adherent and suspension processes. 3) Assess the impact of adherent vs. suspension on process characteristics including impurity profiles and specific virus production per cell. Our results indicate adherent processing offers advantages in terms of productivity, process control, downstream processing, and potential improvements in vector quality. As technology has advanced, the scalability challenges traditionally associated with adherent cultures are becoming a thing of the past. We show scalability from roller bottle to large scale fixed-bed bioreactor with excellent repeatability in our adherent system.

542. Comprehensive AAV Characterisation Using Orthogonal Methodologies for Empty/Full Capsid Analysis and Genome Integrity

Colin Guy[1], Hazel Staton[1], Rose Andryszewska[1], Martin Squires[1], Annahita Sherkat[1], Jennifer Courtney[2], Calvin VonOpstall[2]
[1]BioPharmaceutical CMC Solutions, Labcorp Drug Development, Harrogate, United Kingdom,[2]Genomics, Labcorp Drug Development, Indianapolis, IN

Adeno-Associated Virus (AAV) remains the viral vector of choice for *in vivo* gene therapy. However, there continues to be safety concerns regarding AAV therapeutics. The empty:full ratio of AAV vectors has been highlighted as an important critical quality attribute (CQA), with high proportions of empty capsids resulting in reduced genome titre, increased total protein load and potential toxicity. It is also important to characterise the integrity and purity of the viral genome, to ensure the safety and efficacy of the product. Despite advancements within the field, there remain challenges for the CMC analytical characterisation of AAVs. There are a variety of analytical methods employed for product characterisation and to monitor AAV CQAs. However, these methods are unable to provide a complete picture of the quality of the product, with each method having limitations that need to be fully understood so the data is interpreted appropriately. Therefore, it is important to implement a panel of orthogonal methods to enable comprehensive analysis of the AAV and ensure product safety and efficacy. We have assessed a portfolio of analytical methodologies and technologies to assess the viral genome payload, the viral vector proteins and the empty:full capsid ratio. We acquired commercial research grade AAV2-eGFP samples; two lots of 'full' capsids and 1 lot of 'empty' capsids. Samples were analysed using CryoEM, which is considered one of the gold standard techniques, to determine the empty:full ratio. The data confirmed that one lot of AAV contained a high full capsid content (>90% full), while the second lot contained more empty capsids (74% full). This data was then used to evaluate other methodologies including transmission electron microscopy (TEM), analytical ultracentrifugation (AUC), capillary isoelectric focusing (cIEF), mass photometry and UV-Vis/DLS. While these methods provided valuable data, each method presented challenges, including limitations on sample throughput, concentration and composition, implementation of the methodology and interpretation of the data. While these methodologies are important tools for AAV characterisation, they are unable to determine the quality of the viral genome. Therefore, these methods need to be used in conjunction with a suite of analytical methods including q/ddPCR, capillary electrophoresis, and next

generation sequencing (NGS). This combined analytical package enables a comprehensive dataset which can demonstrate the consistent manufacture of AAV therapeutics and, in conjunction with pre-clinical and clinical data, ensure the product safety and efficacy.

543. Quantitative Analysis of Capsid Loading in Gene Therapies with High-Precision Tunable Laser Spectroscopy

Bryan Hassell

Nirrin Technologies, Billerica, MA

In the rapidly evolving field of gene therapy, the successful delivery of genetic material via capsids is paramount. A critical quality attribute in the development and manufacturing of these therapies is the accurate determination of the ratio of empty to full capsids, a metric that directly impacts their efficacy and safety profiles. Traditional methods for quantifying these ratios often fall short, being laborious, time-intensive, and lacking in precision, particularly when handling crude samples. Our novel technology harnesses the power of tunable laser spectroscopy, providing an unparalleled blend of accuracy and robustness, specifically designed to tackle the challenges of capsid loading analysis. Our advanced methodology utilizes a unique tunable laser system, capable of dissecting the spectral signatures of capsids with remarkable resolution. Leveraging sophisticated signal processing algorithms, we can distinguish between empty and full capsids with extraordinary precision, in real-time. The robustness of our platform ensures consistent performance, even when processing heterogeneous, crude samples, thereby simplifying the workflow from sample preparation to final quantification. Our technology not only streamlines the analytical process but also boosts the speed and reliability of measurements. By delivering a rapid and accurate evaluation of capsid loading, we enable researchers and manufacturers to make timely, informed decisions, thereby enhancing process development and formulation optimization in gene therapy. The presentation will provide a detailed overview of our technology, demonstrate its application in measuring the ratio of empty to full capsids from crude samples, and explore its significant implications for the gene therapy sector. We will also present comparative data underscoring the superiority of our approach over traditional techniques, emphasizing our technology's potential to redefine the standard in capsid analysis.

544. Replacing Vector Genome Titer Readouts with NanoDrop UV-Based Concentration Metrics for Short Turnaround Time (STAT) Testing

Darren W. Begley[1], Libby McClelland[1], Dave Foster[1], Ginny Knight[1], Nelson Reticence[1], Allison Fleck[1], Patrick Starremans[2]

[1]Analytical Development - Gene Therapy Franchise, Resilience, Inc - Gene Therapy Franchise, Waltham, MA,[2]Resilience, Inc., Waltham, MA

Fast or short turnaround time (STAT) testing is a critical support parameter for biologic drug manufacturing unit operations. The concentration of genome-containing capsids have a direct impact on preparations for specific unit operations in adeno-associated virus (AAV) manufacturing, including buffer exchange by tangential flow filtration (TFF) and capsid enrichment by column chromatography. STAT titers are often deployed using quantitative or droplet digital PCR methods to assess AAV concentration in preparation for these unit operations, challenging methods to run under the time constraints required for manufacturing. As important as PCR-based methods are for final drug substance/drug product quantitation, it is questionable if such methods are absolutely required to get ready for TFF buffer exchange or column packing. In this study, we look at PCR and UV absorption data acquired by NanoDrop for several serotypes of AAV, to see if a suitable correlation could be used to permit the use of the latter for STAT concentration metrics. Predictive models utilizing a machine-learning algorithm shows that for certain sample types, a quick NanoDrop can provide sufficient information to permit unit operations to proceed, at a fraction of the time, cost and effort used for genome titer methods.

545. Introduction to Genevoyager's One-Bac 4.0 System: Addressing the Bottlenecks and Challenges of Adeno-Associated Virus (AAV) Manufacturing

Hui Wang

Genevoyager, Philadelphia, PA

Viral vectors play a crucial role in gene therapy, with Adeno-Associated Virus (AAV) emerging as the preferred vector for *in vivo* gene therapy due to its safety profile and the ability to sustain prolonged gene expressions. AAV offers advantages such as low immunogenicity, broad tropism, and a variety of serotypesGenevoyager's proprietary One-Bac 4.0 system marks a novel development in large-scale AAV production platforms, addressing the bottlenecks associated with commercial development of gene therapy products.Key Features of the One-Bac 4.0 system:1) Optimizing the Cap and Rep expression cassettes to increase the AAV full capsid ratio and enhance infectious activity;2) Achieving stability and yields ranging from 1E+15 to 5E+15 vg/L during scale-up manufacturing (500L-2,000L);3) Ensuring a safety profile with minimal impurities, and the absence of replication competent AAV (rcAAV);4) Delivering high potency through increased infectivity and high full capsid ratios (>70% in crude harvest), enhancing the therapeutic efficacy;5) Providing high accessibility with reduced manufacturing costs, enabling an affordable pricing model that addresses the medical needs of a broad patient population.The innovative One-Bac 4.0 system effectively tackles the challenges in AAV manufacturing by offering high yields, sustained stability, elevated full-capsid ratios, enhanced infectious activity, reduced cost, and accelerated production timelines. This system introduces new approach for scalable and efficient production of AAV vectors.

546. A Comparison Between CDMS and Q-Exactive UHMR for the Determination of AAV Empty/Full Ratios

Daniel Haq

National Resilience, Waltham, MA

During the production of AAV considerable amounts of capsids with no genome, often referred to as empty capsids, are produced. Due to potential immunogenic effects, empty capsids are considered

undesirable and need to be quantified. Most of these empty capsids are purified out, but a percentage of final product still contains residual levels. Determination of levels of empty and full capsids is required for release of material. Capsids are very large, rendering many commonly used analytical techniques not suitable. Currently the preferred method for the determination of empty and full levels is analytical ultracentrifugation (AUC). While AUC is reliable and accurate, it is not without drawbacks. AUC requires significant amounts of product and suffers from non-compliant software for the analysis. Charge detection mass spectrometry (CDMS) is another method that produces very reliable results that are consistent with AUC but currently is not commercially available. Here we investigate the feasibility of using the Thermo Q-Exactive Ultra High Mass Range (UHMR) mass spectrometer with Direct Mass Technology as an alternative to CDMS, while using levels determined by AUC as the benchmark. With a mass range up to 80,000 m/z, the Q-Exactive UHMR proved to be a suitable alternative to the commonly preferred methods for determination of capsid empty/full ratios without.

547. Optimal Drug Product Presentation and Container Closure Selection for AAV-Based Genomic Medicines

Madhura Som, Varna Kanapuram, Diego Vargas, Katelyn Esposto, Benson Gikanga, Sandeep Yadav
Formulation & Fill/Finish, Sangamo Therapeutics, Brisbane, CA

The majority of current AAV-based commercial drug products and clinical drug candidates are stored at frozen conditions due to the potential impact on stability associated with long term 2-8°C storage. While conventional container closure systems such as glass vials offer unique benefits like low product adsorption and impermeability to moisture and gas exchange, the risk of glass breakage with frozen AAV storage necessitates the assessment and implementation of alternative container systems. Polymeric vials are a great option to address these challenges, however, they may not provide chemical durability needed to ensure the drug product remains stable and efficacious throughout the drug product life cycle. In addition, the physical durability of the inner surface of the vials is crucial and needs to be assessed for any corrosion or delamination. This study evaluates the compatibility of AAV-based products and related manufacturing stresses in commercially available polymeric vials. The drug products were subjected to thermal and freeze/thaw stability stress. The samples were assessed for changes in critical quality attributes (CQAs) such as pH, osmolality, appearance, aggregation, free DNA, vector genome, and capsid titer. The physical durability of the inner surface of the vials was assessed using light microscopy and Scanning Electron Microscope/Energy Dispersive Spectroscopy. A side-by-side comparison of CQAs impacted as a function of stress and container type is presented to enable a rational selection of container closure system for AAV based products with long term frozen storage.

548. Improving Transient AAV Manufacturability through Model Driven Process Development

Anna Prendeville, Jeff B. McMahan, Kate Dray, Omar El-Ghouch, Kevin D. Smith
Asimov, Boston, MA

Triple transfection of AAV plasmids into HEK293 cells is one of the most widely used production methods for AAV vector generation. However, this approach is met with long development timelines that typically require plasmid ratio optimization, upstream process optimization, and scalability testing. The process parameters are often evaluated one at a time as the transfection process is scaled up from a shake flask to large-scale bioreactors. One option for faster process optimization is the use of a high-throughput bioreactor system. While this high throughput system can be beneficial, it can be difficult to manually parse the results to evaluate the impact of each independent variable (for example, agitation speed, dissolved oxygen percentage) on each dependent variable (titer and percent full) and to determine subsequent experiments to run. To overcome the experimental complexity, we have developed a data-driven approach for experimental design by using Partial Least Squares (PLS) regression to analyze the results of each experiment and choose conditions for the next iteration. This approach has enabled prioritization of experimental exploration in the highest-performing regions of the process design space, leading to rapid optimization of the process in a small number of experiments. We have combined this model-guided experimental design approach with a superior performance clonal HEK293 host cell line, sequence-optimized plasmids, and a high-throughput bioreactor method with integrated metabolite analysis to significantly accelerate the HEK293 AAV triple transfection process development timeline. Cell line aggregation has been addressed through the generation of a clonal, serum-free, suspension HEK293 cell line. This cell line grows with an average of 23 hour doubling time, minimal nutrient consumption, minimal clumping, and is shown to produce a high percentage of fully packaged capsids in a transient system. Using our model-guided approach, optimized growth conditions for the improved host were established at the ambr250 and the 2L scale. This was followed by transfection and process optimization at the ambr250 scale and shown to apply across 5 different serotypes and 3 genes of interest (GOIs), including eGFP and two approved AAV gene therapy GOIs. The harvested clarified lysate across all GOIs and serotypes showed titers of ≥1e11 or higher (measured via primer/probe specific ddPCR) and percent fully packaged of ≥20% (measured using Capsid ELISA and confirmed using analytical ultracentrifugation). Lastly, we demonstrated that optimization of the GOI coding sequence increases the expression of the genetic payload in transduced cells. Overall, by integrating a robust host cell line, sequence optimization tools, and an improved triple transfection process, we showcase an effective system for producing clinical material for new AAV based gene therapies.

549. Enhancing AAV Productivity through Plasmid Design and Plasmid Ratio Optimization

Laure Robert[1], Eric Mauro[2], Jonathan Havard[3], Solène Jachez[1], Mathieu Porte[4], Sylvain Julien[1], Patrick Erbacher[4]

[1]Polyplus, Illkirch-Graffenstaden, France,[2]Polyplus, Illkirch, France,[3]Polyplus-Transfection, Illkirch-Graffenstaden, France,[4]Polyplus-Transfection, Illkirch Graffenstaden, France

Harnessing rAAVs as viral vectors for therapeutic transgene delivery still requires improvements in yields and specificity to lower vector doses, and therefore manufacturing cost, as well as to improve patient safety. To this end, our research is focused on developing novel technologies to ensure manufacturing of high yielding rAAV particles using transient transfection, as well as enhancing viral particles quality and specificity. Here we present our state-of-the art approach to design new helper plasmids (pHelpers) with the aim of improving both the genomic titers and the infectivity (TU/mL) of the viral particle obtained from suspension cultures. We took the opportunity to exploit our proprietary DNA assembly method technology to explore the synergies of multiple genetic features modularly assembled in synthetic plasmids. We then explored how plasmid ratio optimization could further enhance AAV productivity through a Design of Experiment with a mixture design approach. This work led us to identifying plasmid ratios improving both VG titers and Empty to Full ratios.

550. Optimizing Triple Transfection Production of AAV in Upstream Manufacturing Using Design of Experiment

Rebecca Reese, Jennifer Swanson, Austin Storck, Laura Juckem

Mirus Bio LLC, Madison, WI

Recently there has been a surge in the number of registered clinical trials examining the use of recombinant adeno-associated virus (AAV) in gene therapy. There are five AAV gene therapies approved by the FDA to date, three of which were approved in the past two years alone. As AAV therapies move to broader patient populations and systemic indications, the need for highly efficient production processes greatly increases. The upstream production of these therapies is largely driven by transient transfection of suspension HEK293 cells with multiple plasmid DNA constructs encoding essential virus proteins. There are a multitude of factors that can be considered when optimizing upstream manufacturing processes for AAV production including, but not limited to, cell density, plasmid ratios, DNA dosage, transfection reagent-to-DNA ratio, complex formation time, and more. Optimizing these factors individually is time-consuming and costly; instead, Design of Experiments (DOE) provides method of quickly examining the relationship between multiple variables simultaneously. DOE is a branch of applied statistics which allows for the study of multi-variable interactions while reducing the necessary number of runs or experiments. Here we describe efficient experimental designs which take advantage of DOE to model and predict the conditions that will yield the highest AAV titers when using the *Trans*IT-VirusGEN® Transfection Reagent. To streamline the DOE process, we utilized a scale-down system with non-treated 6-well plates containing 2 mL of culture, thereby reducing the necessary reagents and overall cost per condition tested. Using multiple genes of interest (GOIs), we first show that a DOE mixture design is effective in determining the optimal Transfer:RepCap:Helper plasmid DNA ratios under recommended starting transfection conditions. Using these improved plasmid ratios, we then compare two types of response surface methodologies (RSM), a 3-factor central composite design (CCD) and a Box-Behnken design, to determine the DNA dosage, the *Trans*IT-VirusGEN:DNA ratio, and cell density that will yield the highest AAV titers. These conditions were then tested in combination with a range of the *Rev*IT™ AAV Enhancer to further increase genome titers by >2-fold. Taking advantage of DOE allows for rapid and cost-effective optimization of transfection conditions to maximize titers and generate more patient doses per manufacturing run.

551. Standardization and Reproducibility in Gene Therapy Analytics Using Digital PCR-Based Determination of AAV Reference Standard Genome Titers

Jonathan Thorn[1], Anida Mesihovic[2], Dana Holzinger[3], Katharina Hammer[3], Andreas Hecker[2], Domenica Martorana[1], Francesca Di Pasquale[1]

[1]R&D, QIAGEN GmbH, Hilden, Germany,[2]Product Management, QIAGEN GmbH, Hilden, Germany,[3]PROGEN, Heidelberg, Germany

Recombinant adeno-associated virus (rAAV) vectors are widely used as vehicles for gene therapy in both clinical and research applications. To help ensure product safety, reference standard materials (RSMs) are key. The use of viral vector RSMs is particularly important to normalize laboratory internal controls, for the calibration of medical products and procedures and as best practices for manufacturing and testing of ATMPs. Additionally, AAV RSMs are often used for assay validation and optimization. All in all, the use of well-characterized AAV RSMs and orthogonal methods is indispensable to meet current authority guidelines. Since the production of such is not achievable for a large number of companies, it is important to resort to commercially available AAV RSMs for the most commonly used AAV serotypes. These standards underlie a comprehensive characterization process including determination of vector genome and capsid titer, filling grade, purity and aggregation. One of the critical quality attributes needed for AAV reference materials is the viral vector genome titer which is usually determined using qPCR. Here we demonstrate a digital PCR-based streamlined workflow for quantification of genome titers of commercially available, high-quality rAAV reference standards that allows to complete the characterization with increased precision, accuracy and robustness. Additionally, the integrity of the viral vector genome can be assessed ensuring quality and safety of the final product.

552. Simple and Rapid Adeno-Associated Virus Genome Titration with CRISPR-Cas12a Diagnostics

Zach Hetzler[1], Stella Marinakos[2], Noah Lott[3], Noor Mohammad[1], Agnieszka Lass-Napiorkowska[4], Jenna Kolbe[1], Lauren Turrentine[1], Delaney Fields[1], Laurie Overton[3], Helena Marie[5], Angus Hucknall[2], Oliver Rammo[5], Henry George[4], Qingshan Wei[1]

[1]Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC,[2]Biostealth, Durham, NC,[3]Biomanufacturing, Training, and Education Center (BTEC), North Carolina State University, Raleigh, NC,[4]Millipore-Sigma, St. Louis, MO,[5]Merck KGaA, Darmstadt, Germany

The field of gene therapy has seen significant growth in clinical applications, with adeno-associated virus (AAV) achieving much clinical and research interest due to its widespread use as a vector. AAV manufacturing faces several challenges to meet clinical demand, one of which is a lack of robust process analytical technology (PAT) for AAV product quality monitoring. To address this need, we developed a CRISPR-Cas12a assay for quick and simple and accurate at-line determination of the AAV genome titer within 30 minutes. The **CR**ISPR **AAV** **E**valuation (CRAAVE) assay was developed to function universally across genetic constructs by targeting the inverted terminal repeats (ITRs) of the AAV genome. The CRAAVE assay was demonstrated on constructs from AAV samples from three different serotypes as well as accurately titrating samples. The CRAAVE assay was developed for maximum sensitivity with limits of detection below 5e8 vg/mL for all serotypes in 30 minutes. The CRAAVE assay was also translated to a diagnostic chip format with pre-deposited lyophilized reagents with similar analytical metrics and analytical power as the traditional microplate assay format. We believe this new analytical technology with a flexible format and readout will minimize sample-to-result time, particularly for high throughput analysis in process development.

553. Advancing Plasmid Impurity Analysis through Comprehensive, Fragment and Full-Length Kanamycin (*nptII*) Gene Quantification in Gene Therapy Products by Multiplex Digital PCR

David Dobnik[1], Nejc Košir[2], Nejc Jakoš[3], Rebecca Vollmeier Kovačić[2]

[1]National Institute of Biology, Ljubljana, Slovenia,[2]Niba Labs, Ljubljana, Slovenia,[3]Niba Labs Ltd., Ljubljana, Slovenia

Recombinant adeno-associated virus (rAAV)-encapsidated plasmid impurities pose challenges in vector manufacturing, comprising heterogeneous DNA fragments from production plasmids. Regulatory agencies necessitate precise quantification of DNA impurities. However, currently used PCR-based methods used for detection focus on quantifying small fragments, offering limited insights into the actual length of encapsidated plasmid DNA fragments. The potential risk of horizontal gene transfer of resistance genes is not yet completely understood, as current analytical methods were not able to quantify the number of full-length genes packed in rAAV capsids. This is also the area of current scrutiny of regulatory agencies. In response, we present an innovative approach to assess the fragment populations of plasmid-derived antibiotic resistance gene (*nptII*) impurities in rAAV products. This method expands the scope of impurity analysis by providing quantitative information on four different kanamycin fragments, including the full-length gene. We've developed a triplex digital PCR assay and evaluated the performance of the assay against a standard set of criteria (e.g. repeatability, precision, accuracy, range, LOD, LOQ). We've addressed the current limitations in existing techniques and provided a method that offers a valuable enhancement to quality control measures in vector manufacturing, ensuring a more thorough evaluation of plasmid impurities and, consequently, advancing the safety and regulatory compliance of gene therapy products.

555. Potency Method Development Roadmap for AAV Gene Therapy

Kimberley Beattie, Michael MacArthur, Martha Nattyaba, Eugenia Fandunyan, Kalyani Natu

Analytical Development, Bioassays, Oxford Biomedica (US) LLC, Bedford, MA

Potency method development for AAV gene therapy products is complex due to product-specific mechanisms of action. Assessment of biological activity often requires multiple assays. Here we describe a platform approach to potency method development for AAV gene therapy. Product lifecycle-specific implementation of assays and examples of common challenges are discussed.

556. Single-Use Process with In-Line Cell Lysis in Plasmid DNA Purification Using a Single Chromatographic Step for Cell and Gene Therapy

Vicky Argyropoulou[1], Matthias Craig[1], Wu Huacheng[2], Bhargavi Kondragunta[2], Julien Glibert[1], Sebastien Gillet[1], Kistler Christopher[2], Victor Vinci[3], Nuria Gomez Santos[1]

[1]Catalent Pharma Solutions, Gosselies, Belgium,[2]Catalent Pharma Solutions, Baltimore, MD,[3]Catalent Pharma Solutions, Bloomington, IN

Plasmid DNA (pDNA) serves as a critical starting material for the production of advanced therapy medicinal products (ATMPs) in the field of cell and gene therapy. The growing demand for pDNA requires the development of manufacturing processes that can meet aggressive timelines while maintaining cost-effectiveness. Here we present a robust single-use process that enables seamless scale-up from research and development (R&D) to good manufacturing practice (GMP) grade. This process involves bacterial cell growth in fermenters, alkaline cell lysis, and a streamlined chromatographic step. A key aspect of this approach is the implementation of a controlled cell lysis step, ensuring the production of high-quality pDNA that complies with regulatory agency standards. Notably, this process achieves efficient purification and polishing with just a single chromatographic step, simplifying the manufacturing process as well as reducing costs. We will provide an overview of the platform process and share results on the quality attributes obtained through its implementation. Our findings demonstrate the effectiveness of this approach in generating pDNA that meets quality requirements set by regulatory agencies.

557. Quality Control for Adeno-Associated Virus Genome Preparations Using Nanopore Sequencing

Hayley Greenfield, Heather Jeffery, Phill James
Applications, Oxford Nanopore, Littlemore, United Kingdom

The adeno-associated virus (AAV) is a small replication deficient virus that is currently a favourable gene therapy vector. As it stands, five AAV based gene therapies are already approved by the FDA and in 2022, 134 AAV based clinical trials were being undertaken for the treatment of 55 differing diseases. However, to ensure efficacy in downstream clinical applications it is crucial to gain an accurate view of the composition of AAV preparations as part of the quality control (QC) of these vectors. Plasmids containing the transgene of interest, flanked between inverted terminal repeats (ITR) signifying the ends of the AAV genome, are co-transfected into packaging cells with *rep/cap* and helper plasmids allowing for virus and AAV vector propagation. However, truncated AAV genomes, host DNA and both *rep*/*cap* and helper plasmid DNA can result naturally in the process of rAAV manufacturing but can contaminate final rAAV preparations. These contaminations can affect the efficacy of the AAV-based therapy and also cause downstream adverse effects including hepatotoxicities and neuropathological issues, displaying why the QC of rAAV is crucial to contribute to Good Manufacturing Processes (GMP) before use in downstream treatments. At present, there is no standardized QC method, with PCR-based assays, that are unable to identify truncations and contamination, being the most utilized methods. Long read sequencing has been identified as an exciting opportunity for the improvement of GMP in the QC of rAAV preparations. Here, we demonstrate the ability to sequence full length (ITR-ITR) native rAAV vectors and detect both contamination and truncated AAV genomes using a released end-to-end protocol from Oxford Nanopore. A quick library preparation turnaround time of less than five hours allows six AAV samples to be multiplexed on our MinION™ and GridION™ devices. Optimisations of protocol steps including viral extraction and annealing methods were carried out to facilitate the accurate characterisation of rAAV preparations. Additional to the released protocol, a workflow from EPI2ME™ labs, wf-aav-qc, facilitates fast and concise analysis for the easy assessment of contamination and truncation events, as well as the number of full length AAV genomes and proportions of self-complementary AAV (scAAV) and single stranded AAV (ssAAV). We have also successfully sequenced plasmids used for transfections, contributing an additional level of quality control prior to the creation of rAAV. In summary, we demonstrate rapid comprehensive sequencing of native rAAV vectors, identifying contamination, truncated genomes, and optimizing protocols for accurate characterisation.

558. Adventitious Viral Agents Control Through Robust rAAV Production Platform

Ashish Sharma, Thomas Thiers, Luke Mustich, Richard Gilmore
Oxford Biomedica (US) LLC, Bedford, MA

The ICH released its latest revision of ICH Q5A [R2] guideline on viral safety evaluation of biotechnology products derived from cell lines of human or animal origin in 2023. The revised guideline calls for a risk-based approach to ensuring viral safety of biological products such as adeno-associated virus (AAV). This presentation will first introduce a full spectrum control strategy highlighting how Oxford Biomedica's transient transfection platform, implemented across multiple AAV serotypes, de-risks endogenous and adventitious virus contamination through raw material control, single-use closed aseptic processing throughout the platform, dedicated viral intermediate testing, and the final drug product characterization. We will then share a rAAV9 viral clearance case study demonstrating robust upstream inactivation kinetics and downstream viral removal performance to deliver significant log reduction value (LRV) clearance throughout the process (Detergent Inactivation->4.67 LRV, Affinity-4.69 LRV, AEX-4.38 LRV), utilizing orthogonal chromatographic modes of separation, namely affinity capture and AEX polishing. We utilized the small-scale model of manufacturing process at current operating parameters, for spiking studies using enveloped Xenotropic murine leukemia virus [XMuLV] and non-enveloped Minute Virus of Mice [MVM] as representative AAV model viruses. We selected MVM due to similar morphology and physicochemical properties as AAV, which may pose competing challenge to be virally cleared from the process. We will showcase that negligible viral particle were detected in the intermediate product pools and no discernible difference in load and elution chromatographic profile was observed, compared to chromatographic profile with no model virus spike [only AAV9]. We will also showcase how this intensified platform is not affected by process feed stream conditions within normal operating range and the data is robust in nature to potentially offer similar LRVs across multiple serotypes, without the need for retentive viral filters (viral filters need process feed adjustments post polishing and pre UFDF steps, impacting product yield). To our knowledge this could be the first showcased dedicated study from a drug developer utilizing both upstream enveloped viral inactivation and downstream non enveloped viral clearance for a "process evaluation" Phase I/II stage of a rAAV platform. Finally, we will outline the value proposition offered by Oxford Biomedica's rAAV platform for drug developers in four distinct ways: robust controls put in place provide barriers to entry and carryover of adventitious agents in the final product, robust viral clearance from orthogonal chromatography unit operations without the need for retentive viral filters, a robust regulatory IND/BLA package which adheres to the newly released guidance, and the advantage to treat patient populations in a safe and economical manner, with negligible immunotoxin profile.

559. ELEVECTA™ Transient Cell Line: Enabling rAAV Production with Low-Level hcDNA Encapsidation

Ben Hudjetz[1], Sabine Schmidt-Hertel[1], Tamara Grabeck[1], Keerthana Srinivasan[1], Michael Mühle[1], Christian Niehus[1], Michelle Hussong[1], Kelly Cybulski[2], Tala Calvi[2], Anne MacIntyre[2], Akshay Gutha Ravichandran[2], Adam Hejmowski[2], Michelle Olson Peabody[2], Nikola Strempel[1], Markus Krieger[1]
[1]Cytiva, Cologne, Germany,[2]Cytiva, Westborough, MA

The landscape of recombinant adeno-associated virus (rAAV) manufacturing is witnessing a transformative shift, with stable producer cell lines emerging as the cell factories of the future. However, the move towards producer cell lines is a nuanced journey, requiring the use of

transient transfection cell lines in the research and development phases. This dual approach acknowledges the key role of transient transfection in the early stages of candidate AAV development. To address this need, we developed a novel HEK293 cell line, ELEVECTA™ transient cell line. Parental suspension HEK293 cells were genetically modified to decrease hcDNA encapsidation during rAAV production. Then, single-cell cloning resulted in a clonal cell line, which was adapted to a new animal-component-free, chemically defined, and transfection-ready culture medium. The resulting ELEVECTA transient cell line grows in single-cell suspension mode to high density and supports high-titer rAAV production for multiple serotypes with significantly reduced levels of encapsidated hcDNA compared with common HEK293 cell lines used for this purpose. In tandem with ELEVECTA producer cell line, ELEVECTA transient cell line effectively addresses the market demands for high-titer manufacturing of excellent-quality rAAV products from R&D through commercial manufacturing. With their distinctive attributes, these cell lines are poised to contribute significantly to advancing the field of gene therapy and meeting the ever-growing expectations for efficacy and quality.

560. A Modular Approach of AAV Process and Analytical Development Expediated FIH CMC Timeline by 30%

Xiaotong Fu[1], Oliver Meade[1], Colin Haws[1], Darren W. Begley[2], Mukesh Mayani[3], John Kerwin[1]

[1]Resilience, Waltham, MA,[2]Resilience, Inc., Waltham, MA,[3]National Resilience, Inc., Chelmsford, MA

The lengthy timeline from molecule development to phase I trials remains a notable bottleneck in the gene therapy field. The prolonged duration and substantial resources required for clinical material manufacture not only impede patients' timely access to innovative therapies but also expose gene therapy innovators to financial risks as more investors withdraw funds from the biotech sector. Consequently, to expedite the path to clinical with cost-efficiency, we propose to redefine the AAV manufacturing "platform". Here we introduce a modular approach that departs from a singular platform solution, allowing for development at the unit operation level. Each module undergoes an assessment of critical process input against corresponding output, presenting potential tools to address platform permutations arising from different serotypes or consumable availability. This approach allows maximum platform flexibility while constructing an accelerated timeline heading into IND filing. In this work, we want to highlight several modular tools we developed at the unit operation level that helped to reduce development timeline and resource requirements by 30%: **1.** Identification of critical transfection elements led to productivity and packaging efficiency improvement by 2-4 folds. We also established a high-throughput purification workflow that enables at-line product quality monitoring during cell culture development. **2.** Discovery of various levers to improve AAV empty and full separation with different polishing resins. Successfully raised the %full from 15% to 70% for several AAV serotypes, even surpassing 90%. **3.** Establishment of an analytical toolkit for AAV empty and full characterization, incorporating AUC, CDMS, Mass photometer, and SEC-MALS. With a comprehensive understanding of each tool's capabilities and confirmed comparability, the AAV platform can selectively implement the right tool at different phases, ensuring development speed and process robustness. **4.** Identification of key scale independent parameters allows good process robustness during scaling up, leading to consistent batch yield from PD to manufacturing.

561. Manufacturing of a Broad Range of AAV Capsids Using a Suspension Platform Process

Rupert Derler, Martina Ohme, Sandra Henze, Elisabeth Schweigert, Florian Dunker, Markus Hoerer, Andreas Schulze, Florian Sonntag

Ascend, Planegg, Germany

A broad range of naturally occurring and synthetically engineered adeno-associated virus (AAV) capsid sequences have been reported until today and are applied in AAV gene therapy programs to target specific tissues and organs with high efficiency. There is an increasing demand to manufacture high quality AAV vectors based on various AAV capsids in large quantities for pre-clinical and clinical trials as well as approved therapies. Yield and quality parameters can differ from one AAV capsid to another, impacting the manufacturing processes and requiring modularity of a manufacturing platform to be able to address product dependencies. Based on our proprietary HEK293 cell line and split two plasmid system, we have developed a robust suspension platform process, that is optimized towards yield at best possible quality with full scalability. We present here the comparison of a range of AAV capsids (amongst others AAV1, AAV2, AAV3B, AAV8 and AAV9) produced using our suspension platform process. We analyzed yield and quality parameters that are known to be strongly impacted by the design and choice of the biological starting materials and the upstream process. A panel of analytical methods was applied to enable a comprehensive and detailed comparison of many different attributes. Manufacturability of the AAV capsids was demonstrated in the Ambr® 15 platform which has been shown to be predictive for the larger production scales of our manufacturing offering (currently up to 200 L). Overall, very similar vector genome yields within a 3-fold range were observed for the majority of AAV capsids. Serotype-dependent release of AAV particles from cells into cell culture media during production as described in literature was seen. Therefore, our manufacturing platform is designed to recover both, cell-attached vectors and those released into the cultivation medium. A high-throughput one-step affinity purification protocol was developed to allow screening in small-scale format. Despite the same vector genome cassette used for all productions, differences in the levels of plasmid- and host cell DNA-derived mispackaging were determined in a serotype-dependent manner. These data are highly valuable as they inform on specific development needs and underpin the need of a modular platform. Our results demonstrate the applicability of our suspension platform rAAV production process for a broad range of AAV capsids. We continuously invest in further CMC innovation to optimize yield and quality for all capsids.

562. Engineered Cell Lines Can Improve Crude Lysate Titer and Non-Empty Capsid Percentage of AAV Vectors

June Deng, Nannan Jia, Shiliang Hu, Yinxing Chen, Noel Walsh, William Lee, Kayla Iozza, Lauren Drouin, Matthias Hebben, Mariana Nacht, Jing Liao

Alexion Genomic Medicine, AstraZeneca Rare Disease, Lexington, MA

Manufacturing cost of goods is a significant challenge in the gene therapy field. At Alexion, we utilize transient transfection of suspension HEK293 to produce our AAV products. This approach provides scalability and flexibility for swapping therapeutic genes. To optimize the upstream process, we employ optimal transfection conditions determined by the design of experiment (DOE) to achieve high vector yields. Additionally, the generation of a super producer cell line through cell line engineering could potentially reduce manufacturing costs. To achieve this goal, we initially subcloned the polyclonal HEK293F cell line through a series of dilutions in semi-solid media. We selected the top-performing cell line, #159, from 300 cell lines. Subclonal cell line #159 exhibited a 2 to 4-fold enhancement in crude lysate titer over the parental cell line when producing different AAV serotypes (LK03, sL65, DJ, AAV2, and AAV9) using the transfection reagent PEI in the conventional 3-plasmid system. We further enhanced cell line #159 by overexpressing or knocking down target genes using lentivirus. Our previously reported novel 2-plasmid design, mAAVRx, demonstrated high-yield, high-quality, and potent AAV vectors in scalable suspension HEK293 cells. Therefore, we compared different plasmid systems, mAAVRx and the conventional 3-plasmid system, in our engineered cell line with different serotypes. Using mAAVRx, the engineered cell line with target gene knockdown improved the crude harvest titer for different serotypes by approximately 1.5-fold while maintaining the non-empty capsid percentage. Notably, the enhancement of crude harvest titer was found to be transgene, capsid, and plasmid system dependent. For instance, the super producer cell lines for different transgene cassettes were different, even with the same capsid. In summary, we have successfully combined subcloning and cell line engineering to generate a high producer cell line for AAV manufacturing. Our new cell lines can be applied to different plasmid transfection systems and capsids from multiple serotypes. The AAV vector titer of most capsids can be improved. Consequently, the increased vector titers and improved vector purity may reduce the cost of goods per patient and enhance product safety, respectively.

563. AAV Capsid Titer Determination of AAV Standard Material by Orthogonal Methods

Lisa Mutz[1], Robert Mildner[2], David Golonka[2], Francisco Ramirez[3], Chris Heger[3], Katharina Hammer[1], Caroline Odenwald[1], Dana Holzinger[1]

[1]PROGEN, Heidelberg, Germany,[2]Waters | Wyatt Technology, Dernbach, Germany,[3]Bio-Techne, San Jose, CA

Today, AAV-based gene therapy is one of the most promising and fastest growing branches of modern medicine. Moving forward to a more clinical focus in this field, the use of reliable AAV standards and orthogonal methods is indispensable to meet current regulatory guidelines. Since the production of comprehensively characterized, internal standard material is not feasible for many companies and a lack of commercially available reference material, we now offer AAV standard material for the most used AAV serotypes. We have established a comprehensive characterization process for these commercially available standards. Besides testing several characterization parameters (e.g., vg and capsid titer, filling grade, purity, and aggregation), this process also contains the analysis with a set of suitable orthogonal methods ensuring the utmost level of accuracy and reliability of the data. In terms of capsid titer determination, our ELISA still represents one of the most frequently used industry standards due to its robustness and accuracy of measurement. However, the evolution of new biophysical methods such as Size Exclusion Chromatography Multi-angle Light Scattering (SEC-MALS) and Dynamic Light Scattering (DLS), or alternative immunoassays, e.g. Biolayer-Interferometry (BLI), and Capillary Electrophoresis (CE) enables the obligatory, orthogonal measurement of AAV capsid titers. Here we present data of blind measurements of our eGFP-filled AAV standards with the methods mentioned above for comparison of independently determined total capsid titers and to underline the reliability of our established characterization process. The capsid ELISA, SEC-MALS, and BLI showed low assay variation resulting in CVs of around 5%, while CE and DLS showed higher CVs of 6-14% and 10-35%, respectively. Compared to the capsid titer determined by ELISA, each method showed a serotype-dependent average deviation. In summary the average deviation across serotypes compared to ELISA was 13% for SEC-MALS, 46% for DLS, 16% for BLI, and 19% for CE. The use of reliable standards within diverse orthogonal methods ensures not only the most accurate result but also the safety of the final product in compliance with current regulatory requirements. Therefore, we present the reliability of our characterization process for AAV standards, thus demonstrating their reliability and suitability for biophysical and immunological applications, respectively.

564. Next Gen rAAV Production: A Scalable Platform to Revolutionize Gene Therapy Manufacturing

Chris Brown[1], Cameron Fulco[1], Rick Niu[2], Ann-Sophie Reinshagen[3], Anne Wolters[3], Jing Zhu[2], Xiaojun Liu[1]

[1]Process Sciences, ReciBioPharm, Boxborough, MA,[2]Process Sciences, ReciBioPharm, Watertown, MA,[3]ReciBioPharm, Cuxhaven, Germany

Recombinant adeno-associated virus (rAAV) vectors have been proven to be effective tools in delivering therapeutic payloads designed to address various debilitating genetic disorders. Nonetheless, the manufacturing process for these vectors can pose significant challenges, resulting in elevated costs and hindering commercialization. There is a critical need to expedite the development and optimization of rAAV manufacturing processes to meet the growing demand for gene therapies. Here, we demonstrate the development of a robust and scalable rAAV production platform, yielding rAAV8 at a concentration >2.0E15 GC/L and achieving greater than 50% full capsids at harvest, some of the highest reported yields so far using transient transfection. Our introduction of a novel transfection enhancer and optimization of various upstream process parameters has allowed us to increase rAAV yield and % Full capsids by greater than 20-fold and 3-fold, respectively, over a traditional transient transfection process using PEI. The design of experiments (DOE) methodology was employed to identify optimized transfection conditions including cell density, enhancer concentration,

DNA amount, and DNA to transfection reagent ratio, resulting in additional enhancement of rAAV yield and quality. The broad applicability of this optimized platform has been further validated via testing across various wild-type and engineered rAAV serotypes and across various transgenes representative of therapeutic programs seen in the market today. In addition, we have demonstrated our capability to successfully scale-up using Sartorius single-use bioreactor systems, demonstrating the scalability of this platform when used with various serotypes, transfection reagents, and transgenes. The broad applicability of this platform will allow us to reduce process development time and expeditiously ramp up the manufacturing of new products. In summary, this work demonstrates our ability to efficiently increase rAAV yield and quality via optimization of upstream process parameters, an approach which is easily transferrable across rAAV serotype and gene-of-interest. The development of this robust and scalable platform offers a promising and innovative approach to enhance rAAV manufacturability, offering increased yield, reduced cost, and an acceleration of the drug development timeline.

565. Optimizing Transfections for Increased AAV Titers and Effective Media Evaluations

Jessica J. Haywood[1], Kim Schrag[2], Kate Hellman[1], Nathan Stegemann[1], Katelyn McLaughlin[1], Charlotte Javalet[3]

[1]Upstream R&D, MilliporeSigma, Lenexa, KS,[2]MilliporeSigma, Lenexa, KS,[3]MilliporeSigma, Meylan, France

Adeno-associated virus (AAV) has become a prominent vector for gene delivery to treat life-threatening genetic diseases. One of the major limitations for product development and treatment availability is the need for high levels of infectious AAV particles, therefore, media and process optimization is vital to achieving high AAV titers. To maximize AAV titers across multiple HEK293 cell lines, media development for AAV production was performed with three HEK293 cell lines with different growth rates. Following media development, transfections for AAV production were optimized with Design of Experiment (DoE) studies in small scale and confirmed with bioreactor runs. In addition, optimized transfection parameters and smaller DoE designed transfection parameters were applied to cell cultures grown and transfected in media from other vendors. Each media formulation required different transfection parameters to achieve optimized AAV titers.

566. Development and Characterization of a Rhabdovirus-Free Clonal Sf9 Cell Line for Enhanced Safety and Enhanced AAV Production in the Baculovirus Expression Vector System

Gina Zhang[1], Michelle Martin[1], Brendan Leung[1], Chinmay Patkar[1], Christopher Casey[2], Jeffrey Kan[2], Ting Yang[2], Robert Lu[3], Yiling Fang[1]

[1]Analytical Development, Frontera Therapeutics, Bedford, MA,[2]Process Development, Frontera Therapeutics, Bedford, MA,[3]Technical Operation, Frontera Therapeutics, Bedford, MA

The widespread use of *Spodoptera frugiperda 9* (Sf9) cell lines in biological product manufacturing has revealed the presence of a novel rhabdovirus identified in 2014. While downstream purification successfully eliminated rhabdovirus, the desire for a virus-free cell line prompted the development of an in-house rhabdovirus-free clonal Sf9 cell line. The rigorous characterization employing a comprehensive panel of safety assays, established in house, confirmed the absence of rhabdovirus. Evaluation of the cell line's performance in baculovirus generation and Adeno-Associated Virus (AAV) production demonstrated superior yields and product quality compared to the conventional Sf9 cells, spanning multiple AAV serotypes (AAV2, AAV5, AAV8, and AAV9). The successful development of this rhabdovirus-free cell line marks a significant advancement, ensuring heightened safety and efficiency in future process development and Good Manufacturing Practice (GMP) productions.

567. Correlation Analyses of the Quality Attributes of Adeno-Associated Virus Vectors Revealed the Positive Impact of the Viral Protein 2 Ratio on Transduction Efficiency

Takahiro Maruno[1,2], Mitsuko Fukuhara[1,2], Yasuo Tsunaka[2], Aoba Matsushita[2], Kiichi Hirohata[2], Karin Bandoh[2], Megumi Onaka[1], Risa Shibuya[2], Yuki Yamaguchi[2], Yoshiki Nagashima[3], Daisuke Higo[3], Toshie Kuwahara[2], Tomoko Ueno[2], Masaharu Maeda[4], Guirong Kanai-Bai[2], Noriko Yamano-Adachi[2], Tetsuo Torisu[2], Takeshi Omasa[2], Susumu Uchiyama[2]

[1]U-Medico Inc., Suita, Japan,[2]Graduate School of Engineering, Osaka University, Suita, Japan,[3]Thermo Fisher Scientific, Yokohama, Japan,[4]Osaka Consolidated Laboratory, Manufacturing Technology Association of Biologics, Suita, Japan

Adeno-associated virus (AAV) vector capsids, composed of viral proteins (VPs), not only protect the therapeutic nucleic acids but also play an important role in gene delivery and transduction. Among the three types of VPs (VP1, VP2, and VP3), the amino acid sequence of VP3 is identical to that of the VP3 region in VP1 and VP2. VP2 and VP1 have an extended region at the N terminus of VP3 and VP2, respectively. VP1 and VP2 are considered to play important roles in the gene transduction, whereas the VP3 and VP3 regions in VP1 and VP2 are essential for capsid formation. The typical stoichiometry of VP1:VP2:VP3 is roughly estimated to be 5:5:50. The VP ratio is considered to be one of the quality attributes (QAs) that require evaluation. In fact, a higher ratio of VP1 and VP2 leads to higher gene transduction. However, there is limited information on how the QAs affect the transduction efficiency of AAV vectors manufactured using the HEK293 suspension cell line system. In this study, we performed an investigation into how the QAs affect the transduction efficiency of the AAV vectors manufactured using the HEK293 suspension cell line system. To evaluate various QAs of AAV vectors with the same serotype and the same GOI manufactured under different conditions, we manufactured 10 lots of AAV vectors (AAV8-CMV-EGFP) with different conditions modulated within an empirical range in the upstream process and with unified conditions in the downstream process. We monitored the in-process results regarding the cell viability in the upstream process, affinity chromatography (AC) peak area,

A260/A280 after AC, density gradient ultracentrifugation (DG-UC) peak area, A260/A280 after DG-UC, yield, and A260/A280 of the product in the downstream process. Next, we evaluated various QAs including the particle size, particle distribution, full-empty ratio, VP purity, VP stoichiometry, deamidation ratio of VPs, host cell protein, host cell DNA, and transduction efficiency of manufactured AAV vector samples. Remarkable variations in VP stoichiometry were observed; wide variation was observed in VP2 and VP3, whereas little variation was observed in VP1. By performing correlation analyses among the QAs, we identified several attributes that could affect the transduction efficiency. To give one example, the significant positive correlation between the number of VP2 molecules and the transduction efficiency. We plan to discuss correlation between upstream and downstream in-process results and QAs of the products in our presentation.

568. Continuously Improving Quality and Efficiency in AAV Manufacturing

Mathias Kahl

IDT Biologika GmbH, Dessau, Germany

This work explores continuous improvements of quality and efficiency in AAV manufacturing processes. Operating within non-GMP and research-focused environments, the study places a significant emphasis on consistently optimizing both the quality and efficiency of AAV processes, which can be transferred easily into a GMP setting. Utilizing IDT Biologikas' proprietary HEK293 cell line, which we benchmarked against commercially available AAV manufacturing solutions, we are investigating how our platform approach is responding to ongoing advancements in AAV manufacturing. While implementing a generic manufacturing platform for AAV´s we evaluate a broad set of upcoming technologies and trends that might potentially redefine quality attributes, safety profiles and contribute to reducing serious adverse effect. In this Talk we will report about IDT´s journey in implementing a generic concept for AAV manufacturing and about the current standards in production processes, giving a future perspective on the upcoming activities, while evaluating new available technologies from a GMP manufacturer point of view. The exploration of new technologies in AAV manufacturing strives to set new benchmarks. A ready-to-use platform, along with cutting-edge techniques and a proprietary cell line, become instrumental in achieving sustained quality and efficiency. In particular we will allude on further optimization in AAV manufacturing by integrating the use of small molecules and plasmids technologies and the implementation of post-translational modifications characterization by use of analytical solutions beyond the current regulatory requirements. Thus potentially supporting the definition of standards for quality and efficiency for gene therapeutic products.

569. High-Throughput Chromatography Studies to Improve Process Understanding and Reduce Risks in Gene Therapy Clinical Manufacturing

Jeff Plegaria, Matthew Petroff, Qi Lin, Jonathan Palmer, Mi Jin

Spark Therapeutics - Philadelphia, PA, Philadelphia, PA

AAV downstream processes face significant challenges due to high levels of product variants and complex characterization of these impurities. These challenges are further compounded by timeline pressures to generate clinical lots and limited historical information in AAV manufacturing. To address these issues, we present high-throughput case studies for early assessment of platform fit. Using Design-of-Experiments and miniaturized chromatography, we optimized downstream performance while simultaneously identifying key process parameters for control, to ensure manufacturing success. These studies contribute to early process understandings, risk assessment, and control strategy, thereby reducing the risks associated with process changes during early-phase clinical manufacturing.

570. Design of Experiments Approach to Increase rAAV Yield through Optimization of Transfection Conditions

Igor Alves Mancilla[1], Maria Nicla Loviglio[2], Megane Denu[2], Paul Giroud[2], Jean-Thomas Issenhuith[2], Irene Ferreira[3], Carl J. Christel[3], Kimchi Audrey Strasser[1]

[1]Process Development, Revvity Gene Delivery (SIRION Biotech Legacy), Graefelfing, Germany,[2]Polyplus-Transfection, Illkirch, France,[3]Cell & Gene Therapy Platform Innovation & Management, Revvity Gene Delivery (SIRION Biotech Legacy), Graefelfing, Germany

A widely employed method for large-scale production of rAAV vectors involves co-transfecting HEK293-derived producer cell lines with three plasmids. The producer cell line and production plasmids are manufacturing starting materials, which not only influence rAAV Critical Quality Attributes (CQAs) for purity and potency, but also positively affect manufacturability parameters like productivity. Increasing process productivity is an effective strategy to reduce the cost of goods in preclinical and clinical development, as well as in the commercialization phase of a gene therapy product. Concurrently, another avenue to explore involves selecting starting materials that offer optimal cost-effectiveness. Here, we report the application of a design of experiments (DoE) approach to simultaneously increase productivity and investigate a more cost-effective transfection reagent, FectoVIR®-AAV (Polyplus®). An initial DoE was designed by Polyplus® using the MODDE® software, where the factors tested were: amount of plasmid DNA, ratio of DNA to the transfection reagent, complexation time, and day of cell seeding. We produced pAAV-Rep2Cap8 in 26 independent 3-plasmid transient transfections in the Viral Production Cell 2.0 (Gibco™), a HEK293F-derived subclone, with Viral Production Medium (Gibco™), in 30 mL cultures in shaker flasks. We harvested 3 days later and clarified by alluvial filtration. The clarified lysate was measured by ITR-qPCR, where the result was expressed in VG/mL of cell culture. Titers varied from 5.3E11 to 2.6E9 VG/mL. By fitting the results to a response surface model and performing a ridge analysis, we

could determine that higher concentration of DNA and lower ratio of DNA to FectoVIR®-AAV are key factors. However, complexation time and day of cell seeding were not significant factors for this model. To validate these results and to precisely determine the optimal values of plasmid DNA and ratio of DNA to FectoVIR®-AAV, we conceived a follow-up experiment which included 3 different cell concentrations and 4 different complexation buffers. This design was developed through an R-based DoE pipeline using optimal design of experiments. We produced the same vector as previously in 32 independent 3-plasmid transient transfections. The method of production was similar to the one previously described, but with 10 mL cultures in 50 mL mini-bioreactors. We found that the narrower range of DNA amount and ratio of DNA to FectoVIR®-AAV did not excessively impact productivity, showing the robustness in the range tested. Taken together, these results demonstrate the possibilities of utilizing a DoE approach for process development before proceeding with non-GMP productions for preclinical studies. Thus, a DoE approach should not only be considered for developing a cost-effective GMP manufacturing process, but also as a cost-effective non-GPM manufacturing process for preclinical batches.

571. Tackling AAV Manufacturing Process Industrialization Challenges Using a Standard Platform Approach

Emilie Gateau, Andy Reniers, Camille Lemaigre, Solène Arnoult, Claire Favre-Felix, Tiago Albano, Julien Caballero-Montes, Antonio Salgado-Somoza, Kassandra Watillon, Sylvain Fauquenoy, Vasily Medvedev, Hanna P. Lesch

Exothera, Jumet, Belgium

AAV is considered as one of the most versatile tools for gene therapy applications, with its target tissue specificity and its non-replicating property benefits. Therefore, a growing number of AAV clinical trials are initiated each year and increasing safety concerns come with the need to strengthen our understanding of the manufacturing process and its impact on the final product quality. For this purpose, we developed a standardized manufacturing process following QbD principles ensuring sustainable yields for commercial stages and compliance with CMC requirements. Supportive analytical tools are instrumental in the delivery of a high-quality product and a key component of our proprietary platform. With our CMC roadmap, we defined upfront a set of advanced analytical methods to characterize AAV products. We have developed an integrated methodology to evaluate total residual DNA and encapsidated residual DNA on multiple serotypes using ddPCR, targeting host cell DNA and residual plasmid sequences. For infectious particle titration, we also rely on an alternative method to the recognized TCID50. To optimize the comparability with TCID50, general aspects of the cell culture approach have been retained. Our strategy is based on the titration of internalized genomes 24 hours after infection, by ddPCR, using a system of helper virus-free and HeLa cells. We focused our upstream development on key current AAV bottlenecks, *i.e.* delivering high yields and scalable manufacturing processes. Working toward starting material selection and supplementation strategies, we managed to increase our titers by a 4.5-fold factor going from 1E11 VG/mL to 5E11 VG/mL at AAV2 harvest. We have determined how a cell line could also impact quality attributes such as the percentage of full and empty capsids, or the host cell impurity level having drastic consequences on downstream operations. We worked on a methodology to ensure right-first-time process scale up to 1000L bioreactor scale with consistent titers. We then improved our downstream toolbox to deliver optimal product purity. To target the removal of impurities acceptable for highly concentrated AAV product, we used design of experiment methodology to optimize our endonuclease treatment, chromatography and filtration steps leading to a reduction of up to 2 logs of host cell DNA, 5.5 logs of host cell proteins and 2.7 logs of endonuclease. Thanks to this development, our platform reaches overall recoveries of 30-40% after polishing and enrichment up to 90% of full particles determined by AEX-HPLC, directly aligned with latest CMC needs. In conclusion, we demonstrated that a systematic approach can address CMC needs to achieve product quality objective. Through the development of this process, we have successfully engineered a standardized platform that consistently delivers according to industry expectations.

572. Demonstration in Scalability of a Flexible, High-Producing Triple-Transfection rAAV Platform

Cameron Fulco[1], Chris Brown[1], Yeonji Kim[1], Rick Niu[2], Ann-Sophie Reinshagen[3], Anne Wolters[3], Jing Zhu[2], Xiaojun Liu[1]

[1]Process Sciences, ReciBioPharm, Boxboough, MA,[2]Process Sciences, ReciBioPharm, Watertown, MA,[3]ReciBioPharm, Cuxhaven, Germany

The use of recombinant adeno-associated virus (rAAV) is still a key method for DNA delivery to target cells in patients with over 100 companies working on rAAV in some capacity. Depending on numerous factors such as the target disease and route of administration, typical doses can range from 5e8 GC/eye to 1e14 GC/kg. The higher dosing strategies can prove challenging for some companies or institutions to be able to target specific diseases or maintain a broad portfolio of targets. While triple-transfection methods are among the more expensive production processes, their flexibility in product generation and streamlined development timelines can allow for a truly established platform capable of delivering multiple products. Development of a scalable, high-producing upstream platform for AAV generation is paramount for treating more patients with fewer batches. Having fewer batches in turn reduces the manufacturing costs therefore greatly offsetting the costly transfection plasmids and transfection reagent in addition to a reduction in GMP suite time and manufacturing timelines from the larger vessel size. We demonstrate here our capability to scale-up a variety of difference upstream conditions including different capsid serotypes, transfection reagents, and transgene lengths with comparable harvest yields and quality. In addition, through cell health metrics along with harvest yield, we demonstrated the scalability of this HEK293-based Upstream process from shake-flask to 50L scale, while generating harvest yields >1.0E15 GC/L with >40% full capsids.

573. Enhancing AAV Purification: Strategies for Improved Recovery and Quality for Downstream

Yeonji Kim[1], Chris Brown[1], Cameron Fulco[1], Rick Niu[2], Ann-Sophie Reinshagen[3], Anne Wolters[3], Jing Zhu[2], Xiaojun Liu[1]

[1]Process Sciences, ReciBioPharm, Boxboough, MA,[2]Process Sciences, ReciBioPharm, Watertown, MA,[3]ReciBioPharm, Cuxhaven, Germany

In gene therapy manufacturing, proper extraction of adeno-associated virus (AAV) material from transfected cell cultures during the upstream process is pivotal for downstream success. However, challenges in AAV manufacturing include limited titer production and difficulties in high recovery in the extraction of full capsids. This study aims to optimize the initial stages of the downstream process using high-titer AAV material with diverse analytical methods. An exceptionally high titer of >1E12 vg/mL, surpassing the normative threshold post-lysis, as the starting post-lysis material is noteworthy. High-quality AAV purification is crucial to establish a preliminary downstream purification standard before chromatography. Filtration steps eliminate residual host cell proteins and DNA while preserving viral particles. A comparative analysis of three vendors was conducted with each vendor providing the top three of their best performing depth filters. Following filtration, various Tangential Flow Filtration (TFF) methods were assessed to pinpoint the optimal approach for maximizing recovery and purification. During these tests, multiple analytical methods quantified viral particle counts of AAV. Among the methods is ddPCR, a robust method to quantify DNA and viral particles. The comparison involved evaluating each filtrate against pre-filtered materials, examining turbidity reduction, reduction in host cell protein and DNA levels, viral particle recovery, aggregation, and considering factors like time and throughput. The selected methodology concluded from the filtration and TFF study, supported by advanced analytics, demonstrated >95% recovery, even with lysate titer of 1E12 vg/mL, generating pure AAV materials ready for chromatography. The total throughput calculated was ~180 L/m2. Data from viral titer recovery using ddPCR and assays gauging residual host cell proteins and DNA provide valuable insights for optimizing AAV purification processes. This research enhances efficiency and quality in AAV production, advancing gene therapy applications.

574. Knowledge Management Tool for Fast Tech Transfer from Client to IND

Yvette Valenzuela, Catherine Siglin

Resilience, Waltham, MA

Rapid progress is paramount in the development of a promising drug candidate, particularly when navigating the stages of GMP manufacture and IND approval. The challenges are amplified by the inherent uncertainties that accompany early-stage drug development, where process developers operate with limited insights into a scale-appropriate and representative process, compared to what was initially developed in the laboratory. The success of a program hinges on avoiding misinterpretations of critical parameters, order of operations, or materials, as any mistranslation can lead to significant setbacks. Within the Gene Therapy Franchise at Resilience, we've implemented a streamlined tech transfer workflow process to efficiently transfer knowledge from clients to the process development team and seamlessly integrate it into clinical manufacturing. Our innovative approach involves merging the process definition and facility fit exercises, resulting in an impressive 50% reduction in cycle time. This accelerated timeline allows us to assess gaps, identify and track mitigation actions, and facilitate necessary revisions to the process description. The self-service tool we've implemented serves as a versatile platform for knowledge transfer, extending its benefits to supporting functions such as Manufacturing, Supply Chain, Engineering, QC, and Regulatory functions. The result has been the successful maintenance of program timelines, ensuring the timely execution of manufacturing batches and meeting stringent regulatory filing requirements. In essence, our strategic and integrated approach expedites the development process and enhances the precision and reliability of each stage, contributing to the program's overall success.

575. AAV Harvest Lysate After Varying Upstream In-Process Steps Show Comparable Yield and Product Quality

Kenneth J. Cunningham, Hsuan-Ni Lin, William Kaiser, Shohanuzzaman Shohan, Vini Sheth, Sadie Alperson, Elliot Jersild, Clarissa Tjoanda, Sarah Kudman, Adnan Arnaout, Stanley Ng, Jessica Cheng, Mary Ng, John Chan

Prevail Therapeutics (A Wholly Owned Subsidiary of Eli Lilly and Company), New York, NY

During adeno-associated virus (AAV) production, several methods are available for preparation of column load prior to chromatography purification, including clarification, concentration (UF), or diafiltration (DF) of crude cell lysate. The method used can depend on several factors, such as process differences between manufacturing platforms or differences in developmental stage. At the small-scale screening and development stage, the cell lysate volume is not typically large enough to go through the full UF/DF processing, while during large-scale manufacturing it is preferable to concentrate cell lysate volumes to reduce downstream column loading time. However, it is not well understood how differences in upstream processing after cell lysis and the subsequently different column load matrices will impact AAV yield and quality. This study aims to evaluate the impact of using column load sourced from different upstream in-process steps which can support process flexibility and characterization.AAV cell lysate produced for this study was divided into five groups with equal viral genomes (vg) per study condition. These five groups aim to evaluate the impact on the affinity capture step after loading bulk cell lysate prepared by centrifugation, depth filtration, concentration (UF-1), concentration and diafiltration (UF-1/DF), or additional concentration and diafiltration (UF-2/DF). To ensure process intermediate stabilities were comparable, After the five column loads were purified, eluates were split into two groups and either frozen immediately or frozen after a 7-day hold at 2-8C. Affinity chromatograms of the five different column loads were compared and analytics were performed to measure yield, product quality, and residuals. Step yields across each upstream arm and downstream purification were determined by ddPCR. Product quality was evaluated by measuring expression potency. Dynamic light scattering (DLS) was used to measure the AAV size distribution and aggregation. ELISA was used to measure host cell protein (HCP)

levels and clearance across each study condition. This study concludes that there is no significant difference from using varying column loads from different upstream in-process steps post-cell lysate clarification on purification performance. This study further characterizes upstream in-process steps and provides additional downstream hold time stability data. Taken together, these findings support additional process flexibility, allowing AAV from platforms with different matrices to be compared equivalently.

576. Adapting a Hybrid Analysis for AAV Characterization by Combining Long and Short Read Sequencing Techniques

Brianna C. McManus, Rebecca A. Bova, Christina Kalinyak, Maggie Luce, Servane Orozco, Bradley Hasson

BioReliance Corporation (MilliporeSigma/Merk KGaA), Rockville, MD

Adeno-associated viruses (AAV) are one of the most widely used viral vector-based gene therapy delivery systems in the industry. The manufacturing of such products commonly uses a series of 3-4 plasmids that are transfected into a producer cell line in order to assemble the viral particles. Each plasmid contains a critical element for viral production, whose product is an AAV particle containing the gene of interest flanked by two inverted terminal repeat (ITR) regions. NGS has become a widely used tool for biosafety testing for a variety of viral and gene therapy products. Short-read techniques such as Illumina, while effective, can be improved upon. PCR based library preparation methods can introduce levels of biases, and the highly repetitive ITR regions are difficult to sequence due to their repetitive nature, sequencing read length is limited by the number of cycles per sequencing kit. While long-read sequencing technology is a relatively new platform, it aims to mitigate these risks, however, in comparison, it can have reduced quality scores. To analyze these types of samples more effectively, a hybrid-analysis approach can be employed. By combining short and long read methods a genome assembly can be created with deeper coverage, longer reads, and retaining both quality and accuracy. Long-read sequencing with Oxford Nanopore technologies gives direct sequencing data without PCR, genome fragmentation, or limitations on read lengths. There are a wide variety of kits available with ranges of sample inputs. Testing has been performed to optimize the process of extraction through sequencing for these challenging primarily single-stranded molecules. We have tested various serotypes of AAVs through both long and short read NGS methods that were developed. The samples are first treated with Benzonase® endonuclease to remove any non-encapsidated sequences. This allows for the sequencing and analysis of the contents of the capsid DNA. The samples are extracted, amplified, then libraries are prepared using the Ligation Sequencing Kit V14 for sequencing on the GridION instrument. These generated sequences can be analyzed using currently used bioinformatic approaches and combined with short-read data for increased depth of coverage, characterization approaches, and can aid in genome assembly. The reads generated by both methods are directed into an analysis pipeline to assess known residual sequences. Through the analysis, reads can be mapped to multiple sequences that are used in the manufacturing process including plasmids and cell line genomes. It has been observed that by combining the two sequencing methods this would allow for deeper coverage of difficult to sequence regions such as ITRs than short read methods alone. Utilizing NGS to assess these known residuals that may be contained with the capsid allows for an overall understanding of what is seen within various capsid profiles. These methods mentioned above can be used to characterize the contents of capsid whether the capsid is expected to be full, empty or even partially full. By knowing what is in each payload this can help ensure a safer product.

577. A Rapid and Robust Construct Manufacturability Screen Enhances Therapeutic Candidate Selection

Tim Brantley, Samantha Gall, Leah Machlin, Samantha Lasater, Nick Wessler, Charles Pugh

Kriya Therapeutics, Durham, NC

Recombinant AAV vectors are defined by quality attributes that should remain consistent throughout the product development lifecycle, irrespective of process scale. A screening model to assess manufacturability of preclinical candidate constructs can eliminate product candidates with quality attribute challenges early in the development process and avoid the high costs associated with failure at scale. This work demonstrates the comparability of a staged construct screening and selection approach utilizing deep well plates, shake flasks and 1L bench scale bioreactors. A 24-well deep well plate upstream transfection process was developed to rapidly screen transgene constructs for their productivity and ability to package complete transgenes as measured by vector genome integrity capillary electrophoresis. At the cell culture harvest stage, packaged genome and capsid titer were within 21% and 18%, respectively, when comparing plates, shake flasks, and 1L BioBlu bioreactors across 3 transgene constructs. To measure vector genome integrity, a 24-well filter plate affinity chromatography system was developed to purify the culture harvest sufficiently for measurement of vector genome integrity. The 24-well filter plate affinity system was compared to column chromatography on an Akta chromatography system and step yields were between 75-80% for both systems. Once the number of therapeutic constructs has been narrowed down, we compared the final 2 transgene constructs in AAV1 across multiple batches using a scaled-down process designed to mimic our large-scale production processes. This process begins with inoculating and transfecting 1L BioBlu single-use bioreactors, cell lysis and filtration, followed by a two-column purification process. The two transgene constructs were evaluated for their upstream productivity, %full ratio of affinity eluate, AEX column step yield, % full ratio, and vector genome integrity in the final drug substance. The results demonstrated comparability of a staged construct screening and selection approach utilizing deep well plates, through shake flasks to1L bench scale bioreactors. This inclusion of rigorous analytical testing, uncommon at early stage of rAAV product development, ensures that the selected clinical candidates meet critical quality attributes and manufacturability needed to support successful product development.

578. Building a Chromatography Platform for Purification and Full Capsid Enrichment on AAV8

Rick Niu[1], Ann-Sophie Reinshagen[2], Yeonji Kim[3], Anne Wolters[2], Cameron Fulco[4], Chris Brown[3], Jing Zhu[1], Xiaojun Liu[3]

[1]Process Sciences, ReciBioPharm, Watertown, MA,[2]ReciBioPharm, Cuxhaven, Germany,[3]Process Sciences, ReciBioPharm, Boxboough, MA,[4]ReciBioPharm, Boxboough, MA

In the creation of AAV as a gene therapy drug product, empty capsids are formed. These are capsids that do not contain the gene of interest yet can increase the chance of immune responses in patients[1]. Therefore, different methods have been used to remove empty capsid and increase full capsid percentage. The most widely used are cesium chloride ultracentrifugation, iodixanol gradient ultracentrifugation, and anion exchange chromatography (AEX). Given the numerous benefits of chromatography over ultracentrifugation in terms of scalability, this mechanism was chosen to be further developed. The material utilized in this development was made in-house. AAV8 was grown on suspension HEK293 cells with GFP as the gene of interest. Following lysis, it was clarified via depth and sterile filters, then concentrated and diafiltered through tangential flow filtration (TFF). Purification on the TFF retentate was performed by affinity chromatography. To optimize this process, different resins, loading conditions, buffers, and run parameters were compared. Success criteria were evaluated by comparing recovery percents determined by digital droplets polymerase chain reaction (ddPCR). Following the optimized affinity chromatography, the elution was utilized as the starting point for AEX development. Four different columns were screened with varying buffers, load conditions, and modalities. Full capsid enrichment was primarily analyzed with DLS via Stunner by Unchained Labs and cross referenced with SV-AUC and TEM. Promising conditions were then analyzed by ddPCR to determine recovery percentage. Several approaches produced very high full capsid enrichment (95%) while also recovering the majority of product.

579. Evaluating AAV Production Platforms Using Commercially Available Host Cells and Plasmid Systems

Pratima Cherukuri, Lily Ong

Genezen Laboratories, Indianapolis, IN

Adeno-associated viruses (AAVs) are an effective gene delivery platform for gene replacement, regulatory and gene editing applications. However, the challenge of efficient vector manufacturing persists, exacerbated by numerous serotype options and capsid variants. Additionally, a robust and scalable manufacturing process often requires a significant amount of time and investment. Rather than customize the process for each transgene and serotype, a platform process would be most impactful especially for rare and ultrarare indications. To tackle this complexity, we applied a design-of-experiment (DOE) methodology to systematically optimize a production process that is capable of producing high quality, full capsid enriched harvest with downstream purification process agnostic to capsid/serotype variations. Our approach involved evaluating multiple production parameters, including two different suspension-based host cell lines and three commercially available helper plasmid systems with variations in the plasmid DNA-to-cells ratio. By simultaneously adjusting transgene, packaging, and helper plasmid ratios, total DNA concentration, and cell density, we systematically examined their combined effects to enhance titers and achieve superior empty-to-full ratios. Additionally, we evaluated the integrity of the genomes using single genome analysis to ensure the highest quality and potency. The data from the initial DOE studies were used to assess the scalability of the platform at the 50L scale.With an attempt to stay agnostic to capsid and serotype variations, we introduced ultracentrifugation to agnostically improve empty versus full ratios, along with Anion Exchange Chromatography (AEX) in downstream processing. Mass photometry served as a rapid and efficient analytical tool for aggregate and empty-to-full AAV capsid ratio analysis. The results of this approach will be discussed in this presentation. This integration of commercial upstream materials and a serotype-agnostic downstream processing (DSP) process will be used to establish a universal high-quality manufacturing process for AAV viral vectors.

Wednesday Posters: Metabolic Diseases

580. Defining Clinical and Laboratory Biomarkers in Propionic Acidemia to Guide AAV Gene Therapy Clinical Trial Endpoints

Carolina I. Galarreta Aima, Oleg Shchelochkov, Jennifer L. Sloan, Camryn Hall, Abigael Gebremariam, Susan C. Ferry, Carol A. Van Ryzin, Irini Manoli, Charles P. Venditti

Organic Acid Research Section, National Human Genome Research Institute, Bethesda, MD

***Background*:** Patients with propionic acidemia (PA), caused by a deficiency of propionyl-CoA carboxylase alpha (PCCA) or beta (PCCB) subunits, display diverse clinical symptoms and laboratory abnormalities. Currently, clinical management is limited to carnitine supplementation, restricted dietary protein intake, and, for severe patients, elective liver transplantation to restore propionyl-CoA carboxylase activity. Systemic AAV9 gene therapy is being developed as an alternative to organ transplantation for both *PCCA* and *PCCB* PA by the NCATS Platform Vector Gene Therapy (PaVe-GT) and the Bespoke Gene Therapy Consortium (BGTC). Similar to other rare metabolic disorders, the evaluation of outcome measures and surrogate biomarkers important for protocol development and regulatory approval remains challenging. ***Methods*:** To help define clinical parameters and laboratory biomarkers associated with disease severity and response to treatment, we have conducted a prospective study at the NIH Clinical Center (**ClinicalTrials.gov Identifier: NCT02890342**). Protocol visits included a battery of subspecialty evaluations, nutritional assessments, imaging studies, laboratory and molecular genetic testing, including trio exome analysis, the collection of biospecimens, and the generation of EBV and/or fibroblast cell lines from all participants. Cardiac, renal, hematological, hepatic, ophthalmic, neurological and metabolic parameters were systematically examined. Patients underwent a 1-^{13}C-propionate oxidation breath test (POBT), a non-invasive whole-body measurement largely reflective of

residual propionyl-CoA carboxylase activity in the liver, expressed as cumulative percent isotope dose metabolized in 60min. ***Results:*** 42 PA patients, including 23 females (54.7%) were evaluated, with a median age of 12.0 (Interquartile range 6.0, 23.5). Molecular confirmation was obtained for all patients (17 harbored pathogenic variants in *PCCA* and 25 in *PCCB*). Eight patients underwent organ transplants: 5 had isolated liver transplant (LT) (median age 1.5yo, range 1-20), 1 had an isolated kidney transplant (at age 42 years), 1 had a combined liver kidney transplant (21.5 years), and 1 had combined liver/heart transplant (7.8 years). POBT obtained in 29 (14 *PCCA*, 15 *PCCB*) patients showed a median 1-^{13}C-propionate oxidative capacity of 3.79% (IQR 2.0, 13.52). In comparison, 21 healthy volunteers had a median value of 32.9% (IQR 31.0, 34.2), and 3 post-liver transplant PA patients had a median value of 29.8% (IQR 18.1, 41.3). 1-^{13}C-propionate oxidization at 60 minutes negatively correlated with Full-Scale Intelligence Quotient ($p<0.001$), height Z score ($p=0.005$) and left ventricular ejection fraction ($p=0.016$) in untransplanted patients. Plasma concentrations of disease-specific metabolites, including 2-methylcitric and 3-hydroxypropionic acid, were measured (N=35 pre-transplant and 5 patients after LT), showing a reduction in levels post-transplant. Both metabolites negatively correlated with 1-^{13}C-propionate oxidization at 60 minutes ($r=-0.548$, $p<0.001$, and $r=-0.662$, $p<0.001$ respectively) and_correlated negatively with kidney function. ***Conclusion:*** Our study further elucidates the natural history of propionic acidemia and provides a basis for studying surrogate biomarkers that could serve as informative pharmacodynamic endpoints in future phase I/II clinical trials.

581. AAV Gene Therapy Corrects Metabolic Profile and Extends Survival in a Mouse Model of Argininosuccinic Aciduria

Yuchen Zhou, Jill Thaisz, Craig Smith, Christina Chang, Veronica Viramontes, Donald Chun, Yogendra Shrestha, Franziska Graf Leifer

Insmed Incorporated, Bridgewater, NJ

Argininosuccinic Aciduria (ASA) is a rare pediatric-onset urea cycle disorder caused by loss-of-function mutations in the argininosuccinate lyase (ASL) gene. ASL is one of five enzymes in the urea cycle, catalyzing the conversion of argininosuccinate to arginine and fumarate[1]. Reduction in or loss of ASL function leads to rapid buildup of ammonia in the blood stream that, left untreated, can cause coma and death[1]. The current ASA standard of care regimen of nitrogen scavenger therapy, low protein diet, and arginine supplementation may leave patients at continued risk for recurring hyperammonemic crises, and many experience long-term cognitive impairment[1]. Adeno-associated virus (AAV) gene therapy is a promising treatment modality for therapeutic targeting of the liver[2], however, the single-dose paradigm of current AAV gene therapy treatments is not suitable for early-onset pediatric ASA patients due to the expected loss in transgene expression in the growing liver over time[3]. To potentially overcome this limitation, Insmed is developing a redosable AAV gene therapy platform that could enable durable therapeutic benefit. As part of that work, we have developed a series of therapeutic AAV transgene constructs encoding the human ASL gene and evaluated their in vivo efficacy in ASA$^{neo/neo}$ mice, an early-onset mouse model of ASA. We delivered the hASL transgenes encapsulated in wildtype recombinant AAV particles via a single dose ranging from 1e13 vg/kg to 1e14 vg/kg. We monitored clinical outcomes and biomarkers including body weight gain, survival, blood ammonia, and urea cycle intermediates. Following treatment, blood ammonia levels of ASA$^{neo/neo}$ mice dropped from above 400 μM prior to treatment to the normal range below 100 μM and plasma argininosuccinate, the urea cycle intermediate metabolized by the ASL enzyme, was significantly reduced. At the same time, bodyweight gain of ASA$^{neo/neo}$ mice accelerated up to ten-fold from 0.046 g per day predosing to 0.460 g per day the week after dosing and the median survival of ASA$^{neo/neo}$ mice increased from less than 20 days to over 100 days. These results indicate that Insmed's ASA AAV gene therapy constructs can successfully ameliorate the metabolic state of ASA$^{neo/neo}$ mice and could be a promising treatment for ASA if delivered as a redosable AAV gene therapy. References [1]Nagamani, SC, Erez, A, Lee, B. Argininosuccinate lyase deficiency. *Genet Med.* 2012; 14(5):501-7. doi: 10.1038/gim.2011.1. [2]Pipe, SW, Leebeek, FWG, Recht M, et al. Gene Therapy with Etranacogene Dezaparvovec for Hemophilia B. *N Engl J Med* 2023; 388:706-718. doi: 10.1056/NEJMoa2211644. [3]Baruteau, J., Perocheau, D.P., Hanley, J. *et al.* Argininosuccinic aciduria fosters neuronal nitrosative stress reversed by *Asl* gene transfer. *Nat Commun* 2018; 9(1):3505. doi: 10.1038/s41467-018-05972-1.

582. Deep Phenotyping of a Cobalamin B-Type (*cblB*) Methylmalonic Acidemia Patient Cohort Guides the Selection of Biomarkers and Outcomes for AAV Gene Therapy Clinical Trials

Abigael Gebremariam, Camryn Hall, Samantha McCoy, Jennifer L. Sloan, Susan C. Ferry, Carol Van Ryzin, Carolina Galarreta-Aima, Oleg Shchelochkov, Irini Manoli, Charles P. Venditti

National Human Genome Research Institute (NHGRI), Bethesda, MD

Introduction: Cobalamin B-type (cblB) methylmalonic acidemia (MMA) is an ultra-rare autosomal recessive inborn error caused by pathogenic variants in the MMAB gene, which encodes for the enzyme ATP:cob(I)alamin adenosyltransferase (ATR). ATR catalyzes the synthesis and delivery of 5'-deoxyadenosylcobalamin (AdoCbl) to the methylmalonyl-CoA mutase enzyme (MMUT). MMAB-MMA results in life-threatening metabolic instability and progressive multisystem disease with limited therapeutic options outside elective liver transplantation offered to severely affected patients. In collaboration with NCATS via the Platform Vector Gene Therapy (PaVe-GT) project we aim to develop AAV9 gene therapy for MMAB-MMA. In this study, we document genotype-phenotype associations, biomarkers of disease severity and effects of liver transplant in MMAB patients in preparation for a future AAV gene therapy clinical trial. **Methods:** We evaluated 17 cblB patients, 12 male (70.5%), ranging from 2 to 46 years of age, including eight with a liver or combined liver and kidney transplant (LT/LKT) under a natural history protocol (NCT00078078). We examined genotype-phenotype correlations; measured canonical metabolites, including methylmalonic acid (sMMA) levels, and plasma FGF21, a marker of hepatic mitochondrial dysfunction; and conducted a 13C-propionate oxidation breath test (POBT), as a safe, non-invasive, indirect measure of MMAB enzymatic activity. In preparation for gene therapy, we evaluated AAV neutralizing antibody (NAb) titers measured using a Huh7 cell-based assay with a AAV-CMV-lacZ reporter after incubation with serially diluted serum

from 8 study participants (PMID: 19133809). **Results:** In our cohort, 58.8% of alleles (8 homozygous) were the c.556C>T p.(Arg186Trp) variant, which is associated with a severe phenotype. Five patients were compound heterozygotes for c.700C>T p.(Gln234Ter), a nonsense variant associated with a higher residual enzymatic activity and B12 responsiveness in vitro. Half of the homozygotes for the p.(Arg186Trp) variant presented in the neonatal period, while 80% of the patients carrying the p.(Gln234Ter) allele were late onset. The median age of chronic kidney disease stage 3 onset was 3 years old for severe patients compared to the median age of 31 years for the mild variant. The POBT test confirmed low enzyme activity in a patient homozygous for p.(Arg186Trp) (16% of the isotope dose oxidized in 120 min) in contrast to near normal enzymatic activity (47%) in a patient compound heterozygous for p.(Arg186Trp) and p.(Gln234Ter) variant. Mild patients and recipients of a liver or liver/kidney transplant maintained lower serum metabolites, including sMMA and plasma FGF21, than those with severe disease. The total seroprevalence of NAbs against AAV2 or AAV9 was 25% (n=2/8) and 37.5% (n=3/8), respectively, while 66% were co-seropositive. Seropositive patients were older than 18 years (n=2) or had received solid organ transplantation (n=2). All untransplanted pediatric individuals were seronegative for AAV9. **Conclusions:** Genotype-phenotype correlations have emerged from the study of patients with MMAB-MMA, and directly inform the generation of murine models for preclinical proof of concept studies to enable AAV9 gene therapy. We also document that the more severely affected, younger patients have a predictable pattern of biomarker changes and are seronegative against AAV9, which should help facilitate translation of gene therapy for these children.

583. Prevalence of Neutralizing Antibodies Against AAV2 and AAV9 Capsids in Propionic Acidemia (PA): Implications for Systemic Gene Therapy

Camryn S. Hall, Jennifer L. Sloan, Carolina I. Galarreta, Oleg Shchelochkov, Irini Manoli, Charles P. Venditti

National Human Genome Research Institute, Bethesda, MD

INTRODUCTION: Propionic Acidemia (PA) is a severe inborn error of metabolism marked by life-threatening metabolic instability, multiorgan involvement, and poor long-term prognosis. PA is caused by biallelic variants in PCCA or PCCB resulting in a deficiency of alpha or beta subunits of propionyl-coA carboxylase, respectively, which catalyzes the conversion of propionyl-CoA to methylmalonyl-CoA. The limited treatment options for the disorder, which may involve invasive procedures like liver transplantation in severe cases, underscore the need to develop systemic AAV gene therapy as a novel treatment approach. Studies have demonstrated the efficacy of systemic AAV gene delivery in mouse models of PA, and initiatives by the Platform Vector Gene Therapy (PaVe-GT) program and Bespoke Gene Therapy Consortium (BGTC) are underway to advancing systemic AAV9 gene therapy for the patients. Thus, our focus was on evaluating neutralizing antibody (NAb) titers against serotypes 2 and 9 in a well-characterized group of PA patients, aiming to assess the feasibility of future gene therapy clinical trials with AAV9 vectors. METHODS: Participants and biospecimens were accrued via NIH study "The Natural History, Physiology, Microbiome and Biochemistry Studies of Propionic Acidemia" (clinicaltrials.gov identifier: NCT02890342). NAb titers were measured using an Huh7 cell-based assay with a AAV-CMV-lacZ reporter after incubation with serially diluted serum from the patients (PMID: 19133809). One sample from a previous study that had been processed in identical fashion was included as a positive control (PMID: 26790480). RESULTS: Archived serum samples from 36 patients (16 PCCA, 20 PCCB) aged 2 to 53 years were analyzed. Four patients have had organ transplants (3 liver, 1 kidney). The total seroprevalence of NAbs against AAV2 or AAV9 was 22.2% (8/36) and 25% (9/36), respectively; 70% of patients were co-seropositive. Most individuals with AAV9 NAbs were either adults or had undergone solid organ transplantation (55.5%, n=5/9). In the cohort of PA patients under 18 years without solid organ transplants, 10% (1/10) PCCA patients and 30% (3/10) PCCB patients were seropositive against AAV9, however, seropositive patients had titers less than or equal to 1:40, and 16/20 (80%) were seronegative for AAV9. CONCLUSIONS: In our study, the prevalence of AAV NAbs in PA was lower than observed in historical controls where AAV9 seropositivity ranged from 31-47%, with a lower limit of detection of 1:30 in adults and 1:50 in pediatric participants (PMID: 20095819, 29064732). Of special note, in our cohort, the younger patients who had not been transplanted were largely seronegative (16/20, 80%). Whether this reflects an underlying immune irregularity is unknown but is unlikely caused by decreased exposure to AAV given the frequent hospitalizations experienced by the patients. The Nab profile against AAV, especially AAV9, has encouraging implications for the clinical development of systemic gene therapy, especially for pediatric patients with propionic acidemia.

584. Using Genomic Databases to Estimate Rare Disease Prevalence: Application to Disorders of Propionyl-CoA Oxidation

Jennifer L. Sloan[1], Camryn S. Hall[1], Irini Manoli[2], Charles P. Venditti[2]

[1]National Human Genome Research Institute, Bethesda, MD,[2]NIH, Bethesda, MD

Introduction: Inborn errors of propionate metabolism are a group of autosomal recessive, genetically heterogenous, severe and potentially lethal disorders detected via elevated propionylcarnitine on newborn screening (NBS). As part of public and private partnerships, including the PaVe-GT and Bespoke Gene Therapy Consortium efforts, the development of AAV-gene therapy is underway for isolated methylmalonic acidemia caused by variants in *MMUT* or *MMAB* and propionic acidemia due to deficiency of propionyl-CoA carboxylase, encoded by *PCCA* or *PCCB*. Data from NBS studies suggest these conditions should be classified as rare diseases however the exact prevalence of each disorder is unknown and complicated by genetic heterogeneity and the lack of a nationwide NBS registry. *Methods*: We first interrogated genome sequencing datasets, gnomAD v2.1.1 [125,000 exomes] and SVs 2.1 [15,000 genomes]. Variants in *MMUT, MMAB, PCCA* and *PCCB* were downloaded and pathogenic or likely pathogenic (P/LP) ClinVar variants were analyzed. Variants that were conflicting, of uncertain significance, or not annotated were further reviewed as well as those found in literature through the Human Genome Mutation Database (HGMD) Professional version 2022.3 to determine variant pathogenicity using expert curation informed by guidelines (PMID: 25741868). To augment classification of variants, we included additional disease-causing mutations present in participants

evaluated at the NIH Clinical Center (NCT00078078, NCT02890342). Gene carrier rate (GCR) was determined by the sum of the minor allele frequency (MAF) of all P/LP variants. Carrier frequency and predicted incidence were calculated using the Hardy-Weinberg equation. *Results*: For *MMUT*, 153 P/LP variants were identified with GCR of 0.0024, equating to an estimated carrier frequency of 1/209 and predicted incidence of 1/175,849. Variants were individually rare and missense changes accounted for about half. We identified 37 P/LP variants in *MMAB*, yielding a GCR of 0.0007. We calculated an estimated carrier frequency of 1/677 and prevalence of 1/1.8 million. The c.556G>T p.Arg186Trp, was the most common variant, consistent with observations that it accounts for ~30-40% of alleles in MMAB patients. For propionic acidemia, we identified 93 variants in *PCCA* and 85 variants in *PCCB* with similar GCRs of 0.0013 and calculated a combined prevalence of ~1/319,000. Most variants in *PCCB* were missense whereas 70% of variants in *PCCA* were loss of function. One commonly reported insertion/deletion variant in *PCCB* may be underestimated given poor alignment and incorrect annotation. *Conclusions*: To our knowledge, there is no official regulatory guidance on the use of genomic databases, which are massively expanding across the globe, to assist with population estimates of rare disease prevalence and incidence, which are required to report to the FDA during the development of rare disease therapeutics. Although genome-based analysis of the prevalence of *MMUT, MMAB, PCCA* and *PCCB* pathogenic variants represents a conservative estimate of disease allele frequency, the subsequent predicted birth incidence calculated using the Hardy-Weinberg equation closely agrees with the number of infants diagnosed with MMA and PA in a cohort of ~4.5 million babies who underwent NBS in California (PMID: 32778825). For example, we estimate a prevalence of *MMUT*-type MMA as ~1/175,849 while NBS data reports ~1/110,000. Extending this approach to other rare genetic disorders that lack data from newborn screening but have promising therapies under development is feasible. Whether genomic analysis may more widely be accepted by regulatory bodies as a primary data source to support orphan designation and ultrarare disease classification is supported by our analyses.

585. Precise Base Editing of Hyperlipidemia-Associated Genes by the Transformer Base Editor to Reduce Blood Lipid Levels

Lang Zhang[1], Lijie Wang[1], Peixue Li[1], Huanyu Li[1], Tao Wei[1], Jinyan Zuo[1], Yuchen Zhang[1], Jing He[1], Bingbing Wu[1], Jia Chen[2], Xiaodun Mou[1]

[1]CorrectSequence Therapeutics Co., Ltd, Shanghai, China,[2]ShanghaiTech University, Shanghai, China

Hyperlipidemia refers to abnormal elevated levels of low-density lipoprotein cholesterol (LDL-C), triglycerides and/or lipoprotein (a) in the blood, which is associated with increased risks of atherosclerotic cardiovascular diseases (ASCVDs). Gene editing of certain hyperlipidemia-associated targets provides a promising strategy for lifelong reduction of blood lipids. We have previously reported a novel transformer base editor (tBE), which was elaborately designed to eliminate both sgRNA-dependent and sgRNA-independent off-target editing. The tBE is a cytidine base editor which converts C•G to T•A pairs, and therefore, enables gene silencing by conversion of CAA (Gln), CAG (Gln), CGA (Arg), TGG (Trp) to stop codons. In this study, tBE was employed to knock out genes associated with lipid metabolism, including *PCSK9*, *Angptl3*, *ASGR1*, *ApoC3* and *LPA*. Different pairs of hsgRNA (helper single-guide RNA) and msgRNA (main single-guide RNA) were screened for each target gene in hepatic cell lines. The highest editing efficiency reached above 80% for all the target genes *in vitro*. Next, the candidate sgRNA pairs for each target gene were tested *in vivo*, with PCSK9 as an example here. The four components of tBE, including Locator (L) mRNA, Effector/Key (EK) mRNA, hsgRNA and msgRNA, were encapsulated into lipid nanoparticles (LNPs), and injected into mice via tail vein at a dose of 0.5-3.0 mg/kg (n = 5). Gene editing efficiency, plasma PCSK9 levels and LDL-C levels were determined 7 days after LNP administration. The whole hepatic *PCSK9* knockout efficiency increased in a dose-dependent manner, and reached a maximum of 57% as determined by next-generation sequencing (NGS). Compared with the vehicle group, the plasma PCSK9 protein levels were reduced by over 90%, while the LDL-C levels decreased by about 50%. No off-target editing was detected at the three potential off-target sites predicted by Cas-Offinder. Furthermore, *PCSK9* editing occurred specifically in the liver, while little editing was detected in other tissues. These results demonstrated the feasibility of hepatic delivery of tBE by LNP for efficient gene editing. Since certain patients showed a combined increase of LDL-C, triglycerides, or lipoprotein (a), gene editing of one single target may not be sufficient to eliminate all the risks of ASCVDs. We will evaluate multiplex editing of different hyperlipidemia-associated genes in future studies.

586. A New Class of Circulating Hepatic Biomarkers Derived from Aberrant Post-Translational Modifications in Isolated Methylmalonic Acidemia

PamelaSara Head[1], Lisa M. Jenkins[2], Tapan K. Maity[2], Charles P. Venditti[3]

[1]Metabolic Medicine Branch, NHGRI, National Institutes of Health, Bethesda, MD,[2]Mass Spectrometry Resource, Center for Cancer Research, NCI, National Institutes of Health, Bethesda, MD,[3]Metabolic Medicine Branch, NHGRI, NIH, Bethesda, MD

Background: Organic acidemias (OAs), such as methylmalonic acidemia (MMA), are a heterogeneous group of inborn errors of metabolism that largely arise from impaired catabolism of amino acids. OAs are difficult to treat and have multisystemic manifestations, leading to increased morbidity and mortality. Successful elective liver (LT), kidney (KT), and liver-kidney transplantation (LKT) can provide metabolic stability, but patients can still manifest significant metabolic perturbations, with massively elevated circulating methylmalonic acid levels despite total hepatic correction. Therefore, there is a need to define circulating biomarkers that might correlate with the extent of *in vivo* hepatic correction after liver directed mRNA, gene editing and/or AAV gene addition therapy. In previous work (PMCID: PMC10468269), we discovered that aberrant methylmalonylation characterizes the hepatic proteome in MMA mice and human patients, and therefore hypothesized that a differential pattern of modification(s) on a subset of circulating proteins would be present in MMA patients that have received a LKT compared to untransplanted patients or those that have received a KT. Methods: Affinity anti-methylmalonyllysine antibody columns were prepared

and used to enrich for methylmalonylated targets from liver, kidney, and brain extracts from *Mmut*$^{-/-}$;*TG*^{MCKMmut} mice, an animal model that recapitulates the hepatorenal cerebral syndromes of MMA. An expanded LC MS/MS discovery methymalonylome dataset containing >300 aberrantly acylated targets was generated and then subject to an *in silico* analysis to identify circulating proteins that could be routinely measured in a standard chemistry panel. Aspartate aminotransferase (AST) was nominated and studied after validation of methylmalonylation via immunoprecipitation (IP) from MMA mouse and human liver tissues. Enzyme activity of AST was also measured in MMA and control mouse livers. AST was then examined in plasma from *Mmut*$^{-/-}$;*TG*^{MCKMmut} mice, control *Mmut*$^{+/-}$;*TG*^{MCKMmut} mice, transplanted (LKT, KT) and untransplanted patients with MMUT type MMA, and healthy controls. Results: AST in *Mmut*$^{-/-}$;*TG*^{MCKMmut} mice and human patient liver tissues was heavily methylmalonylated as determined by LC MS/MS and by direct validation in liver extracts from mice and MMA patients. Despite an increased level of immunoreactive AST in MMA, enzymatic activity was not elevated in *Mmut*$^{-/-}$;*TG*^{MCKMmut} mice as compared to controls suggesting that methylmalonylation could inhibit AST activity. AST was methylmalonylated in plasma from *Mmut*$^{-/-}$;*TG*^{MCKMmut} mice but not *Mmut*$^{+/-}$;*TG*^{MCKMmut} controls. Patients with MMA also demonstrated methylmalonylation of plasma AST that was not seen in a healthy control. Methylmalonylation of plasma AST was absent in patients that received a LKT, but persisted after KT. Conclusions: Traditional biomarkers typically rely on protein abundance or measurement of enzymatic activity. This proof-of-concept study has identified a promising class of new biomarkers derived from a disease-associated aberrant PTM - methylmalonyllysine - which is readily detected on a routinely measured enzyme, AST. The complete correction of PTMs on AST in MMA patients who have received an LKT, but not KT, establishes the utility of directly measuring the PTM load as a dynamic hepatic biomarker to assay the *in vivo* response to liver-directed therapies in MMA. Extension of this approach to other IEMs, specifically organic acidemias and FOADs, is envisioned.

587. rAAV-Mediated Gene Therapy for Cytochrome C Oxidase 20 Deficiency

Peiyi Guo[1], NhatAnh B. Nguyen[1], TramAnh M. Nguyen[1], Morgan Mooney[1], Chuanjiang He[2], Guangping Gao[1,3,4], Dominic J. Gessler[1,4]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Department of Laboratory Medicine, The First People's Hospital of Kashgar, Kashgar, China,[3]Li Weibo Institute for Rare Diseases Research, UMass Chan Medical School, Worcester, MA,[4]Co-corresponding Authors, Worcester, MA

Cytochrome c oxidase 20 (COX20) deficiency is a rare autosomal recessive genetic disorder resulting from mutations in the *COX20* gene. The encoded protein is a chaperone stabilizing the COX2 subunit on the inner mitochondrial membrane and, thereby facilitates the assembling of complex IV and the proper function of the mitochondrial respiratory chain. Twenty-seven out of the 30 documented cases of this newly described disease have been reported within the past five years, suggesting the existence of a potentially larger patient population. Individuals with COX20 deficiency exhibit clinical features including sensory neuropathy, early onset ataxia, dysarthria, hypotonia, dystonia, and ophthalmoplegia. Despite the growing awareness of this disorder, an effective therapeutic intervention for COX20 deficiency has yet to be established. In this study, we developed a recombinant adeno-associated virus (rAAV)-based gene therapy for the treatment of COX20 deficiency using the ubiquitously active chicken beta-actin (CB) versus endogenous human COX20 promoter. In a proof-of-concept study rAAV9.CB-hCOX20 was delivered intravenously in neonatal wild-type mice expressing one of the two isoforms of the human *COX20* gene (201 and 203). The chaperone activity of COX20 relies on its mitochondrial localization. Therefore, we examined the subcellular localization of the hCOX20 using co-immunofluorescence staining, and observed the co-localized of hCOX20 with mitochondria markers in the brain, liver, and heart, supporting the proper function of the expressed hCOX20. To assess the safety of the rAAV-based gene therapy for COX20 deficiency, we monitor mouse weights and blood markers. Interestingly, we found that although treated wild-type mice show significant body weight loss compared to untreated mice, no pathologic findings were observed in these mice using complete blood count (CBC) and basic metabolic panel (BMP) blood tests, as well as multiple tissue H&E staining conducted at 6 months after treatment, indicating a well-tolerated and minimal toxic response to AAV9-COX20 vectors. With western blot, we found that endogenous COX20 protein levels remain consistently low in both mice and human HEK293 cells. In contrast, hCOX20 driven by CB promoter exhibits high expression in mouse tissues, including the liver, muscle, and heart, as well as in HEK293 cells. This heightened protein level may contribute to additional energy consumption, potentially leading to weight loss of rAAV9.CB-hCOX20 treated WT mice. To avoid the risk of unintended side effects resulting from excessive protein expression, we generated the second generation of AAV9-COX20 vectors driven by the human COX20 promoter and conducted examinations of protein expression and toxicity of these vectors in WT mice. There is currently no Cox20 mouse model available. To overcome this gap, we generated a COX20 mouse model mimicking the human compound heterozygous variant and a conditional COX20 knockout mouse model, which enables the ongoing evaluation of our newly developed rAAV vectors for COX20 deficiency. In summary, our study supports the safety of rAAV-based gene therapy for COX20 deficiency but also suggests that improved specificity with an endogenous promoter might be advantageous. Additionally, this work will provide valuable in vivo efficacy information for this therapeutic approach.

588. Design of the Prospective Study and the Consideration of the Clinical, Laboratory, and Dietary Determinates of Outcomes in Purine and Pyrimidine Metabolism Disorders

Shannon Haines[1], Oleg Shchelochkov[2], Katharine Chaillet[1]

[1]Purine and Pyrimidine Unit, NHGRI, Bethesda, MD,[2]Purine and Pyrimidine Unit, National Institutes of Health, Bethesda, MD

Introduction: Disorders of Pyrimidine and Purine Metabolism (DPPMs) is a group of inborn errors of metabolism characterized by a range of clinical presentations and significant unmet need. The overall prevalence of DPPMs in the general population is not known. The purine and pyrimidine metabolism pathways encompass >29 and >12 genes, respectively, which are involved in their synthesis,

transport, and degradation. Manifestations of DPPMs can affect any system including skeletal, neurological, immune, hematological, and renal. Some DPPMs can shorten life expectancy and lead to severe disabilities. With few exceptions, there are currently no effective gene-specific therapies. The lack of established diagnostic biomarkers, surrogate endpoints and poor understanding of the natural history slows progress in the development of new biologics for DPPMs. **Methods**: To help close knowledge gaps and to develop strategy for the discovery and validation of predictive biomarkers, we initiated a natural history study of DPPMs at the National Human Genome Research Institute aims. The study aims to improve our understanding of genomic, metabolomic, and environmental correlates underlying outcomes of DPPMs and biobank blood, urine, and stool samples (ClinicalTrials.gov ID NCT06092346). To optimize our recruitment strategy, design the clinical protocol, and to delineate prevalence of DPPMs, we reviewed the pattern of inheritance, the range and severity of DPPM symptoms in the published literature and reviewed variant databases. **Results**: All twelve of the pyrimidine disorders are known to be inherited in an autosomal recessive manner. In contrast, 3/29 (10%) of the purine disorders are inherited in an X-linked recessive pattern, and 2/29 (6%) purine disorders are inherited in an autosomal dominant pattern. The remainder of the purine disorders 23/29 (79%) show an autosomal recessive pattern. Among disorders of synthesis, transport and breakdown of pyrimidines, neurological manifestations are seen in 4/12 (33%) conditions, skeletal/dysmorphic features in 4/12 (33%), hemolytic anemia in 3/12 (25%), disorders of inflammation 2/12 (10%), renal manifestations in 1/12 (8%). Among disorders of synthesis, transport, and breakdown of purines, neurological manifestations are seen in 14/29 (48%) conditions, renal manifestation 8/29 (27%), disorders of inflammation 5/29 (17%), hemolytic anemia 5/29 (17%). Two purine disorders, AMPD1 and IMPDH1, are known to have isolated disorders outside of the typical spectrum (isolated rhabdomyolysis and isolated retinitis pigmentosa, respectively). **Conclusions**: We anticipate this study will garner information to expand our understanding of the phenotypic spectrum of DPPMs as well as identify novel diagnostic biomarkers. The long-term aim is to support future clinical trials and development genomics therapies for this group of rare of disorders.

589. Gene Replacement Therapy Targeting the Root Cause for the Treatment of MPS IIID in Mice

Pratikshya Adhikari[1], Neil Sud[1], Xinyu Cao[1], Tierra Bobo[2], Haiyan Fu[2]

[1]The University of North Carolina at Chapel Hill, Gene Therapy Center, Chapel Hill, NC,[2]Division of Genetics and Metabolism, Department of Pediatrics, The University of North Carolina at Chapel Hill, Gene Therapy Center, Chapel Hill, NC

Mucopolysaccharidosis (MPS) IIID is a devastating ultra-rare neuropathic lysosomal storage disease caused by autosomal recessive defects in N-acetylglucosamine-6-sulfatase (GNS), a lysosomal enzyme crucial for breaking down Heparan Sulfate (HS), a biologically important Glycosaminoglycan (GAG). The disease manifests in all organs with severe progressive neurological impairment, leading to high mortality and premature deaths. There are no treatments currently available for MPS IIID. To address the unmet needs, we developed a self-complementary (sc) AAV gene replacement vector MPS IIID, employing the trans-BBB-neurotropic AAV9 vector to deliver the human GNS (h*GNS*) cDNA via the systemic route. The scAAV-h*GNS* vector construct mediated efficient expression and secretion of rGNS when tested *in vitro* in human MPS IIID skin fibroblasts. The scAAV9-h*GNS* vector was further tested in preclinical studies in 1-2-month-old MPS IIID mice via systemic delivery. As a result, a single IV injection of scAAV9-hGNS at doses of 5e12 vg/kg - 5e13 vg/kg resulted in the rapid restoration of functional rGNS enzyme and clearance of lysosomal GAG storage in the CNS and peripheral tissues in MPS IIID mice at 1m pi. Long-term experiments are ongoing, and animals are scheduled for behavior testing at age 8m. Given the highly reproducible efficacy and safety profiles of systemic AAV9 gene delivery, our preliminary data support the therapeutic potential of scAAV9-h*GNS* for the treatment of MPS IIID.

590. Delivery of scAAV9.co*SLC6A8* for Restoration of Creatine Transporter Function in a Creatine Transporter Deficient Mouse Model: A Dosage Study

Chiara E. Sawilla[1], Troy B. Webster[1], Robyn T. Binsfeld[1], Ilona Tkachyova[2], Melissa Mitchell[3], Andreas Schulze[2,4], Jagdeep S. Walia[1,3]

[1]Queen's University, Kingston, ON, Canada,[2]The Hospital for Sick Children, Peter Gilgan Center for Research and Learning, Toronto, ON, Canada,[3]Department of Pediatrics, Queen's University, Kingston, ON, Canada,[4]Departments of Pediatrics and Biochemistry, University of Toronto, Toronto, ON, Canada

Creatine deficiency syndromes describes inborn errors of creatine metabolism, impairing affected individuals' synthesis and transport of creatine. One such disease is creatine transporter deficiency (CTD). CTD, an X-linked disorder, results from mutations in the *SLC6A8* gene and renders creatine transporters in affected patients dysfunctional. Under normal physiological conditions creatine is synthesized endogenously and released into the blood stream to provide cellular energy. Creatine uptake is reliant on Na^+/Cl^- dependent creatine transporters as they deliver the synthesized creatine against a large concentration gradient into cells. Mutations in *SLC6A8* present clinically as intellectual disability, developmental delays, behavioural abnormalities, and speech and motor impairments. There are currently no treatments for CTD; creatine supplementation has shown limited success in restoring creatine levels in the brain of those affected. Thus, therapies focus on behavioural and seizure management strategies. The delivery of a codon optimized *SLC6A8* transgene via self-complementary adeno-associated virus 9 (scAAV9.co*SLC6A8*) has been proposed as a treatment for CTD. Previously, delivery of *SLC6A8* transgene via intracerebroventricular injection at 1 day of age in a *Slc6a8*$^{Y/-}$ murine model showed increased creatine levels in the brain, a decrease in hyperactivity, and an increase in muscle strength. This study aims to investigate the dose-response of the scAAV9.co*SLC6A8* using intrathecal route in a *Slc6a8*$^{Y/-}$ murine model. 5 cohorts (n=6 mice) received either vehicle, low dose ($3.5e^{11}$ vg/mouse), medium dose ($7.5e^{11}$ vg/mouse), or high dose ($1.1e^{12}$ vg/mouse), as well as a daily administration of immunosuppression with rapamycin and prednisone beginning at 5 weeks of age and injections at 6 weeks of

age. Both short term (13 weeks) and long term (24 weeks) cohorts undergo grip strength testing at 13 weeks. Long term cohorts will also undergo blood collection at 13 weeks, hyperactivity analysis in a digitally ventilated cage at 22-24 weeks, and a final grip strength test at 24 weeks. At the endpoint- serum, peripheral organs, and central organs will be collected for biochemical analysis. Data will be shown for short-term and long-term analysis. Our hypothesis is that the medium and high dose cohorts will show greater levels of creatine in the central nervous system, greater grip strength, and less hyperactivity when compared to vehicle control groups. Results of this study will provide important information for a future clinical trial.

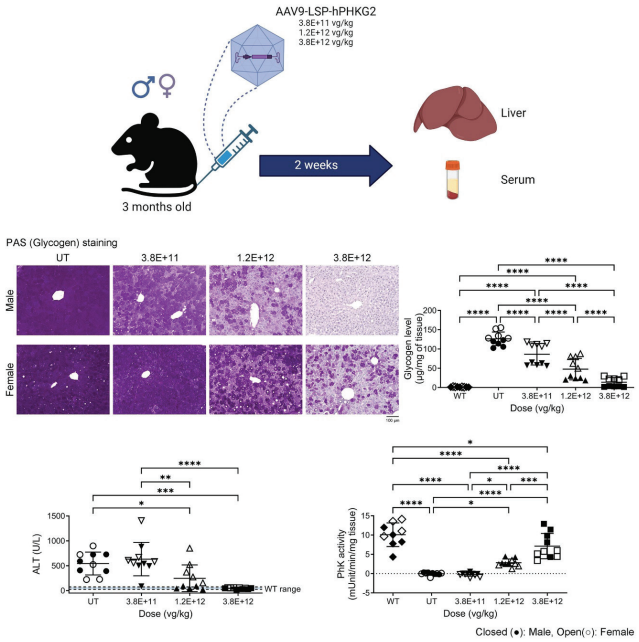
Wednesday Posters: Liver Genetic Diseases

591. Assessing the Minimum Effective Dose of a Clinical Candidate AAV Vector in a Mouse Model of Glycogen Storage Disease Type IXγ2

Jeong-A Lim[1], Rebecca L. Koch[1], Su Jin Choi[1], Andrea Pappas[2], Priya S. Kishnani[1]

[1]Duke University Medical Center, Durham, NC,[2]Kriya Therapeutics, Research Triangle Park, NC

Glycogen Storage Disease type IX (GSD IX) is caused by a deficiency in phosphorylase kinase (PhK), which is responsible for initiating the breakdown of glycogen into glucose. In the liver, this complex enzyme is composed of four subunits, with α2, β, and δ modulating the activity of the catalytic γ2 domain. Deficiency of PhK in the liver disrupts glycogenolysis, leading to glycogen accumulation and subsequent metabolic complications observed in individuals affected by hepatic PhK deficiency. Pathogenic variants in *PHKG2* encoding the γ2 subunit cause GSD IX γ2, constituting approximately 25% of liver GSD IX cases. Patients with GSD IX γ2 typically exhibit symptoms such as hepatomegaly, fasting ketotic hypoglycemia, hypertriglyceridemia, and elevated serum ALT and AST levels. More than 95% of GSD IX γ2 patients develop liver fibrosis, which can progress to cirrhosis, elevating the risk of liver failure and death. Our research group recently characterized the GSD IX γ2 mouse model (Phkg2 KO), revealing its striking resemblance to clinical manifestations observed in patients. This mouse model recapitulates key symptoms and pathophysiological features, making it a valuable tool for studying the disease mechanisms and potential therapeutic interventions. Moreover, in our previous studies, we evaluated the efficacy of AAV gene therapy for GSD IX γ2. For this purpose, we delivered murine Phkg2 (mPhkg2) and human PHKG2 (hPHKG2) under a liver-specific promoter (LSP). We systematically treated Phkg2 KO mice with AAV9-LSP-mPhkg2 or AAV9-LSP-hPHKG2 at various ages and treatment durations to establish proof of concept. Both the short- and long-term treatments successfully reversed the disease phenotypes in Phkg2 KO mice. In this study, we assessed the minimum effective dose of the clinical candidate, AAV9-LSP-hPHKG2, in the Phkg2 KO mice. Male and female Phkg2 KO mice received intravenous injections of AAV9-LSP-hPHKG2 at three different doses (3.8E+11, 1.2E+12, and 3.8E+12 vg/kg) and were evaluated 2 weeks later. Liver tissue and serum were collected for assessment of disease indicators. The study results revealed that male and female mice responded differently to varying doses of AAV treatment. At the lowest dose (3.8E+11 vg/kg), male mice exhibited minor effects, whereas female mice demonstrated no noticeable impact. Alternatively, male mice treated with a dose of 1.2E+12 vg/kg displayed improvements in disease phenotypes, while the effects in females were minimal. Notably, treatment with a dose of 3.8E+12 vg/kg produced significant improvement across disease indicators in both male and female mice. These improvements included decreased liver size, normalized expression of PhK subunits, increased PhK enzyme activity, decreased liver glycogen levels, and normalized serum ALT and AST levels. Collectively, our findings suggest that the minimum effective dose of AAV9-LSP-hPHKG2 capable of producing positive effects in both male and female Phkg2 KO mice is 3.8E+12 vg/kg. These findings have significant implications for the development of definitive treatments for patients with GSD IX γ2.



592. Exploration of Improvement Opportunities for Liver-Directed Gene Therapy in Glycogen Storage Disease 1a (GSD1a) through Protein Engineering and Sequence Optimisation

Robert Murren, Parisa Rahimnashat, Sophie Snow, Sid Sridharan, Erald Shehu, Su Kit Chew, Tihomir Dodev, Alex Dimokov, Nisha Nair Dastidar, Eliza Bujor, Sylvia Podda, Thangavelu Kaliyappan, Carlos Bueno Beti, Raquel Nunez Toldra, Dario Pilla, Mario Buono, Jaminder Khinder, Miriam Canavese, Rose Sheridan, Henning Stennicke, Eileen McNeill

Freeline Therapeutics, Stevenage, United Kingdom

Glycogen storage disease 1a (GSD1a) is a monogenic metabolic disease arising from the loss of functional glucose-6-phophatase protein (G6pase) expression in the liver. Lack of functional G6PC

leads to impaired gluconeogenesis leaving patients at risk of severe hypoglycaemia under fasting conditions. A successful gene therapy for GSD1a should offer widespread expression across liver hepatocytes as G6pase is an intracellular membrane protein. Current therapeutic efforts express wild-type G6Pase protein from a codon-optimised sequence driven by large portion of the endogenous promoter, placing the total transgene cassette outside the optimal transgene packaging size. In our approach, we sought to promote robust and regulated G6Pase expression, while reducing packaging size to 4kb. In addition to full sequence optimisation for optimal codon use with removal of CpGs and splice signals, we also carried out protein engineering to increase stability, thus increasing expression of G6Pase. Finally, we benchmarked basal and insulin-regulated expression of our novel promoter design using the proximal G6Pase gene promoter (2.86kb). Protein engineering approaches included homology and rational structure-based design. Compared to wild-type G6Pase, our novel protein variant showed a 2.5-fold expression improvement in liver cells. Screening of sequence-optimised variants yielded another 2.5-fold increase in expression compared to the natural human gene sequence. In addition, we also applied a bioinformatics approach to condense the endogenous promoter while retaining its activity. Candidate regulatory elements for G6PC were identified from epigenetic and transcriptional features in the wider G6PC genomic locus. These were combined with the core G6PC promoter to generate a condensed sequence of ~2.2kb. Our condensed promoter has similar basal expression to the large endogenous variant, and preliminary results indicate that it is sensitive to insulin treatment *in vitro*. Taken together, our AAV cassette engineering has yielded the optimised elements required for broad G6Pase expression in the liver of GSD1a patients, driven by an endogenous promoter within a 4.0kb transgene cassette.

593. Dissecting the Challenges of AAV-Mediated Liver Gene Transfer to Fibrotic Livers

Gemma Bruno[1], Rosa Ferriero[1], Agnese Padula[1], Iolanda Boffa[1], Marco Gargaro[2], Simone Pisano[1], Teresa Imperatore[1], Edoardo Nusco[1], Luigi Ferrante[1], Elena Polishchuk[1], Roman Polishchuk[1], Severo Campione[3], Nicola Brunetti-Pierri[1,4], Francesca Fallarino[2], Pasquale Piccolo[1]

[1]Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli, Italy,[2]Department of Medicine and Surgery, University of Perugia, Perugia, Italy,[3]Pathology Unit, "A. Cardarelli" Hospital, Naples, Italy,[4]Department of Translational Medicine, "Federico II" University of Naples, Naples, Italy

Liver fibrosis, characterized by the accumulation of scar tissue in response to various chronic insults, poses a significant challenge for liver-directed gene therapy. Patients with overt liver damage and fibrosis are currently excluded from gene therapy clinical trials based on the postulate that intact liver architecture is necessary for safe and efficient gene transfer to hepatocytes using adeno-associated viral vectors (AAV), currently the platform of choice for in vivo gene therapy. While preliminary evidence suggests a potential hindrance to AAV-mediated gene transfer in the presence of liver fibrosis, further investigations are needed. In this study, we explored the biodistribution and hepatocyte transduction of AAV-based vectors in the context of liver fibrosis. Analysis of three mouse models, representing induced and genetic liver fibrosis and characterized by different fibrosis patterns, revealed that fibrotic livers exhibit reduced transduction efficiency by AAV vectors. This reduction primarily arises from diminished vector uptake by the liver rather than vector genome loss due to hepatocyte proliferation. Additionally, liver fibrosis led to alterations in blood vector clearance and vector biodistribution in extra-hepatic organs, with a model-specific impact on increased viral particle uptake by the spleen, lung, and kidney. At the cell-type level, fibrosis resulted in decreased vector genome content in hepatocytes and increased content in non-parenchymal liver cells, splenic macrophages, and dendritic cells. Ongoing investigations are focused on understanding the implications of these changes on the innate and adaptive immune response against AAV. These findings underscore that liver fibrosis impedes AAV-mediated gene transfer to hepatocytes, emphasizing the need to overcome fibrotic barriers for the successful implementation of safe and effective gene therapy to fibrotic livers.

594. Use of an Oversized AAV8 Vector for CPS1 Deficiency Results in Long-Term Survival and Ammonia Control

Wesley Zhou[1], T. Diep[1], M. Nitzahn[1], I. Day[1], P. Patel[1], G. Makris[2], L. Lueptow[3], I. Zhuravka[3], J. Gangoiti[4], H. Padaon[4], B. Barshop[4], J. Haberle[2], Gerald S. Lipshutz[1]

[1]Surgery, Pharmacology, UCLA, Los Angeles, CA,[2]Metabolism, Univ Children's Hospital Zurich, Zurich, Switzerland,[3]Psychology, UCLA, Los Angeles, CA,[4]Metabolism, UCSD, San Diego, CA

Background: Carbamoyl phosphate synthetase 1 (CPS1) is the 1st enzyme in the urea cycle (UC), utilizing ATP to catalyze the reaction of bicarbonate & ammonia to carbamoyl phosphate, the 1st intermediate in the UC. CPS1 deficiency, a UC disorder, defined as the partial or total loss of the CPS1 enzyme, reduces ureagenesis & alters nitrogen metabolism, leading to hyperammonemia, initiating a sequence of adverse events that can lead to coma & death if not intervened upon rapidly. The development of an AAV-based gene approach for CPS1 deficiency is challenged by the large cDNA size (4.5 kb) & high protein requirement (~20% of mitochondrial matrix). Methods: We utilized an AAV 8 vector as a gene therapy approach to treat CPS1 deficiency. In order to constrain the AAV genome size, we used a small active liver specific promoter (LSP) & enhancer with a minimal synthetic polyA; this resulted in an ITR-to-ITR genome size of 5.3 kb. Using a murine conditional knockout model of Cps1, AAV-CPS1 was injected IV into 2-month- old male & female mice; controls received a null vector. Starting on day 14, n-carglumic acid & dextrose were added to their water along with carbohydrate to their cages; no nitrogen scavengers though were given. Blood was sampled monthly for amino acid profiling & $NH4^+$ determination. Surviving animals underwent behavioral testing at 8 months & $NH4^+$ challenging at 9 months after AAV-CPS1 administration. Ureagenesis was measured by ^{13}C-acetate administration. Results: Alkaline gel electrophoresis of the oversized AAV-vector demonstrated a dominant band at ~5.2 kb with some evidence of fragmentary genome packaging. Long-term survival (9 months, end of study) with $NH4^+$ control was achieved in AAV-CPS1 administered floxed Cps1 mice while null vector-injected controls all died by day 36. When compared with untreated knockouts, treated females demonstrated improved survival ($p < 0.0001$) over treated males ($p = 0.0020$). While plasma glutamine was elevated compared to wild type controls in both sexes, plasma $NH4^+$ was well-controlled

in surviving animals. Mice maintained their body weights & were not sarcopenic. Male mice (not females) exhibited a significant weight difference from controls; at necropsy this was revealed as lack of obesity in treated Cps1 male mice where controls had intra-abdominal adiposity that was often abundant. To test the physiologic response to exogenous $NH4^+$, mice were challenged with 3mM/kg ammonium chloride. In general, wild type mice had a controlled response (i.e., plasma $NH4^+$ peak at the 20-minute time point with subsequent decline) while AAV-treated mice had an exaggerated response at 20 minutes followed by declining plasma ammonia at 60 minutes; no seizures occurred. Behavioral phenotyping of treated Cps1-deficient mice demonstrated no statistically significant differences compared with controls. Plasma transaminases between AAV-CPS1 treated mice & controls exhibited no statistically significant differences between the two groups. At necropsy, livers appeared normal without evidence of fibrosis or malignancy. Conclusion: The development of an oversized AAV serotype 8-vector expressing CPS1 can be achieved & is functional. While the dominant genome size present is ~5.2 kb with this single vector, there is some evidence of vector fragmentation. Nevertheless, administration to a Cps1-deficient murine model results in long-term survival, $NH4^+$ control, normal behavior, & an appropriate response to an exogenous ammonia challenge. These findings demonstrate the utility of a gene therapy approach for a very challenging urea cycle disorder where high-level hepatic protein is necessary for survival.

595. Restoration of Ornithine Transcarbamylase Function with Endless RNA (eRNA)

Inbal Rahamin, James Sefton, Alexander Garruss, Jack Kwon, Richard Wilson, Ki Young Paek, Jason Wade, Russell Miller

Sail Biomedicines, Somerville, MA

Ornithine transcarbamylase (OTC) is an essential hepatic enzyme in the urea cycle, a metabolic pathway crucial for detoxifying ammonia in the liver. Patients with a mutation in the OTC gene causing deficiency in OTC enzymatic activity can present with an inability to produce urea and hyperammonemia, resulting in neurological issues and even death. Genetic approaches to restore hepatic OTC protein and enzymatic activity with AAV-based OTC gene delivery and exogenous OTC mRNA delivery are being developed for therapeutic treatment of OTC-deficient urea cycle disorders. mRNA-based protein replacement therapy has the potential to address the cause of disease but is hampered by several challenges, including the short half-life of exogenously administered mRNA and subsequent protein production. Endless RNA™ (eRNA™) has emerged as a promising platform due to its unique covalently closed, circular structure, which confers resistance to exonuclease degradation, and an extended half-life as compared with traditional mRNA. In this study, we demonstrate that eRNA-expressing human OTC (hOTC) with optimized lipid nanoparticles (LNPs) can be used to treat OTC deficiency successfully in mice, thereby restoring the impaired urea cycle. To select eRNA sequences yielding maximal OTC protein expression, we conducted comprehensive screens optimizing coding and non-coding sequence elements. We identified novel combinations of optimized open reading frames (ORFs) and diverse untranslated regions (UTRs), which resulted in significantly higher OTC expression levels compared to both endogenous protein and control mRNA. These optimized eRNAs were delivered intravenously with LNPs to OTC-deficient Spf^{ash} mice and resulted in a lowering of urinary orotic acid to wild-type animal levels, consistent with a rescue of the OTC deficiency. Collectively, our findings present a promising new therapeutic approach for OTC deficiency, demonstrating the potential of hOTC eRNA as an effective strategy to restore urea cycle function.

596. Using a Semi-Mechanistic Model to Predict Efficacious Dosing Regimens and Optimal Drug Design Properties for a LNP-Delivered mRNA UGT1A1 Replacement Therapy in Crigler-Najjar Syndrome Type 1 Patients

Jamie Nosbisch, Marc Presler, Saheli Sarkar, David Bassen, Carter Johnson, Diana Marcantonio, Joshua Apgar, David Flowers

Applied BioMath, LLC, Concord, MA

PURPOSE: Crigler-Najjar syndrome type 1 (CN1) is an autosomal recessive disease where bilirubin accumulates to toxic levels in the blood as a result of the reduced enzymatic activity of glucuronosyltransferase family 1 member A1 (UGT1A1). With no approved drugs for CN1, patients are often treated with blue light, but as they age, this is insufficient to control bilirubin levels. While liver transplants can be used to restore UGT1A1 activity, an alternative approach considers using gene therapy to restore UGT1A1 activity by delivery of mRNA encoding UGT1A1 protein via lipid nanoparticle (LNP). Utilizing a semi-mechanistic model, we explored the feasibility of dose amounts and frequencies that could sustain reductions in bilirubin levels over time and what properties could be optimized. METHODS: A semi-mechanistic model was developed to describe an mRNA therapy delivered to the liver via a LNP. The model includes LNP delivery to the liver, endosomal escape of the mRNA, translation of the target protein UGT1A1, and UGT1A1 catalysis of bilirubin to bilirubin monoglucuronide. To illustrate the usage of the model for a feasibility assessment, no parameters were derived from clinical or preclinical studies, but rather from the literature. RESULTS: Model simulations predict that prohibitively large doses are required to maintain the bilirubin reduction target of 25% of baseline over time. The model predicts that doses larger than 0.07 mg/kg achieve sustained reduction in bilirubin concentration at Q1W dosing frequency; however, increasing the dosing interval requires increasing doses, and at Q3W doses need to be prohibitively high to maintain desired bilirubin levels throughout the dosing period. This is because the mRNA is cleared at an exponential rate inside the cells, creating a nonlinear relationship between dosing frequency and sustaining the reduction. To understand requirements to the desired bilirubin reduction at 0.5 mg/kg dosed Q3W, 3 drug parameters were scanned over a realistic range. The efficiency of mRNA escape from endosomes is known to be a major bottleneck in intracellular drug delivery. The model predicts that optimizing endosomal escape efficiency may provide some benefit, but not enough to attain the feasibility criteria at 0.5 mg/kg dosed Q3W. Similarly, optimizing translation does not result in enough benefit to reach feasibility. The stability of the mRNA, on the other hand,is a key design variable that impacts the feasibility for

UGT1A1 replacement and its pharmacology. The model predicts that as the mRNA half life increases, the dose required for a given amount of bilirubin reduction is reduced substantially. With an mRNA half life of 30 hours, the drug achieves 25% reduction at all times at 0.5 mg/kg. Increasing the half life to 40 hours reduces the required dose five-fold to 0.1 mg/kg. CONCLUSIONS: A semi-mechanistic model was developed and parameterized to predict efficacious dose and dosing intervals and to help prioritize optimization of liver-targeted mRNA enzyme design to treat CN1. Using this model, we determined that it is feasible for liver UGT1A1 to restrict bilirubin to non-toxic levels at frequent (Q1W-Q2W) dosing intervals, but dose amounts would be prohibitively high at less frequent intervals (Q3W). With further model analysis, we identified UGT1A1 mRNA stability as the primary design variable that may be modified to enable a longer dosing interval at reasonable dose amounts. This case study highlights how mechanistic modeling can be used for making early feasibility assessments for novel gene therapy treatments and how modeling can help inform the drug design targets for optimal efficacy of these therapeutics.

597. RNA Gene Writers Drive Therapeutically Relevant Levels of Correction of Monogenic Disease Mutations Expressed in the Liver

Anne Bothmer, Luciano Apponi, Xiaolong Dong, Zsanett Jancso, Raed R. Ibraheim, Mugdha Bedekar, Annie Chivatakarn, Alan Mendoza, Florence Caro, Daniella Lu, Selina Sun, Elizabeth English, Rahul Nair, Gulen Tonga, Erin Spofford, Carol Cai, Jeff An, William Querbes, Cecilia Cotta-Ramusino, Michael C. Holmes

Tessera Therapeutics, Somerville, MA

Monogenic diseases are caused by mutations in a single gene, and although most monogenic diseases are rare, together they affect millions worldwide and result in high morbidity, mortality, and economic burden. RNA Gene Writers are designed to introduce a broad range of alterations to the genome, from writing in a transgene, inserting an exon, or introducing single nucleotide changes, providing an attractive toolkit for the treatment of monogenic diseases. RNA Gene Writers are inspired by the biology of non-LTR retrotransposons, a class of mobile genetic elements that can edit the genome through target-primed reverse transcription, or TPRT. RNA Gene Writers are designed to be delivered as all-RNA composition, which is intended to facilitate *in vivo* delivery by lipid nanoparticles, or LNP. Here we explored the application of RNA Gene Writers to correct mutations leading to various monogenic diseases expressed in the liver, including classic phenylketonuria (cPKU), alpha-1 antitrypsin deficiency (AATD), and Wilson's disease (WD), at therapeutically relevant levels *in vivo*. We developed an RNA Gene Writer designed to target the R408W mutation in the *PAH* gene causing cPKU and observed an average of 47% rewriting efficiency after a single dose in the liver of a novel humanized PKU R408W mouse model. We observed this level of correction led to normalized blood Phe levels. We also developed an RNA Gene Writer intended to correct the PiZ allele of *SERPINA1* carrying the E342K mutation that causes AATD. We observed LNP-RNA delivery of an RNA Gene Writer corrected this mutation in an existing high copy transgenic mouse model (*NSG-PiZ*). Separately, we engineered a humanized mouse model where the PiZ allele was inserted into the Rosa26 locus to better approximate humans with the homozygous PiZZ genotype. LNP-RNA delivery of an RNA Gene Writer achieved an average of 47% correction after a single dose in this diploid mouse model, and a corresponding increase in serum AAT was observed. We next developed an RNA Gene Writer designed to correct the H1069Q mutation in the *ATP7B* gene leading to WD. We engineered a humanized mouse model containing the human H1069Q mutation with its flanking sequence in the analogous mouse *Atp7b* gene. Through LNP-RNA delivery of an RNA Gene Writer, we achieved dose dependent levels of correction of the H1069Q mutation and observed a substantial reduction in copper accumulation at the highest dose tested. Taken together, we demonstrated that our RNA Gene Writing platform can be applied to efficiently correct disease-causing mutations expressed in the liver at therapeutically relevant doses with a single administration of LNP formulated RNAs. Through optimization of the template and RNA Gene Writers, we observed efficient editing *in vivo* across three different monogenic disease targets, supporting our belief that the potential therapeutic applications of our RNA Gene Writers may be broad and wide-ranging.

598. Expressing AAT from Circular RNA-Encoding Vectors as a Promising Gene Therapy Approach for Alpha-1 Antitrypsin Deficiency

Eoghan T. O'Leary[1,2], Sara Vikberg[1,2], Juan Zhang[1,2], Daisy Hjelmqvist[1,2], Erik D. Wiklund[1], Victor Levitsky[1], Thomas B. Hansen[1]

[1]RnD, Circio AB, Huddinge, Sweden,[2]Clintec, Karolinska Institutet, Huddinge, Sweden

Alpha-1 antitrypsin deficiency (AATD) is a genetic disorder that manifests in the lung and liver. The deficiency is driven by pathogenic mutations in the SERPINA1 gene which typically impairs production of the protein in the liver reducing its serum levels and leading to lung emphysema. Despite greater than 100 pathogenic variants of SERPINA1 being described, the most common mutations known to cause AAT deficiency are the Z (Glu342Lys) and S (Glu264Val) variants. Moreover, 95% of individuals with severe AAT deficiency are homozygous for the Z allele mutation. Z-AAT proteins are prone to aggregate and accumulate in hepatocytes, resulting in liver cirrhosis through a toxic "gain of function" mechanism. Except for liver transplantation, there are no curative therapies available for alpha-1 antitrypsin deficiency, and current treatment options are focused on reducing the disease progression. Several gene therapies are in development that aim to elevate the serum alpha-1 antitrypsin level and prevent the progression of lung emphysema, while other approaches aim to deplete the accumulation of toxic Z-AAT in the liver. Circio is developing an alternative therapeutic technology based on our proprietary vectorized circular RNA (circRNA) expression platform, circVec. CircRNA is known to have superior intra-cellular stability compared to mRNA due to resistance to exo-nucleolytic decay, and is rapidly emerging as a promising novel therapeutic modality for disorders where persistent transgene expression is required, such as AATD. Here, we demonstrate that AAT expression from our circVec vector system achieves superior accumulation and out-competes conventional mRNA expression in terms of durability and absolute protein expression over time. Additionally, we demonstrate that our

circVec expression cassette is vector agnostic, facilitating expression from both viral and non-viral DNA-based vectors. Furthermore, we have developed a remove-and-replace system in which the mutant ZZ allele is specifically targeted and significantly depleted using a primary miRNA-like element (PME) while circVec-encoded wild-type AAT remains unaffected in vitro. Based on these results, we propose circVec vector-based circRNA expression as an alternative gene therapy technology for the treatment of AATD. The potential of combining long-term therapeutic protein expression with a mutant-specific depletion element enables the combined treatment of both lung and liver associated AATD pathologies, which has not previously been achieved with conventional expression systems. We foresee deployment of circular RNA-based platforms in the treatment of a variety genetic diseases, and expect that circular RNA will replace mRNA as the expression platform of choice for vector-based therapies.

599. An Improved AAV Vector with High Expression Efficiency for HAE Treatment

Yong Wang, Zhou Yang, Fenglan Wu, Tenghui Yu
Gritgen Therapeutics Co., Ltd., Suzhou, China

Background: Hereditary angioedema (HAE) is a rare autosomal dominant disorder most commonly caused by pathogenetic variants in the SERPING1 gene, resulting in reduced C1-INH function in plasma. HAE is typically characterized by reoccurring swelling in the limbs, the face, the airways, and the intestinal tract, among which upper airway angioedema (UAE) has a lifetime mortality rate up to 40%. Pharmacological management of HAE consists of on-demand treatment aiming to lessen both the severity and duration of symptoms during an HAE attack and prophylaxis to prevent HEA attacks. While different drugs have been approved for prophylaxis, they all require routine administration. C1-INH is expressed mainly in hepatocytes and secreted into blood and thus treatable by restoration of stable C1-INH function through AAV-mediated liver targeted SERPING1 gene transfer. So far there is only one AAV-mediated HAE therapy in clinical trial at a relatively high dose. we aim to develop an AAV vector for HAE treatment at a lower dose. Results: After multiple rounds of screening in different species, we have developed GS1196, an AAV8-based vector expressing human C1-INH for HAE treatment. In mouse models, supraphysiological activity of plasma C1-INH was detected after a single intravenous (IV) injection of GS1196 at a dose of 3e12vg/kg and maintained for at least 28 weeks. We observed a dose and sex dependent effect in mice. GS1196 was specifically distributed and expressed in liver. In NHP, single IV injections of GS1196 at a dose of 1e13vg/kg could produce C1-INH activity at therapeutic concentrations. Toxicological studies indicated that GS1196 was safe and well-tolerated in both species. Conclusion: Our data have shown efficient expression of GS1196 in mice and NHP. Preclinical studies have demonstrated the safety of GS1196 and provided support for its clinical studies.

600. Correction of the Human Wilson's Disease Mutation in an Engineered Disease Mouse Model Using RNA Gene Writers

Raed Ibraheim, Mugdha Bedekar, Luciano Apponi, Elizabeth English, Selina Sun, Mae Caldwell, Carol Zuhua Cai, Gang Sun, Ana Maria Jaimes Castillo, Anne Bothmer, William Querbes, Cecilia Cotta-Ramusino, Michael C. Holmes
Tessera Therapeutics, Somerville, MA

Wilson's Disease (WD) is an autosomal recessive disease characterized by the inability to excrete excess copper due to an inherited mutation in the transmembrane copper-transporting ATPase 2, ATP7B. The predominant WD mutation in North America and Europe is the missense (c.3207C>A) mutation, with a histidine to glutamine amino acid change at position 1069. We estimate that there are approximately 12,000 WD patients in the United States and Europe carrying the H1069Q mutation. Homozygotes or compound heterozygous carriers of this mutation experience ATP7B protein misfolding, abnormal phosphorylation, decreased ATP-binding affinity and abnormal localization to the trans-Golgi network. In WD, the proper transport of copper to the other body parts through ceruloplasmin and the trafficking of excess copper to the bile are dysregulated. This results in toxic accumulation of copper in the liver, brain, and other organs, leading to progressive liver disease and neuropsychiatric symptoms. The disease is primarily managed by copper chelators and/or zinc salts to promote copper excretion in the urine and reduce copper absorption from diet, respectively. Multiple barriers to compliance and adherence exist, including significant side effects, cumbersome dosing schedules, high pill burden, and the requirement of life-long therapy. RNA Gene Writers are inspired by the biology of naturally occurring non-LTR retrotransposons, a class of mobile genetic elements that can edit the genome through target-primed reverse transcription, or TPRT. We have engineered non-LTR retrotransposon proteins that are designed to introduce a broad range of edits to the genome, from writing in a transgene, inserting an exon, or introducing single nucleotide changes. We believe RNA Gene Writers could potentially be applied to WD to restore the H1069Q mutation to wild-type in the *ATP7B* gene. We deployed a high throughput screening approach to identify and engineer an RNA Gene Writer designed to correct the H1069Q mutation in model cell lines *in vitro*. We then developed a new mouse model engineered to contain the human H1069Q mutation with its flanking sequence in the analogous mouse *Atp7b* gene. We observed that this model recapitulated the phenotype of WD, such as elevated copper accumulation in the liver and brain, and increased levels of copper detected in the urine. We tested an RNA Gene Writer formulated in lipid nanoparticle, or LNP, *in vivo* in this humanized mouse model to evaluate correction levels of the H1069Q mutation as well as levels of hepatic copper and urinary copper compared to untreated animals. In summary, we developed a humanized H1069Q murine model of WD and were able to demonstrate correction of this mutation through the *in vivo* delivery of an LNP-RNA.

Wednesday Posters: Heart, Lung, and Kidney Diseases

601. Enhanced Therapeutic Efficacy of Embryonic Stem Cell-Derived Macrophages with Heterozygous Deficiency in PHD2 for the Treatment of Murine Hindlimb Ischemia

Sang W. Han[1], Patricia Alves[2], Sayone Moura[1], Helen Tayor[3], Antonella Fidanza[3], Cristiane Gil[1], Leonardo Martins[4], Alex Nasare[1], Andre Lengert[1], Bianca Garcia[1], Gabriel Guarache[1], Gustavo Pereira[1], Ivan Koh[1], Lesley Forrester[3]

[1]Federal University of Sao Paulo, Sao Paulo, Brazil,[2]University Federal of Sao Paulo, Sao Paulo, Brazil,[3]University of Edinburgh, Edinburgh, United Kingdom,[4]Institute of Medical Biology of Polish Academy of Science, Łódź, Poland

Background: Macrophages are pivotal immune modulators capable of reshaping the ischemic microenvironment by releasing factors that enhance arteriogenesis and facilitate tissue repair. Prolyl hydroxylase domain-containing protein 2 (PHD2) functions as a critical oxygen sensor and regulates hypoxia-inducible factors (HIFs), which are central transcription factors that adapt to decreased oxygen availability within the cellular environment. Previous studies on Phd2+/- mice have indicated that modulation of this pathway may offer therapeutic benefits in treating ischemia. However, studies for the translation of this approach to patient-specific treatments has been hampered by the difficulty of acquiring Phd2 haplodeficient macrophages. This study aimed to overcome such barriers by producing Phd2+/- macrophages from E14IV mouse embryonic stem cells (ESCs) modified to acquire the Phd2+/- genotype and evaluating their therapeutic value in a mouse model of hindlimb ischemia.Methods: The precision of CRISPR-Cas9 genome editing was utilized to establish a mouse embryonic stem cell line with a heterozygous deficiency in Phd2 (E14IV+/-). These E14IV+/- cells were then differentiated into macrophages, designated as C22-E14IV-MØ. Detailed phenotypic characterization was conducted using flow cytometry and RT-qPCR to ascertain cellular marker expression. Functional analyses were carried out, including Western blotting for PHD2 levels, phagocytosis assays, and angiogenesis evaluation through immunoassays and the HUVEC tube formation assay. The interaction between these macrophages and myoblasts was investigated using C2C12 cells. *In vivo* assessment of macrophage therapeutic efficacy was carried out in a murine model of hindlimb ischemia, with perfusion and tissue recovery measured via laser Doppler and histological evaluations.Results: The study revealed that C22-E14IV-MØ macrophages expressed more than a 50% reduction in PHD2 levels and showed an upregulation of specific cell surface markers indicative of an M0-like phenotype, such as CD206 and MHCII. These cells exhibited superior phagocytic function compared to wild-type counterparts. *In vitro*, the macrophages demonstrated a unique angiogenic profile and significantly enhanced capillary structure complexity in cocultured HUVECs. Additionally, the conditioned medium from M2-polarized C22-E14IV-MØ macrophages substantially increased proliferation rates in C2C12 myoblasts compared to control media. *In vivo*, the application of these macrophages into ischemic limbs led to observable improvements in blood circulation, indicated by increased perfusion and the formation of larger arterioles, as documented by laser Doppler and histological studies (Figure 1).Conclusion: The study presents strong evidence of the therapeutic potential of Phd2+/-MØ macrophages for the treatment of limb ischemia. The macrophages derived from Phd2+/- ESCs not only exhibit enhanced functional capabilities *in vitro* but also translate into significant therapeutic benefits *in vivo*.

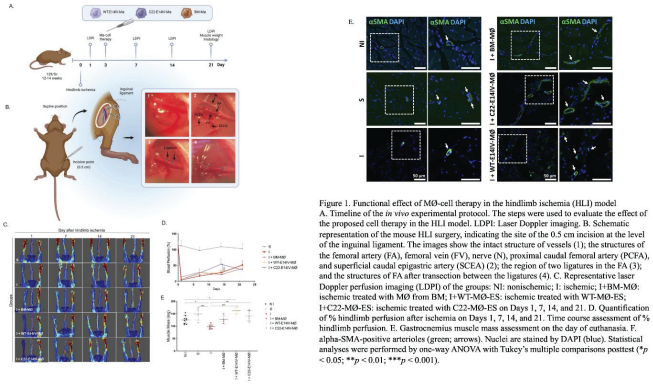


Figure 1. Functional effect of MØ-cell therapy in the hindlimb ischemia (HLI) model A. Timeline of the *in vivo* experimental protocol. The steps were used to evaluate the effect of the proposed cell therapy in the HLI model. LDPI: Laser Doppler imaging. B. Schematic representation of the mouse HLI surgery, indicating the site of the 0.5 cm incision at the level of the inguinal ligament. The images show the intact structure of vessels (1); the structures of the femoral artery (FA), femoral vein (FV), nerve (N), proximal caudal femoral artery (PCFA), and superficial caudal epigastric artery (SCEA) (2); the region of two ligatures in the FA (3); and the structures of FA after transection between the ligatures (4). C. Representative laser Doppler perfusion imaging (LDPI) of the groups: NI: nonischemic; I: ischemic; I+BM-MØ: ischemic treated with MØ from BM; I+WT-MØ-ES: ischemic treated with WT-MØ-ES; I+C22-MØ-ES: ischemic treated with C22-MØ-ES on Days 1, 7, 14, and 21. D. Quantification of % hindlimb perfusion after ischemia on Days 1, 7, 14, and 21. Time course assessment of % hindlimb perfusion. E. Gastrocnemius muscle mass assessment on the day of euthanasia. F. alpha-SMA-positive arterioles (green; arrows). Nuclei are stained by DAPI (blue). Statistical analyses were performed by one-way ANOVA with Tukey's multiple comparisons posttest (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

602. Exosome-Targeted MiR-33a Antagomir is Present in Vascular Medial Cells After *In Vivo* Endothelial Transduction

Goren Saenz De Pipaon Echarren, Brad Wacker, Lianxiang Bi, David A. Dichek

University of Washington, Seattle, WA

Introduction: Atherosclerosis is driven by cholesterol accumulation in cells below the luminal arterial endothelium (i.e., macrophages and smooth muscle cells). MiR-33a is a micro-RNA that downregulates mRNA encoding the cholesterol transporter ATP-binding cassette subfamily A, member 1 (ABCA1; a key mediator of cholesterol efflux from artery wall cells). Antagomir-mediated inhibition of miR-33a can de-repress ABCA1 expression and increase cholesterol efflux in vitro and should reduce vessel wall cholesterol and prevent atherosclerosis in vivo. However, delivery of antagomir-expressing vectors to cells below the luminal endothelial layer is challenging. Here we test whether in vivo transduction of rabbit carotid artery endothelium with vectors expressing exosome-targeted miR-33a antagomirs can deliver the antagomirs to cells below the luminal endothelium. **Methods:** We transduced rabbit carotid endothelial cells in vivo with helper-dependent adenoviral vectors (HDAd) encoding miR-33a-directed antagomirs. The vectors expressed antagomirs either with (n=2 arteries) or without (n=2 arteries) the "X-motif" (an oligonucleotide sequence that directs small RNA preferentially to exosomes). Contralateral carotids in the same rabbits (n=4) received HDAd that express scrambled oligonucleotides (HDAdScr). 3 days after transduction, we used RT-qPCR to measure levels of antagomirs, miR-33a, and ABCA1 mRNA in extracts of FACS-sorted carotid endothelial cells (CD45-CD31+) and non-immune medial/adventitial cells (CD45- CD31-). We also measured levels of antagomirs, miR33a, and ABCA1 mRNA in extracts of laser-microdissected carotid endothelium and media and we measured miR-33a in arterial sections using fluorescence in-situ hybridization (miRNA-scope). **Results:** We detected miR-

33a antagomirs—both with and without the X-motif—in carotid endothelial cells and in medial/adventitial cells sorted from HDAd-anti-miR33a-transduced—but not HDAdScr-transduced—carotids. Analysis of laser-microdissected samples confirmed the presence of antagomirs in both the intimal and medial cells of all HDAd-anti-miR33a-transduced carotids. No antagomir-related changes in miR-33a or ABCA1 expression were detected. **Conclusions:** These preliminary results indicate that HDAd-mediated in vivo transduction of carotid endothelium reliably achieves accumulation of miR-33a antagomirs within both endothelial and sub-endothelial cells. In the coming weeks we will complete this study by increasing "n" (to 11 for both of the antagomir-expressing HDAds; to 22 for HDAdScr). We will measure vector DNA in endothelium and subendothelium to test our assumption that transduction is largely limited to endothelial cells. These new data will also allow us to test our hypotheses that addition of the X-motif promotes antagomir accumulation in vascular media and that downregulation of miR33a and upregulation of ABCA1 in cells below the endothelium can be achieved via HDAd transduction of endothelium.

603. Engineering the CRISPR-Cas13 System for Efficient PHD2 Enzyme Downregulation to Enhance *In Vitro* Angiogenesis

Sara Carolina Vieira Terra

Department of Physiological Sciences, Santa Casa School of Medical Sciences of São Paulo, São Paulo, Brazil

Peripheral Arterial Disease (PAD) arises from the stenosis of blood vessels in the upper or lower extremities. Hypoxia-inducible factor (HIF) plays a pivotal role in the stimulation of angiogenesis, constituting a critical component of cellular response to hypoxia. HIF-1α is degraded by the PHD2 enzyme. The CRISPR-Cas13d system offers a compelling avenue for gene editing. Consequently, we propose the utilization of CRISPR-Cas13d to downregulate PHD2, fostering angiogenesis through HIF-1α augmentation in endothelial cells (HUVEC) under hypoxia (H) and normoxia (N). Firstly, guide RNA (gRNA) design was performed, targeting the EGLN1 gene (encoding PHD2 enzyme). Subsequently, cloning of guide PHD2-KI (knock-in) and guide NTG (non-targeting genome) was carried out, followed by lentivirus production and endothelial cell transduction (HUVEC PHD2-KI and HUVEC NTG). Finally, a hypoxia assay, cell proliferation by MTT, and sprouting assay were conducted.The integration of the Cas13d cassette and its gRNA (NTG or PHD2-KI) was confirmed through RT-qPCR, with Cas13d expression observed in NTG and PHD2-KI groups under N and H. EGLN1 gene knock-in was confirmed by RT-qPCR, showing a 37.5% (N) and 34% (H) reduction in its expression in the PHD2-KI group, compared to the control (CTL) group.The Hypoxia Assay, followed by RT-qPCR, revealed in the PHD2-KI group a 7x (N) and 5.4x (H) increase in VEGFA; a 7.5x (N) and 30x (H) reduction in HIF-1α; and a 3.3x (N) and 4.4x (H) increase in MMP2 expression, all compared to CTL. VEGF ELISA showed a 4x (24h) and 5x (48h) higher expression in the PHD2-KI group, compared to CTL, in both N and H. TNF-α ELISA revealed 26x lower expression in the PHD2-KI group at 24h, in N and H, and no expression in the PHD2-KI group in both conditions at 48h. HIF-1α ELISA for showed a 280% increase in its expression in group PHD2-KI, in N, 24h, compared to CTL. Cell proliferation assessed by MTT over 5 days showed that, starting with 2000 cells, the PHD2-KI lineage exhibited an increase in proliferation on day 4, ending with 1.7x higher absorbance compared to CTL. Finally, to verify whether PHD2 downregulation would lead to an increased angiogenesis, a sprouting assay was performed. Under N, the PHD2 group, compared to CTL, showed approximately 2.4x, 3.4x, and 3.9x more sprouts per bead, respectively, in EGM-All, EGM without VEGF, and EGM SFB medias. Under H, the response was more pronounced, with 4.6x, 5.5x, and 2.1x more sprouts per bead in the PHD2-KI group, in the same medias, respectively. Regarding the length of the sprouts, the PHD2, compared to CTL, exhibited 1.4x and 1.6x longer sprouts, in EGM-All and EGM SFB medias, in N; 1.6, 2.5x and 1.6x longer sprouts, in EGM-All, EGM without VEGF, and EGM SFB medias, in H. In conclusion, this study demonstrated that the CRISPR-Cas13d system was able to downregulate the EGLN1 gene, consequently reducing PHD2 expression and endogenous HIF-1α. There was an increase in angiogenic factors and a decrease in inflammatory cytokines, indicating an anti-inflammatory action of this therapy. Additionally, there was an enhancement in the proliferative and angiogenic capacity of modified endothelial cells.

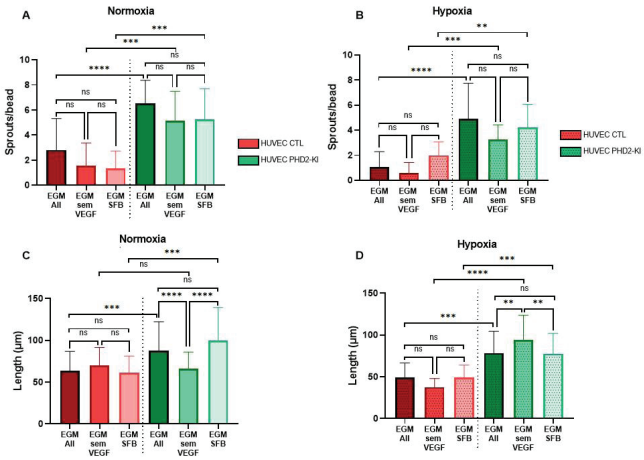


Figure 2 - Sprouting Assay. (A) and (B) number of sprouts per bead. (C) and (D) length of each sprout. EGM: Endothelial Growth Media. EGM-All: EGM media containing all the growth factor. EGM withou VEGF: EGM media containing all growth factors, but VEGF. EGM SFB: EGM media without growth factors, supplemented with FBS only.

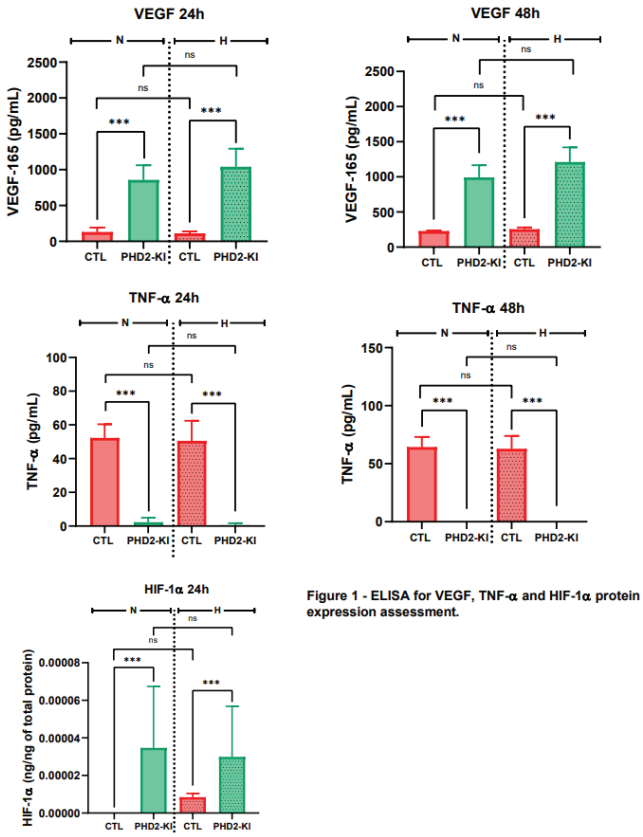


Figure 1 - ELISA for VEGF, TNF-α and HIF-1α protein expression assessment.

604. Engineering Cardiac-Specific Expression Cassettes for High Level Cardiac-Specific Transgene Expression

Neil R. Hackett, Abhishek Bose, William J. Pascal, Ronald G. Crystal

Genetic Medicine, Weill Cornell Medical College, New York, NY

Genes to treat hereditary cardiac disease can be transferred to the heart by intravenous (IV) administration of a cardiac tropic vector such as AAVrh.10. However, some recessive genetic diseases of the heart such as dilated cardiomyopathy type 2A resulting from TNNI3 mutations, may require very high levels of transgene expression since the endogenous gene is in the top 1 percentile of cardiac gene expression. A significant proportion of intravenous vector traffics to the liver, with concomitant high levels of unwanted liver expression so optimal cardiac expression is required. With the goal of maximizing cardiac transgene expression while minimizing liver transgene expression, we used AAVrh.10 vector administered intravenously to C57Bl/6 mice (dose at 4 x 10^{12}gc/kg) to screen expression cassettes with 5 liver-specific miRNA targets (mir122, 148, 192, 194, 208) and 5 cardiac-specific promoters (TNNI3, PLN, TNNT2, MYOZ2, ANKRD1) for the optimal high cardiac / low liver expression. Using the CAG strong ubiquitous promoter as the 100% comparator, screening of the 5 miRNAs yielded the maximum suppression of liver expression with mir122 (liver 0.5% that mediated by CAG), but at the price of suppression of cardiac expression (10.4±16.3% of CAG). In contrast, mir194 mediated the highest cardiac expression (116±26% of the level mediated by CAG) with liver expression reduced to 6.2±0.9% of CAG. Screening of the 5 cardiac-specific promoters demonstrated that all were associated with low liver expression (all <1% that of CAG), but at the price of low cardiac expression (all <8% that of CAG). With the goal of increasing the cardiac expression while minimizing liver expression, we used the TNNI3 cardiac-specific promoter as the example (TNNI3 mediated cardiac expression that was 2.6±0.61% of CAG), and added enhancers MYH6/7, CS-CRM4 or CMV to assess improvement. The highest cardiac expression was with the TNNI3-CMV combination (87.9±57.1% that of CAG), but with higher liver expression (10.2±5.2% of CAG). Two cardiac enhancers (MYH6/7 and CS-CRM4) were added to the TNNI3 promoter and while they respectively provided 2.4 and 7.5-fold enhanced expression in heart compared to TNNI3 promoter alone, the total cardiac expression level was <20% of that provided by the non-specific CAG. We further tested if a combination of cardiac specific promoter and enhancer with a target for a liver-specific miRNA could provide better performance. The CS-CRM4 enhancer combined with the TNNI3 promoter gave 12.0% of the cardiac expression level provided by the CAG promoter and addition of a mir192 target increased this only to 16.0%. However, the addition of a mir192 target suppressed hepatic expression by 5.8-fold. Overall, this study suggests that while cardiac specific promoters can provide cardiac selectivity and high cardiac expression with the addition of enhancer elements, similar levels of cardiac selectivity and higher cardiac expression can also be achieved by liver specific miRNAs.

605. Systemic Administration of a Novel Cardiotropic AAV Vector Encoding BAG3 Rescues Left Ventricle Functional Deficits in a Mouse Model of Myocardial Infarction

Lisa M. Stanek, Bryan Mastis, Charles Gualtieri, Tyler Ironside, Cynthia Pryce, Elizabeth Scott, Emily Grandell, David Ma, Giridhar Murlidharan, Thomas Matthew Edwards, John Reece-hoyes, Roberto Calcedo, Sherry Cao, Charles F. Albright, Laura K. Richman

Affinia Therapeutics, Waltham, MA

Gene therapy with adeno-associated virus (AAV) vectors is emerging as a promising therapeutic platform for the treatment of cardiovascular disease. Unfortunately, effective gene delivery to the heart and safety concerns have been major limitations to clinical translation. We have identified a novel AAV capsid (ATC-0108) by a machine learning guided rational design approach that demonstrates significantly improved cardiac transduction and robust liver detargeting compared with wild-type AAV9. To test the performance of our capsid in a disease-relevant context, we employed a mouse model of myocardial infarction (MI) with diminished left ventricular (LV) function and B-cell lymphoma 2 (Bcl-2) associated anthanogene-3 (BAG3) as a therapeutic transgene. BAG3 has emerged as a promising drug target for patients with heart failure and genetic cardiomyopathies. BAG3 is a 575 amino acid member of the BAG family of proteins that plays a critical role in cardiac contractility and Ca2+ homeostasis (Felman A., 2016). Decreased levels of BAG3 were found in the ventricular myocardium of patients with end-stage heart failure (HF) due to either coronary artery disease or idiopathic dilated cardiomyopathy. Animal models of heart failure including trans-aortic banding and myocardial infarction models in mice and pigs show similar reductions in BAG3

following these procedures. It has previously been shown that AAV9-mediated expression of BAG3 improves LV function in a murine model of myocardial infarction (MI) when delivered systemically at 1e14 vg/kg (Knezevic T., 2016). Here we sought to determine if we could improve LV function in a murine MI model using our novel cardiac capsid ATC-0108 expressing BAG3. Myocardial infarction was surgically induced by a distal ligation of the left anterior descending (LAD) coronary artery creating sustained ischemia and infarction in the LV anterior wall. Cardiac infarction was confirmed by weekly ultrasound echocardiography. Six weeks following surgery, when MI mice showed significantly diminished LV function compared to control mice (that did not undergo surgery), MI mice received IV injection of ATC-0108 expressing human BAG3 under the control of a cardiac promoter (ATV-0134). Based on biodistribution studies performed in unoperated wild type mice we selected an IV dose of 2e12 vg/kg that led to >70% transduction of the mouse myocardium. Following systemic injection of ATV-0134 MI mice demonstrated a 40% improvement in LV function by echocardiography. Histological analysis of heart tissues confirmed the presence of a surgically-induced infarct in all MI mice with no evidence of test article related safety findings in any tissue analyzed (heart, skeletal muscle and liver). Immunohistochemistry demonstrated transduction of 70% of the myocardium and BAG3 expression throughout the heart with little to no expression in liver or skeletal muscle. The results of this study confirm that our novel cardiotropic AAV capsid demonstrates safety and robust therapeutic efficacy in an animal model of cardiac dysfunction. ATC-0108 encoding BAG3 provided significantly improved cardiovascular function in the MI mouse model. A highly cardiotropic liver detargeted may provide a novel therapeutic option for the treatment of cardiovascular disease.

606. Systemic Administration of CBN-1100, a New Recombinant Parvovirus Vector with a Non-AAV Capsid, Shows Favorable Biodistribution and Reduced Liver Tropism in Nonhuman Primates

Salvatore Iovino[1], Samantha Smith[1], Leila Malkoc[1], Melvys Valledor[2], Michael Rak[2], Jeffrey Moffit[3], Sebastian Aguirre[2], Marc Abrams[4]

[1]Translational Biology Team, Carbon Biosciences, Waltham, MA,[2]Platform Development Team, Carbon Biosciences, Waltham, MA,[3]Nonclinical Development Team, Carbon Biosciences, Waltham, MA,[4]Carbon Biosciences, Waltham, MA

Most systemically-administered viral gene therapies which have achieved marketing approval or are in late-stage development are recombinant AAV vectors, representing only one genus (dependoviridae) within the parvovirus family. Despite their strong pharmaceutical attributes universal applicability is limited by high seroprevalence in the human population, limited cargo capacity, and safety and efficacy concerns related to high levels of liver transduction. To overcome many of these limitations, Carbon Biosciences is developing hybrid parvovirus vectors containing capsids from non-primate-derived autonomous parvoviruses and genomes packaged with AAV2 inverted terminal repeats (ITRs). The new recombinant vector CBN-1100 offers the ability to package genomes of at least 5.5 kb, low potential for neutralization by human antibodies, and has been shown to transduce cell types of therapeutic interest. Importantly, CBN-1100 maintains the strong manufacturability and scalability properties of AAV vectors. In this report, we present the tissue distribution, preliminary tolerability, and gene expression profile of CBN-1100 in mice and cynomolgus macaques at several dose levels. We observed strong viral genome copy numbers and transgene expression in tissues of therapeutic interest including kidney and heart. Remarkably, liver distribution and liver gene expression were extremely low in comparison to AAV historical data. CBN-1100 also exhibited a favorable tolerability profile, with clinically insignificant elevation of liver transaminases or complement factors. These compelling pharmacological properties, powered by a wild-type parvovirus capsid that is >80% divergent from AAV vectors at the amino acid level, support further development of CBN-1100 and related vectors for therapeutic applications which are out-of-reach for current gene therapy technologies.

607. Evaluating the Transduction Efficiency of Different AAV Serotypes on hiPSC Derived Cardiomyocytes

Georgios Kosmidis

Ncardia, Leiden, Netherlands

Gene therapy holds great promise for the treatment of a wide range of genetic disorders. Adeno-associated viruses (AAVs) have emerged as versatile and efficient vectors for gene delivery due to their ability to transduce a variety of cell types with minimal immunogenicity. In recent years, the integration of AAV-based gene therapy with human induced pluripotent stem cell (hiPSC) technology has unlocked new possibilities for personalized medicine and disease modeling. The identification of AAV vectors with the highest transduction efficiency, lowest toxicity and best efficacy in ameliorating disease-specific phenotypes, is crucial for efficiently selecting which vectors pass on subsequent preclinical and clinical studies. hiPSC derived cell models allow us to assess all three of these key aspects. In fact using high content screening as a readout, the first two of these parameters, transduction efficiency and cytotoxicity, can be evaluated quickly and cost-effectively prior to the more laborious task of efficacy analysis with the use of other elaborate functional assays. In order to evaluate AAV transduction efficiency in hiPSC derived cardiomyocyte cells (hiPSC-CMs) a panel of AAVs of different serotypes, all expressing GFP, was tested at several MOI values in our bioreactor generated (healthy control) hiPSC-CMs called Ncytes. Fluorescence intensity levels for GFP were recorded daily for a period of 10 days post transduction using a microplate reader. At the final experimental timepoint the nuclei of the transduced hiPSC-CMs were stained with Hoechst dye and the cells were imaged using a high content image confocal microscope. The parameters of cell %viability (number of nuclei/average number of nuclei of vehicle control), %transduction efficiency (number of GFP+ nuclei/number of total nuclei) and average fluorescence intensity were analyzed for all conditions. Most AAV serotypes at their respective MOIs, had an acceptable GFP signal (2-7x fluorescence intensity above local background). The only vector that failed to transduce hiPSC-CMs was AAV4. For all other vectors transduction efficiency increased with increasing MOIs as expected. The most efficient transductions were achieved using AAV1 and AAV5 vectors. Cytotoxicity was observed at higher MOIs (>10.000) for several vectors. Using our high content

imager we now have a suitable platform to evaluate the transduction efficiency and cytotoxicity of test AAVs. This not only helps us to identify which AAV serotypes are most suitable for transducing our Ncyte cells but also allows us to assess the transduction potential of AAVs supplied by our clients cost effectively, prior testing their efficacy in downstream functional assays.

608. Novel Human Heart-Derived Adeno-Associated Viruses Are Cardiac-Specific Gene Carriers for Effective *In Vivo* Gene Delivery to Mammalian Hearts and Human iPS-Derived Cardiomyocytes

Daniel Zeissler[1], Sebastian Grieskamp[1], Dorothea Kehr[1], Stephanie Simon[2], Philipp Schlegel[2], Eric Meinhardt[2], Hugo A. Katus[2], Malte Loos[3], Arne Hansen[3], Martin Busch[4], Andreas Jungmann[1], Julia Ritterhoff[1], Patrick Most[1]

[1]Molecular and Translational Cardiology, Heidelberg University Hospital, Heidelberg, Germany,[2]Cardiology, Heidelberg University Hospital, Heidelberg, Germany,[3]Experimental Pharmacology and Toxicology, Hamburg University Hospital, Hamburg, Germany,[4]AaviGen, Heidelberg, Germany

Introduction: Recombinant adeno-associated viral (rAAV) vectors currently used in clinical trials for cardiac gene therapy (GT) lack myocardial specificity. rAAV9, being clinically probed in human cardiomyopathy GT trials, exerts high hepatic toxicity resulting in fatal liver failure and death in several patients. As such, rAAVs with high cardiac specificity and ability to transduce the human heart would be close to an ideal vector for human cardiac GT. **Hypothesis:** We therefore hypothesized that the human heart may be a source for identifying novel AAVs, therefore capable to transduce human myocardium, with appropriate cardiac specificity to enable safe and effective gene transfer to mammalian hearts in vivo. **Approach and Results:** We identified AAV9 and AAV6 in myocardial biopsies from heart failure patients as serotypes known to effectively transduce myocardium in vivo and in vitro, respectively. Additionally, a group of novel AAVs (AAVcor1-3) with sequences close to rAAV13 were discovered. HEK-293 high-titer producible vectors with cloned rAAVcor1, 2 and 3 and rAAV9 capsid sequences were used for in vivo biodistribution analysis with a CMV-YFP reporter cassette in mice (each n=6 mice). 2 weeks after i.v. injection (per rAAV 1E12 vgc/kg), the rAAVcor group equaled rAAV9s excellent cardiac transduction and YFP mRNA expression abilities by vgc and mRNA levels in left ventricles (LV). In contrast to rAAV9s significantly higher hepatic than cardiac enrichment, all three rAAVcor vectors were almost undetectable in liver and other organs (e.g. brain) with a 1000-fold higher heart/liver vgc ratio than rAAV9. From this group, rAAVcor1s biodistribution was further compared with rAAV9 in mice using a cardiac troponin (cTnT)-YFP reporter system with clinically relevant dosages (5E12, 5E13, 5E14 vgc/kg; n=6 animals). 2 weeks after i.v. injection, rAAV9 showed high dose-dependent hepatic YFP protein IHC and IB expression. In contrast, rAAVcor1 unveiled the desired dose-dependent homogeneous cardiac YFP protein expression in cardiomyocytes (CM) but no detectable hepatic YFP protein expression. Given its high cardiac specificity, rAAVcor1 with the cTNT-relaxin family peptide receptor 1 (rfxp) inotropic response gene, which can be acutely activated using the peptide ligand relaxin (rel), was injected into mice (5E13 vgc/kg; n=6 animals). After 2 weeks, the murine hearts showed high cardiac-restricted rfxp mRNA levels and significant increase in cardiac contractile performance by LV catheterization in response to 50 µg single i.v. rel. injection. Subsequent i.v. injection of rAAVcor1 and rAAV9 in adult farm pigs (1E12 vgc/kg, n=3 animals per vector), each with a CMV-YFP reporter system, confirmed the high cardiac specificity of rAAVcor1 over AAV9 (>1000-fold) and homogeneous LV and RV transduction of the human-sized hearts. In hiPS-CM-EHTs, the ability of rAAVcor1 (1E9 vgcs per EHT) to homogenously transduce a human myocardial tissue equivalent was validated using YFP protein expression analysis via microscopy and FACS from dissociated EHTs. **Conclusion:** rAAVcor1, discovered in human failing myocardium, combines the clinically desirable properties of high and homogeneous *in vivo* transduction of murine and human-sized pig hearts with excellent liver detargeting after systemic injection and producibility. Such characteristics may destine rAAVcor1 as a suitable vector for safe and effective human cardiac GT.

609. Development of Cardiomyopathy Variant Knock-in Mouse Strains for Gene Therapy Proof-of-Concept

Hideki Uosaki[1], Tatsuya Anzai[2], Ryo Kawakami[3], Takeshi Tokuyama[1], Razan E. Ahmed[1], Fuad Gandhi Torizal[1], Yasumitsu Nagao[4], Hirofumi Nishizono[5], Norimichi Koitabashi[6]

[1]Division of Regenerative Medicine, Center for Molecular Medicine, Jichi Medical University, Shimotsuke, Japan,[2]Department of Pediatrics, Jichi Medical University, Shimotsuke, Japan,[3]Department of Cardiovascular Medicine, Gunma University Graduate School of Medicine, Maebashi, Japan,[4]Center for Experimental Medicine, Jichi Medical University, Shimotsuke, Japan,[5]Medical Research Institute, Kanazawa Medical University, Uchinada, Japan,[6]Department of Cardiovascular Medicine, Gunma University Graduate School of Medicin, Maebashi, Japan

Gene therapy is a rapidly expanding field, but the development of gene therapy for cardiomyopathy is still limited. One reason for this is the lack of suitable mouse models to demonstrate proof-of-concept in vivo. However, with recent advancements in genome editing using CRISPR/Cas9, it is now easier and faster to generate mouse models with knock-in variants for cardiomyopathy. Therefore, we aimed to develop these mouse strains and test the effectiveness of gene therapy. We created mutant mouse strains intending to model four types of cardiomyopathy by introducing variant knock-ins in Mybpc3, Lmna, Tnni3, and Bola3 genes. All the strains exhibited reduced heart function and/or shorter lifespan. For example, Tnni3 and Bola3 homozygous mutant mice survived for only two to three weeks after birth. In contrast, Mybpc3 and Lmna mutant mice lived longer despite their impaired heart function.To gain proof of concept, we utilized Mybpc3 mutant strains—nonsense (Q84X) and missense mutations (R824Q). MYBPC3 mutations are frequently associated with hypertrophic cardiomyopathy (HCM), and one clinical trial has recently been initiated for MYBPC3-associated HCM using adeno-associated virus (AAV). HCM often progresses to develop ventricular systolic dysfunction, termed HCM with left ventricular systolic dysfunction

(HCM-LVSD). Thus, it is intriguing to ask if gene therapy could treat HCM or even HCM-LVSD following pathological cardiac remodeling. We first examined the characteristics of Q84X and R820Q. Q84X is the orthologous mutation of Q76X that was a causative gene for fatal congestive heart failure in an infant. Q84X homozygous mice displayed reduced systolic function even at two weeks but survived for almost a year. Meanwhile, R820Q is associated with familial HCM and HCM-LVSD in patients. In mice with R824Q, the equivalent of R820Q, cardiac hypertrophy, and mild systolic dysfunction developed spontaneously at 8 weeks old, recapitulating the HCM-LVSDphenotype.Next, we examined if gene therapy is effective in these HCM models. To this end, we constructed a vector expressing Mybpc3 under the control of a cardiomyocyte-specific, cardiac troponin T promoter and packaged it into AAV serotype 9 capsid. First, we tested the AAV in adult Q84X mice by administering 1 x E+14 vg per head. Although Mybpc3 expression was observed, heart function remained low, indicating that gene therapy may not be effective once pathological cardiac remodeling is completed. Next, we administered the AAV to R820Q mice that began to develop hypertrophy at 8 weeks of age at a dose of 1.76 x E+13 vg per head. Echocardiography and cardiac catheter revealed that AAV gene therapy significantly suppressed the deterioration of cardiac function over time. The overexpression of fetal genes of cardiomyocytes was also significantly suppressed, and mitochondrial gene abnormalities and myocardial fibrosis were attenuated.Our genetically modified mouse strains with cardiomyopathy variants accurately replicated the human cardiomyopathy symptoms. They can be used as models to examine the effectiveness of AAV-based gene therapy. The administration of gene therapy with AAV, even at a stage when cardiac remodeling has already begun, but not yet completed, successfully suppressed cardiac dysfunction and prevented the transition to HCM-LVSD.

610. Developing an AAV-Based Gene Therapy for MYBPC3 Mutation-Associated Hypertrophic Cardiomyopathy

Therese Salameh[1], Hongbin Xu[1], Hong Duan[1], Yogeshwar Sharma[1], Deepthi Yedlapudi[1], Chao Ren[2], Jingsong Cao[1], Zhong-Dong Shi[1]

[1]Frontera Therapeutics, Bedford, MA,[2]Frontera Therapeutics, Shanghai, China

Hypertrophic cardiomyopathy (HCM) is a highly prevalent cardiovascular genetic disorder affecting approximately 0.2% (1:500) individuals worldwide. This disease which potentially results in heart failure or sudden cardiac death, is characterized primarily by left ventricular hypertrophy, diastolic dysfunction, myocyte disarray, and interstitial fibrosis. Loss-of-function mutations in *MYBPC3,* the gene encoding cardiac myosin-binding protein C (cMyBP-C), is one of the primary causes of genetic HCM. Most pathogenic mutations of *MYBPC3* arise via frameshift, nonsense, or conserved RNA splice site mutations on a single allele that results in protein truncation, which is more likely to degrade resulting in lower total cMyBP-C protein levels (haploinsufficiency). Haploinsufficiency of *MYBPC3* contributes to sarcomeric dysfunction and deregulation of contraction and relaxation in cardiac myocytes. Restoration of cMyBP-C haploinsufficiency offers a viable therapeutic approach for the treatment of HCM. We are developing an AAV-based therapy to treat MYBPC3-associated HCM. We have engineered AAV vectors encoding human cMyBP-C protein. We showed that systemic delivery of AAV vectors effectively restores cMyBP-C to cardiomyocytes in a *Mybpc3* knockout (KO) mouse model. AAV vector treatment of neonatal *Mybpc3* KO mice (on P1) caused a significant reduction in heart weight to body weight ratio in comparison to the vehicle-treated group, indicating there was an improvement in cardiac hypertrophy. Our data also showed a correlation between the level of cMyBP-C protein restored in cardiomyocytes and the reduction in heart weight to body weight ratio. In addition, treatment of the *Mybpc3* KO mice with AAV vectors showed a significant reduction in HCM-associated biomarkers in the heart, including the genes encoding atrial and B-type natriuretic peptide and tissue inhibitor of matrix metalloproteinase-1, a marker of fibrosis which is a hallmark of this disease. Biodistribution studies demonstrated that our vector specifically targeted the heart as evidenced by the lack of protein and mRNA expression in liver and skeletal muscle. Furthermore, no clinical abnormalities or safety concerns were observed in our studies. These data demonstrate that our AAV vectors are capable of restoring cMyBP-C deficiency and improving cardiac hypertrophy and associated biomarkers in a murine model that resembles the human condition of HCM and support the continued development of this AAV-based gene therapy.

611. Epicardial Hydrogel for Cardiac Gene Delivery

Gabriella Kabboul[1,2], Luiza DaMotta[3], Christopher B. Doering[4], Andrés J. García[3], Rebecca D. Levit[2]

[1]Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology & Emory University, Atlanta, GA,[2]Division of Cardiology, Department of Medicine, Emory University, Atlanta, GA,[3]Woodruff School of Mechanical Engineering & Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, GA,[4]Department of Pediatrics, Emory University & Children's Healthcare of Atlanta, Atlanta, GA

Introduction: The use of adeno-associated virus (AAV) has emerged as a novel approach to deliver genes to the heart. However, clinical translation of AAV-based cardiac therapies has been largely unsuccessful owing to pre-existing neutralizing anti-AAV antibodies and poor cardiomyocyte transgene expression. An effective delivery method is a significant barrier in cardiac gene therapy. Epicardial hydrogel delivery of AAV may overcome obstacles to cardiac gene transfer. Here we investigate the delivery of an epicardial hydrogel encapsulating AAV. We hypothesize that hydrogels delivered to the epicardial surface through the pericardial space may result in longer tissue residence time, reduced off-target effects, and minimal vascular washout; consequently, shielding the virus from immune-mediated neutralization and increasing cardiac gene uptake. **Methods:** Degradable hydrogels were synthesized with 4-arm poly(ethylene glycol) maleimide-terminated (PEG-4MAL) macromer mixed with a crosslinker. AAV9 encoding mCherry and luciferase driven by the CMV promoter was encapsulated in the gels. A viral dose of 1E12 vector genomes was delivered to male Sprague-Dawley rats by epicardial hydrogels (n=7) or intravenous (IV) injection (n=5). Animals were randomized to receive AAV by only one delivery method. After 28 days, bioluminescence imaging was performed to quantify vector biodistribution and serum was collected to measure anti-AAV9 antibody titers. Bioluminescent expression was quantified from *in*

vivo images with regions of interest drawn around the heart and liver. Histology was performed to assess transgene expression in the heart. **Results:** Animals receiving epicardial hydrogels showed a 9-fold higher average radiance in the heart compared to animals receiving AAV intravenously ($p<0.01$). In the liver, animals that received epicardial hydrogels showed significantly lower average radiance in comparison to animals that received AAV via IV injection ($p<0.05$). In addition, anti-AAV9 IgG titers were 2-folds lower in serum from animals receiving epicardial hydrogels in contrast to IV delivery. Immunofluorescent analysis of cardiac tissue indicated epicardial hydrogels enable epicardial and myocardial transduction, while systemic delivery showed sparse mCherry expression in the myocardium. **Conclusion:** These results support the potential benefits of epicardial hydrogel-AAV delivery to effectively transfer therapeutic transgenes to the heart with minimal off-target transduction and lower humoral immunity generated compared to systemic AAV administration. Future studies will focus on optimizing epicardial hydrogels for AAV release and demonstrating cardiac therapeutic efficacies.

612. Clinical Grade Human Adenovirus 5 Vector Overexpressing TIMP-3 is Safe and Efficacious in a Pig Model of Vein Graft Neointimal Hyperplasia

Stuart A. Nicklin[1], Samantha Carmichael[2], Sarah J. George[3], Collin Berry[4], Alex McConnachie[4], Nawwar Al-Attar[1], Stefano Colloca[5], Rachel Dakin[4], Menzo Havenga[6], Angelique Lemckert[6], Rebecca Bayliss[7], Rebecca Wallace[7], Alan L. Parker[7], Raimondo Ascione[3], Andrew H. Baker[4,8]

[1]University of Glasgow, Glasgow, United Kingdom,[2]Greater Glasgow and Clyde NHS, Glasgow, United Kingdom,[3]University of Bristol, Bristol, United Kingdom,[4]University Of Glasgow, Glasgow, United Kingdom,[5]ReiThera Srl, Rome, Italy,[6]Batavia Biosciences, Leiden, Netherlands,[7]Cardiff University, Cardiff, United Kingdom,[8]Centre for Cardiovascular Science, University of Edinburgh, Edinburgh, United Kingdom

Late failure of saphenous vein bypass grafts remains a major clinical problem. Mechanisms that drive neointima formation after implantation of grafts are thought to be pivotal in creating an environment through which accelerated atherosclerosis can ensue, and cause graft failure. We previously showed that research grade human adenoviral serotype 5 vectors overexpressing the human tissue inhibitor of metalloproteinase-3 (TIMP-3) gene delivered ex-vivo to saphenous vein in a pig model reduced neointimal formation at both 1 month and 3 months post grafting. Here, in advance of a first-in-human clinical study, we generated a clinical lot of Ad-TIMP-3 and assessed safety and efficacy in the pig saphenous vein-to-carotid interposition graft model. We produced Ad5.CMVTO.TIMP-3, a first generation E1/E3 deleted adenovirus serotype 5 vector expressing TIMP-3 under control of the CMVIEP and Tet repressor (TO) transcriptional elements under GMP conditions to perform a preclinical in-vivo trial at the Bristol Translational Biomedical Research Centre (TBRC) facility. A randomised blinded single-dose placebo-controlled trial was undertaken using the established porcine model of right carotid artery interposition of autologous saphenous vein (aSV) (n=11 per group of crossed Large White x Landrace female, 60-65kg pigs, approx 6 months old). Outbred Large White/Landrace pigs were randomised to receive aSV pre-treated with either Ad5.CMVTO.TIMP-3 or placebo (vehicle). Ad5.CMVTO.TIMP-3 or placebo was instilled into the vein lumen. The vein was incubated at room temperature for 30 minutes prior to flushing the lumen and then grafting end-to-end into the carotid artery. The 4-week single-dose toxicology study demonstrated no treatment-associated adverse findings or mortalities. All cardiovascular parameters remained within normal range during graft implantation and resection surgery and no differences in blood clinical chemistry were detected between animals grafted receiving Ad5.CMVTO.TIMP-3-treated or control-treated veins. At 1 month post vein grafting, a significant 26% reduction in intimal area was observed in Ad-TIMP-3 treated grafts using the clinical grade viral vector, compared to the placebo. Based on this, and the prior efficacy studies, we are progressing to a first-in-human study to begin in 2024 to assess the safety of gene therapy for vein graft failure.

Wednesday Posters: Neurologic Diseases

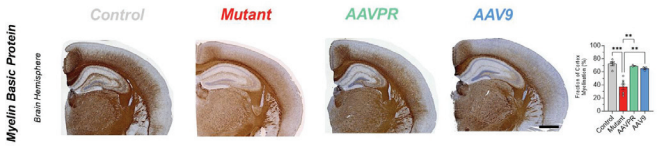
613. Systemic AAV-Delivered Targeted Gene Editing Restores Cerebrovascular Disease and Prevents Associated Neurodegeneration in ACTA2 R179H-Related Multisystemic Smooth Muscle Dysfunction Syndrome (MSMDS)

Vijai Krishnan, Sabyasachi Das, Christiano Alves, Christian Lino Cardenas, Takahiko Imai, James Hai, Aarushi Gandhi, Pazhanichamy Kalailingam, David Chung, Mark E. Lindsay, Benjamin P. Kleinstiver, Patricia L. Musolino

Massachusetts General Hospital, Boston, MA

Objective: MSMDS is an ultrarare genetic smooth muscle (SMC) myopathy with dysfunction in the vascular, respiratory, enteric, and genitourinary systems. The most severe forms are caused by missense variation at arginine 179 of the ACTA2 gene. Neurological manifestations include cerebral small vessel disease (cSVD) and ischemic strokes (IS). IS commonly occur during episodes of hypotension or anesthesia suggesting severe impairment of cerebral autoregulation and neurovascular coupling, mechanisms required for responsive cerebral blood flow (CBF) during during arterial flow. We developed a conditional knock in ACTA2 R178H mouse model where only smooth muscle cells are mutant and found an accelerated form of neurodegeneration with classic histopathological and behavioral features commonly seen in neuronal proteinopathies associated with Alzheimer-related degenerative disorders. Using a custom CRISPR-Cas9 (VRQR+) adenine base editor (ABEs) delivered by two AAV-intein constructs we achieved high levels of correction of the disease-causing G-to-A mutation. Here we characterized the neurodegeneration caused by a SMC-specific ACTA2R179H mutant in a mouse model and determined whether targeted gene correction of SMCs can improve cerebral vascular function (SMC contractility, BBB permeability, CA and NC) and ameliorate neurodegeneration. Methods: Our murine MSMDS model encompasses a whole-body smooth muscle activation

of the mutation using Myh11-cre (Myh11-Cre:Acta2$^{R179fl/+}$). For *in vivo* delivery of the ABE, we cloned an intein-split construct compatible with dual adeno-associated virus (AAV) delivery. The constructs were packaged in two AAV serotypes, including AAV9 and our novel AAVPR capsid, injected systemically by retroorbital single dose injection at P3. Neurodegeneration was evaluated in treated and untreated mice and animals underwent various assessments, including physiology, behavior, and immunohistochemistry 8 weeks after treatment. Results: Examination of myelin basic protein (MBP) expression by immunohistochemistry in 8 week old *Myh11-Cre:Acta2*$^{R179Hfl/+}$ mice show significant loss of MBP in mutant *Myh11-Cre:Acta2*$^{R179Hfl/+}$ mice. Demyelination coincided with neuronal loss revealed by NeuN nuclei staining in various cortical and hippocampal regions. Furthermore, neurofibrillary tangles and Congo red-positive aggregates in the cortex and hippocampus, highlight that a defective neurovascular smooth muscle cell function can trigger characteristic protein aggregates associated with neurodegeneration. Impaired motor and cognitive performance in behavioral tests including Y maze, the rotarod and open field were observed starting at 4 weeks of life. Gene targeted correction significantly rescue myelination (Fig. 1), decreased pathological microglial and astrocytic activation of the *Myh11-Cre:Acta2*$^{R179Hfl/+}$ mice. Additionally, Myh11-cre:Acta2R179Hfl/+ mice treated with gene therapy exhibit improved sensorimotor behavior. Conclusion: Our findings analyzed the start of vascular dysfunction culminating in neurodegeneration in MSMDS. We also present evidence that targeted gene therapy as a promising approach to mitigate neurodegeneration in MSMDS mouse models, offering hope for potential future treatments.



614. Patient-Derived iPSC-Neurons and Microglia for Modeling Friedreich's Ataxia

Anusha Sivakumar[1], Priyanka Mishra[2], Carla Pernaci[1], Avalon Johnson[3], Angelyn B. Solis[3], Rafael A. Badell-Grau[4], Veenita Khare[5], Sydeny Gillette[3], Emily A. Hansen[3], Nicole Coufal[3], Stephanie Cherqui[6]

[1]1Department of Pediatrics, University of California, University of California, San Diego, La Jolla, CA,[2]University of California San Diego, San Diego, CA,[3]Department of Pediatrics, University of California, University of California, San Diego, La Jolla, CA,[4]Department of Pediatrics, Division of Genetics, UCSD, La Jolla, CA,[5]Department of Pediatrics, Division of Genetics, UCSD, San Diego, CA,[6]Department of Pediatrics, Division of Genetics, University of California, San Diego, La Jolla, CA

Friedreich ataxia (FA) is a neurogenerative disorder caused by homozygous GAA expansion mutation in the first intron of frataxin (*FXN*) gene. FXN is a mitochondrial protein, and its deficiency leads to mitochondrial iron overload, defective energy production, and oxidative stress. We previously showed complete rescue of neurologic complications of FRDA in YG8R mice with a single, systemic infusion of wild-type hematopoietic stem and progenitor cells (HSPC), and rescue was mediated by FXN transfer from engrafted HSPC-derived microglia to diseased neurons. We next developed an autologous stem cell transplantation approach using CRISPR/Cas9-mediated excision of GAA repeats in FRDA patients' CD34$^+$ HSPCs that increased frataxin expression and improved mitochondrial functions. The aim of the current study is to understand the mechanism of rescue of the neurodegeneration by HSPC in FRDA and to this end to investigate the complex interaction between microglia and neurons in the disease pathogenesis. Thus, we generated a cohort of four different FRDA patient-derived induced pluripotent stem cells (iPSCs) along with the CRISPR/Cas9-gene edited FRDA iPSCs, two familial carriers and two unrelated control lines. These iPSCs were then used for generating neurons and microglia (iMG). iPSC derived FRDA neurons displayed characteristic apoptotic and mitochondrial phenotype of the disease, such as non-homogenous microtubule staining in neurites, increased caspase-3 expression, mitochondrial superoxide levels, mitochondrial fragmentation, and partial degradation of the cristae compared to healthy controls. These defects were fully prevented in the gene edited neurons. RNASeq analysis of FRDA and gene edited neurons demonstrated striking improvement in gene clusters associated with endoplasmic reticulum (ER) stress in the isogenic lines. Gene edited neurons demonstrated improved ER-calcium release, normalization of ER stress response gene, *XBP-1,* and significantly increased ER-mitochondrial contacts that are critical for functional homeostasis of both organelles, as compared to FRDA neurons. Ultrastructural analysis for these contact sites displayed severe ER structural damage in FRDA neurons, that was undetected in gene edited neurons. Similarly, FRDA-iMGs displayed activated, amoeboid morphology with compromised phagocytosis, mitochondrial dysfunction and attenuated Ca^{2+} release while the edited-iMGs exhibit a phenotype comparable to controls. These studies support that both neurons and microglia are impacted in FRDA, that can be rescued by our gene-editing approach.

615. ZIP Delivery, A Novel Peptide Delivery System for the Brain

Barbara Bailus[1,2], Stephen Scheeler[1], Kizito-Tshitoko Tshilenge[1], Ningzhe Zhang[1], Swati Naphade[1], Sally Mak[1], Kenneth Wilson[1], Robert O'Brien[1], Mahru An[1], Brian Rossmiller[1], Stanislav Moraz[1], Jennifer Suoja[1], Maria Sanchez[1], Tyne McHugh[1], Akos Gerencser[1], Lisa Ellerby[1]

[1]Buck Institute for Research on Aging, Novato, CA,[2]Keck Graduate Institute, Claremont, CA

Delivery of therapies to the central nervous system remains a major challenge in treating neurodegenerative disorders. In an effort to overcome this challenge we developed a novel cell penetrating peptide based on the Zika virus called ZIP-delivery. To test our ZIP-delivery system we focused on Huntington's Disease (HD) a rare neurodegenerative disorder caused by a CAG expansion in Exon 1 of the Huntingtin gene which results in a toxic protein and protein fragments being formed. In the last twenty five years much progress has been made in both understanding the basic genetics of HD and developing tools for treating HD. However, one area that still needs further optimization is the method of delivery to the brain for these novel therapies. We were able to attach ZIP-delivery to Cas9n, creating ZIP-Cas9n. ZIP-Cas9n is able to cross the blood brain barrier and achieve widespread distribution in a murine model of

HD by an intravenous injection. ZIP-Cas9n was also able to achieve high recombination efficiency in Huntington patient derived human neuronal stem cells, and striatal organoids, which makes it a promising technology for both therapeutic and disease modeling applications. Additionally, we attached ZIP-delivery to EGFP. ZIP-EGFP achieved widespread distribution in the murine brain from an intravenous injection. Furthermore, ZIP-EGFP showed widespread distribution and penetration in human cortical organoids. Our presentation will highlight the different applications and promise of ZIP-delivery as a therapeutic delivery model.

616. Phenotypic Rescue of STXBP1 *In Vitro* and *In Vivo* Models Using AAV-STXBP1 Gene Replacement Approach

Sijia Wu, Brian Harvey, Elena Morozova, Ayan Ghoshal, Adam Sheehy, Kevin Ghaemi, Moore Arnold, Manzhao Long, Chris Hinckley, Sharanya Unnikrishnan, Nicholas B. Gallo, Anh Nhu, Ramiro Massol, Chris Ehrenfels, Pete Clarner, Jinkuk Choi, Davide Gianni, Wan-Hung Lee, Kelly Bales

Biogen, Cambridge, MA

STXBP1-related developmental and epileptic encephalopathy (STXBP1-DEE) is a rare genetic epilepsy and neurodevelopmental disorder caused by heterozygous loss-of-function mutations in the STXBP1 gene. STXBP1-DEE is characterized by refractory seizures, developmental delay, intellectual disability, and motor dysfunction. Here, we evaluated effects of AAV-STXBP1 gene replacement in *in vitro* and *in vivo* models of the disorder. We demonstrate a dose-dependent increase in STXBP1 protein in STXBP1$^{-/-}$ iPSC-derived NGN2 neurons treated with AAV-STXBP1, resulting in the rescue of neurotransmission phenotypes. To evaluate effects in vivo, neonatal Stxbp1$^{+/-}$ mice were treated with AAV-STXBP1 and a dose-dependent increase of STXBP1 expression was observed. Importantly, AAV-STXBP1 improved measures of cortical function detectable by electroencephalogram (EEG) as well as motor and cognitive behavioral phenotypes. These results demonstrate that AAV-mediated restoration of STXBP1 expression can robustly rescue a range of disease-associated phenotypes in models of STXBP1-DEE.

617. Delivery of *DARS2* via AAV9 as a Potential Therapeutic Approach for Leukoencephalopathy with Brainstem and Spinal Cord Involvement and Lactate Elevation

Bayley Lindsay[1], Brett Ratajczak[1], Yajie Liang[2], Miroslaw Janowski[2], Piotr Walczak[2], Ali Fatemi[1,3], Christina L. Nemeth[1,3]

[1]Moser Center for Leukodystrophies at Kennedy Krieger, Kennedy Krieger Institute, Baltimore, MD,[2]Department of Diagnostic Radiology and Nuclear Medicine, University of Maryland School of Medicine, Baltimore, MD,[3]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD

Introduction: Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) is a rare neurological disorder caused by mutations in the *DARS2* gene, which encodes for the mitochondrial aspartyl-tRNA synthetase. Despite the wide phenotypic spectrum associated with LBSL - ranging from severe cerebellar hypoplasia and early demise in antenatal- or infantile-onset to mild spasticity and cerebellar ataxia in adult-onset - nearly all patients have compound heterozygous mutations on *DARS2*, the most common of which results in a frameshift mutation, premature termination, and decreased protein abundance. Methods: In this study, we aimed to restore the production of *DARS2* transcripts and functional protein in LBSL patient derived neurons through treatment with Adeno-Associated Viral (AAV) vectors, serotype 9, targeting the CNS. LBSL patient induced pluripotent stem cells (iPSCs) were differentiated into mature motor neurons according to an existing protocol and treated with the human *DARS2*- AAV9 vector under EF1a promoter on *in vitro* Day 11. Ten days after exposure, *DARS2* expression (qRT-PCR), neuronal arborization (high content imaging), mitochondrial energetics (Agilent Seahorse Mito Stress Test), and lactate levels (colorimetric assay) were measured. Results: Expression of *DARS2* transcripts including Exon 3 were increased in a dose-dependent fashion, suggesting the production of functional protein. *DARS2* gene expression was increased in parallel to increased neuronal arborization as measured by high content imaging. Increases in branching were evident before Day 21. On Day 21, mitochondrial analysis revealed increases to baseline oxygen consumption as well as increased max capacity without affecting cell viability. Lactate release from neurons as measured in spent media was decreased in a dose-dependent manner suggesting reduced mitochondrial stress. Together these data suggest that gene delivery is sufficient to induce functional changes to neuronal health which may have more widespread effects with systemic delivery. Conclusions: While further testing is needed to confirm these findings, it is promising that gene delivery increases normal DARS2 protein production, reducing the phenotypic burden *in vitro*. Additional studies are underway to investigate the effect of AAV9 treatment on electrophysical activity, long-term *in vitro* survival, and the effect of AAV9 *in vivo*. To date, studies suggest gene therapies may be a viable treatment for slowing or halting disease progression in those with mitochondrial tRNA synthetase disorders, such as LBSL.

618. The Peripheral Neuropathy of Adrenomyeloneuropathy (AMN)

Yasemin Özgür Günes[1], Andoni Echaniz-Laguna[2], Catherine Le Stunff[1], Romina Aron-Badin[3], Pierre-François Bougnères[4]

[1]INSERM, Le Kremlin-Bicêtre, France,[2]Department of Neurology, Bicêtre Hospital, Le Kremlin-Bicêtre, France,[3]CEA, CNRS, Université Paris-Saclay, MIRCen, Laboratoire des Maladies Neurodégénératives, Fontenay aux Roses, France,[4]Therapy Design Consulting, Vincennes, France

AMN prominently affects the spinal cord. However, peripheral neuropathy is a common feature in AMN although usually surpassed by spinal cord symptoms. Many patients have electrophysiological signs of axonal neuropathy or present with demyelinating neuropathy. We studied electrophysiological signs of axonal neuropathy in Abcd1 knock-out (Abcd1-KO) mouse model together with wild type mice as a control. Measurement of sensory nerve conduction velocity (NCV) was performed with a standard EMG Natus apparatus. Sensory nerve conduction velocity (NCV) measured at 24-month of age. We observed no significant difference between untreated 25.0 ± 5.3 mA (n=18), and

WT group 26.8 ± 5.3 mA (n=9). In contrast, obvious abnormalities of the axons were present in the sciatic nerve with electron microscopy (JM Vallat) in the absence of demyelination or inflammation. AMN seems associated with a structural peripheral neuropathy that does not alter electrophysiological parameters.

619. Novel AAV Gene Therapy Candidates Show Promise *In Vitro* and *In Vivo* for Treatment of PGAP3-CDG Syndrome

Julieth A. Sierra Delgado[1], Matthew Simon[2], Riya Talekar[1], Xiaojin Zhang[1], Abuzar Kaleem[1], Meysam Ganjibakhsh[1], Shrestha Sinha Ray[1], Shibi B. Likhite[1], Cathleen Lutz[2], Kathrin Meyer[1]

[1]Nationwide Childrens Hospital, Columbus, OH,[2]The Jackson Laboratory, Bar Harbor, ME

PGAP3-Congenital Disorder of Glycosylation (PGAP3-CDG) is a devastating, rare neurological condition that disrupts the synthesis of Glycosylphosphatidylinositol (GPI) anchors. PGAP3-CDG patients exhibit a diverse spectrum of symptoms that vary in age of onset and severity including developmental delay, intellectual disability, seizures, hypotonia, and characteristically high levels of serum alkaline phosphatase (hyperphosphatasia). Currently, there is no evidence of a close genotype-phenotype correlation. Current treatment strategies for PGAP3-CDG only focus on management of symptoms and do not impact disease progression. Furthermore, the intricate pathogenic mechanisms associated with PGAP3 mutations, particularly in neurons and astrocytes, remain poorly understood, impeding therapeutic advancements. This underscores the urgent need to unravel the pathophysiology and develop effective interventions for PGAP3-CDG. To achieve that, we have designed four different AAV9-mediated gene therapy constructs to restore PGAP3 function. To comprehensively evaluate these constructs across diverse mutations representative of the disease spectrum, we established an innovative PGAP3-CDG in vitro model. Primary fibroblasts from PGAP3-CDG patients were reprogrammed into induced neural progenitor cells and further differentiated into induced astrocytes (iAs) using a direct conversion protocol. PGAP3-CDG iAs displayed abnormal mitochondrial morphology in immunofluorescence studies, as well as significantly altered mitochondrial activity using the Seahorse XFe96 platform. In co-culture assays with healthy mouse GFP+ neurons, PGAP3-CDG iAs induced a high degree of neuronal death compared to healthy iAs. Moreover, we generated induced neurons (iNs) using the PGAP3-CDG patient fibroblasts via direct chemical conversion, finding reduced neurite length and neuron with neurite percentage compared to controls. We have optimized our in vitro AAV9 transduction protocol in both iAs and iNs, and we have successfully transduced our both of our in vitro models, showing a robust expression pattern. All constructs showed improvement on the cellular phenotype, with transduced iAs showing a remarkable improvement on neuronal support. The constructs are also being evaluated in vivo on a PGAP3 Knockout model, with some showing improvement of biochemical and clasping phenotypes, as well as in wild type mice for an initial safety assessment. Overall our candidates show efficacy on both in vitro and in vivo PGAP3-CDG Models. Collectively, the combined findings of this study will allow us to identify the optimal gene therapy vector for PGAP3-CDG treatment. These findings will support the development of upcoming IND-enabling studies.

620. Exploring Galectin-3 as a Biomarker for Disease Progression and Gene Therapy of Canavan and Other Neurodegenerative Diseases

Morgan Mooney[1], Peiyi Guo[1], Jie Wang[1], Anoushka Lotun[1], NhatAhn Nguyen[1], Guangping Gao[1,2,3], Dominic J. Gessler[1,3]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Li Weibo Institute of Rare Diseases Research, UMass Chan Medical School, Worcester, MA,[3]Co-corresponding Authors, Worcester, MA

Canavan Disease (CD), a fatal neurodegenerative disorder, stems from mutations in the Aspartoacylase (ASPA) gene, culminating in the brain's buildup of N-acetylaspartate (NAA). This disease tragically leads to patient's demise in early childhood. It is marked by severe symptoms including inability to walk, lack of head control, macrocephaly, and failure to meet developmental milestones. At the cellular level, spongiform degeneration and astrogliosis are prominent, yet the exact pathomechanism is unclear, but critical to develop gene therapy and monitor therapeutic response. A pivotal aspect of neurodegeneration is neuroinflammation driven by microglial activation. Probing into CD's cellular dynamics, single-cell RNA sequencing was performed comparing wild type (WT), knockout (KO), and AAV gene therapy-treated KO mouse brains. A striking finding was the substantial elevation of galectin-3 (Gal-3) in KO brains, an intracellular and secreted key protein in immune response. This led to the hypothesis that Gal-3 plays a critical role in CD pathogenesis via microglial activation, potentially allowing to monitor disease trajectory and therapeutic response. Using the Nur7 mouse model, which mirrors CD's severe neurological symptoms yet with a comparatively longer lifespan, we traced the onset and escalation of CNS histopathology. Vacuolization and neurodegeneration were initially observed on post-natal day 14, intensifying rapidly thereafter. Intriguingly, this degeneration localized to specific brain regions, paralleling a surge in activated microglia. Notably, early stages showed Gal-3 positive microglia levels akin to WT, but these levels increased significantly as the disease progressed. These findings prompted us to investigate Gal-3's potential as a biomarker for tracking CD progression and gene therapy response. Early intervention in asymptomatic Nur7 mice with rAAV-mediated ASPA expression achieved WT-level Gal-3 expression and absence of neurodegeneration, suggesting early treatment's potential to prevent disease onset and microglial activation. Furthermore, symptomatic juvenile Nur7 mice displayed a gradual increase in ameboid Gal-3 positive microglia in brain areas with disease progression. Treatment at this symptomatic stage resulted in the absence of neurodegeneration and normalization of Gal-3 positive microglia levels, akin to WT controls, indicating a reversal of microglial activation. To understand Gal-3's role in CD's pathomechanism, we engineered Gal-3/Aspa double-knockout mice, with studies currently ongoing. Our preliminary data suggest that Gal-3 could function as a promising biomarker for monitoring CD progression and gene therapy responsiveness, potentially extending

to other neurodegenerative diseases. Moreover, targeting Gal-3 could enhance rAAV-based gene therapies, offering new hope in modifying disease trajectories.

621. AAV-Mediated Gene Supplementation Provides Thorough Correction in Two Models of Vanishing White Matter Disease

Jessica A. Herstine[1,2,3], Tamara J. Stevenson[4], Sergiy Chornyy[1], Pi-Kai Chang[4], Jessica Rediger[1], Julia Wentz[4], Nettie Pyne[1], Alex Sunshine[1], Erika Scholl[4], Tatyana A. Vetter[1,2], Kevin M. Flanigan[1,2,3], Joshua Bonkowsky[4], Allison M. Bradbury[1,2,3]

[1]Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]Department of Pediatrics, The Ohio State University, Columbus, OH,[3]Center for Clinical and Translational Science, The Ohio State University, Columbus, OH,[4]Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, UT

Vanishing White Matter Disease (VWM) is a progressive childhood leukodystrophy that presents with ataxia, spasticity, neurological decline, and seizures which lead to premature death. There are no treatments. Caused by autosomal recessive, loss of function mutations in the subunits of eukaryotic initiation factor 2B (eIF2B), VWM most commonly originates from pathologic variants in subunit 5 (*EIF2B5*). Due to VWM's monogenic nature and the size of the *EIF2B5* transgene, it is a promising candidate for adeno-associated virus (AAV)-mediated gene supplementation therapy. Prior studies in in vitro and in vivo models of VWM revealed that astrocytes are a critical target for therapy, as their differentiation, morphology, and function are impaired, thus mediating disease progression. To better understand disease mechanisms and to serve as a baseline for correction, we first aimed to characterize the newly identified and severe VWM *Eif2b5*^{I98M} murine model with clinically relevant readouts such as motor function, gait mapping, and myelin loss through magnetic resonance imaging. We found that these mice display significant loss of mobility, ataxic gait, and demyelination all within a strikingly short life span (5.5 months). Molecular characterization indicates that the integrated stress response (ISR)—a molecular pathway regulated by eIF2B that rescues homeostasis during cellular stress—is dysregulated, supporting our findings that these mice are displaying classic and severe VWM phenotypes. To provide targeted correction of disease, we designed gene replacement constructs to drive therapeutic *EIF2B5* transgene expression in astrocytes. We tested multiple iterations of the glial fibrillary acidic protein (GFAP) promoter, including one ***novel*** intermediate form—gfaABCD1405—and found robust astrocyte targeting throughout the neuroaxis. Therefore, we are currently evaluating three astrocyte-specific, and one ubiquitous therapeutic AAV vectors in two murine VWM models, *Eif2b5*^{R191H} and *Eif2b5*^{I98M}, and are monitoring disease progression using molecular, biochemical, behavioral, and imaging readouts. Our data suggests that the astrocyte-targeted gene therapy delays disease progression, partially rescues body weight and motor function, and attenuates myelin loss in both models. The life spans of the *Eif2b5*^{I98M} animals treated with AAV9-gfaABC(1)D-*EIF2B5* ($p<0.0001$), AAV9-CAG-*EIF2B5* ($p<0.001$), and AAV9-gfaABCD1405-*EIF2B5* ($p<0.01$) are also significantly increased, with our current longest extension in life expectancy exceeding 1 year of age in our gfaABC(1)D-treated cohort. Expression analysis through RT-qPCR indicates we achieved increased expression of the *EIF2B5* transgene in the CNS of treated mice. Overall, we anticipate the development of a lead astrocyte-targeted gene therapy candidate in which the data will be strengthened through the evaluation of clinically relevant measures in two murine models of VWM, allowing for expeditious translation to the clinic. Our findings support the importance of targeting the underlying disease mechanisms through the modification and tailoring of the AAV vector design.

622. The miREX Platform Technology Allows for Highly Efficacious X-Reactivation for Treatment of X-linked Disorders

Cassandra Dennys[1], Samantha L. Powers[2], Mohammadreza Khani[1], Song Lou[3], Shibi B. Likhite[2], Lucas Sass[1], Andrea Sierra Delgado[2], Stuart Sater[1], Ostin Arters[4], Sanchita Bhatnagar[5], Bryn Martin[1], Deep Singh[6], Kathrin Meyer[7]

[1]Alcyone Therapeutics, Lowell, MA,[2]Nationwide Children's Hospital, Columbus, OH,[3]UC Davis, Davis, MA,[4]Moscow, ID,[5]UC Davis, Davis, CA,[6]Alcyone Therapeutics, Waltham, MA,[7]Nationwide Childrens Hospital, Columbus, OH

X chromosome inactivation is a developmentally regulated process in females, wherein one X chromosome per cell is inactivated through a complex stepwise process involving the Xist RNA. This process allows for dosage regulation in females for X-linked gene expression. As a consequence, female tissue affected by X-linked genetic disorders, such as Rett Syndrome, will be a mixture of cells expressing the mutated version of the gene or the healthy copy depending on which X chromosome is active in any given cell. This mosaicism complicates gene replacement strategies significantly as the healthy cells are at risk of being overdosed. Notably, all cells that express the loss of function mutation contain a healthy copy on the silenced chromosome which, upon re-expression, could ameliorate the disease phenotype. Thus, mechanisms that regulate X-inactivation may be an effective therapeutic strategy for Rett Syndrome and other X-linked disorders. miR106a is a natural regulator of X-inactivation that binds to Xist. Here, we show that sequestration of miR106a via miREX technology (miRNA sponge for Endogenous Gene Expression by X-Reactivation) allows X chromosome reactivation and expression of target genes such as MeCP2. Furthermore, viral vector-based delivery of miREX showed efficacy in both Rett patient derived astrocytes and neurons, with a broad spectrum of MeCP2 mutations, as well as in a severe female Rett mouse model. Moreover, the technology was also efficacious in several other X-linked disorders when tested in various patient cell lines. This demonstrates that miREX may serve as a platform therapy for multiple diseases. To ensure optimal therapeutic benefit, effective targeting of the central nervous system is critical for success in the treatment of neurological disorders including Rett Syndrome. For this purpose, Alcyone Therapeutics has developed a novel intra-CSF drug delivery platform (FalconTM) allowing to optimize drug delivery to the brain. The Falcon technology allows studying and optimizing drug biodistribution taking into consideration disease related changes in CNS anatomy and CSF flow dynamics. Thus, the Falcon technology

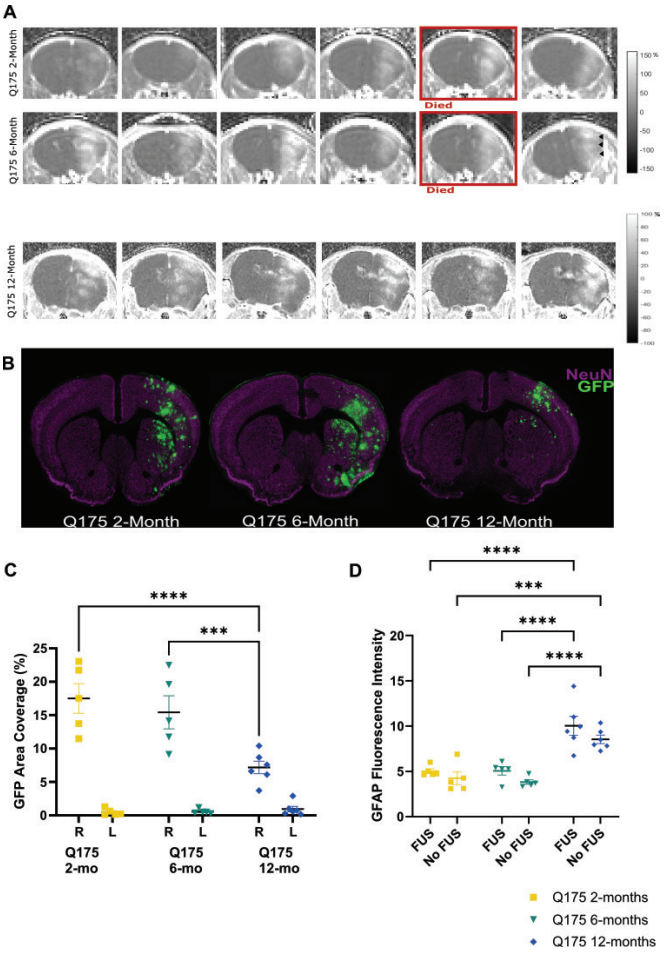
will ensure optimal delivery of miREX in Rett Syndrome patients further improving the therapeutic potential of our novel treatment for X-linked disorders.

623. Focused Ultrasound-Mediated Delivery of AAV9-Based Gene Therapy in Aged Huntington's Disease Mice

Bernie S. Owusu-Yaw[1], Yongzhi Zhang[1], Lily Garrett[2], Alvin Yao[3], Kai Shing[4], Rita Batista[5], Miguel Sena-Esteves[5], Jaymin Upadhyay[6], Kimberly Kegel-Gleason[4], Nick Todd[1]

[1]Brigham and Women's Hospital, Harvard Medical School, Boston, MA,[2]Northeastern University, Boston, MA,[3]Harvard University, Cambridge, MA,[4]Massachusetts General Hospital, Harvard Medical School, Charlestown, MA,[5]UMass Chan Medical School, Worcester, MA,[6]Boston Children's Hospital, Harvard Medical School, Boston, MA

Background: There are currently no cures for Huntington's disease (HD), and lowering HTT mRNA via RNA interference (RNAi) gene therapy appears to be a promising strategy. However, gene delivery to the central nervous system (CNS) is challenging due to the presence of the blood brain barrier (BBB). Focused Ultrasound (FUS) with microbubbles (MB) can be used to temporarily disrupt the BBB in a non-invasive manner to deliver therapeutic genes to the CNS. Safe and reversible BBB opening has been reported from phase one clinical trials in patients with Alzheimer's disease, Parkinson's disease and Amyotrophic Lateral Sclerosis. To date, no studies have been conducted to investigate the safety of FUS-mediated BBB opening in HD patients and there have been no preclinical studies testing the FUS-mediated delivery of adeno-associated viral vectors (AAVs) in mouse models of HD. The breakdown of the BBB has been described in Huntington's disease and other neurodegenerative diseases. Therefore, it is important to determine the impact of HD neuropathology on FUS-mediated BBB opening and subsequent gene delivery. Here, we tested the delivery of a ssAAV9 containing a miRNA (miR1050) targeting HTT mRNA using FUS in combination with MB to the right striatum of 2-month, 6-month, and 12-month-old zQ175 HD mice. **Methods:** This study compares FUS-mediated AAV9 delivery in zQ175 2-month, 6-month, and 12-month-old zQ175 HD mice(N=6 per group). FUS-BBB opening was performed by targeting the right striatum. Mice received four sonications (burst length=10 ms, frequency= 1Hz, total duration= 120s), in the right striatum following the tail vein injection of microbubbles. AAV9-U6-miR10150-CBA-GFP at 5.5 x 10^{11} vg/mouse (2.2 x 10^{10} vg/g) was administered immediately after FUS treatment via tail vein injection. Then gadolinium-enhanced T1-weighted images were acquired to quantify the extent of BBB opening. Mouse brains were harvested after 3 weeks for immunohistochemistry. Brain sections were co-stained with GFP and antibodies recognizing GFAP/Iba1/NeuN/S100β. **Results:** BBB opening was confirmed in all three groups through T1-weighted contrast MRI indicating gadolinium leakage in the brain parenchyma (Figure 1A).Greater GFP expression was observed in the sonicated hemisphere of the three HD cohorts compared to the untreated side (Figure 1B). However, the GFP area coverage was significantly lower in zQ175 12-month mice compared to the younger mice (Figure 1C).It has been reported that FUS-BBB opening can lead to astrocyte and microglial activation. We did not observe any significant differences in astrocytic activation between the FUS-treated and untreated side across the age groups (Figure 1D). There were significantly higher levels of GFAP activation in the zQ175 12-month group in both hemispheres compared to the younger cohorts, which is in line with previous studies demonstrating increased astrocyte activation during HD progression in this mouse model. Staining for Iba1 has been carried out and the slides are being processed. Cell counts are also ongoing to determine the cellular transduction efficiencies across the different age groups.



624. Sandhoff Disease is Corrected in a Murine Model by scAAV9-*HEXM* Gene Transfer

Brianna M. Quinville[1], Alex E. Ryckman[1], Natalie M. Deschenes[1], Melissa Mitchell[2], Kshitiz Singh[2], John G. Keimel[3], William F. Kaemmerer[3], Jagdeep S. Walia[1,2]

[1]Centre for Neuroscience Studies, Queen's University, Kingston, ON, Canada,[2]Department of Pediatrics, Queen's University, Kingston, ON, Canada,[3]New Hope Research Foundation, North Oaks, MN

Sandhoff disease (SD) is an autosomal recessive lysosomal storage disorder characterized by progressive neurodegeneration due to an excessive accumulation of GM2 gangliosides (GM2) in neurons. The resulting widespread neuronal dysfunction eventually leads to death, typically by age 4 in the most common, infantile form. The hydrolysis of GM2 in humans is solely conducted by a single lysosomal enzyme, β-hexosaminidase A (HexA), a heterodimer comprised of an α- and

a β-subunit, encoded by genes *HEXA* and *HEXB*, respectively. SD arises from a deficiency or absence of β-subunits, and consequently, accumulated GM2. Engineered from the human α-subunit, a novel μ-subunit encoded by *HEXM*, homodimerizes to form a stable HexA isoenzyme named HexM. Previously, a proof of concept study demonstrated improved survival in a *Hexb*$^{(-/-)}$ murine model of SD following transfer of the *HEXM* gene packaged in a self-complementary adeno-associated viral vector, serotype 9 (scAAV9). This study assessed the dose response of a scAAV9-*HEXM* treatment in the *Hexb*$^{(-/-)}$ murine model when utilizing a novel method of dual delivery, via intravenous (IV) and intra-cisterna magna (ICM) routes, utilizing osmotic induced flow and ancillary administration of immunosuppressants. A total of 10 cohorts (n = 10/cohort), received simultaneous infusions of vector or vehicle through IV and ICM routes. The study design enables a comparison of treatment efficacy between delivering a total dose through a single route or splitting it between two routes. The researcher who conducted the procedure, behavioural testing, and all analyses was blinded to which infusate each animal received. Behavioural and blood collections were done at specific time points until the mice reached their humane endpoint. Upon termination, tissue and blood samples were collected for analysis of Hex enzyme activity, GM2 accumulation, histology, vector biodistribution, and immune response. Survival results showed a clear dose response and added benefit when a total dose is split between the IV and ICM routes compared to IV alone. While untreated SD mice live only ~16 weeks, the median life span was 88 weeks (>5.5-fold increase) in the IV-only cohort receiving the high dose, and 105 weeks (>6.5-fold increase) in the dual-delivery cohort receiving the same high dose. The heterozygous control group had a median survival of 95.5 weeks. Both high-dose cohorts showed consistent behavioural performance over a two-year lifespan with no observable differences compared to the heterozygous control group. Results of the GM2 accumulation assays and the HexM enzyme activity assays correlated with survival and the added survival benefit of dual delivery. The dual high-dose cohort showed a 92% reduction in GM2 ($p < 0.0001$) compared to untreated SD mice and a >51-fold increase in Hex enzyme activity in sera ($p < 0.005$) compared to heterozygous controls. Hex enzyme activity in both brain and spinal cord regions was significantly higher in cohorts that received an ICM vector infusion compared to the IV-only groups ($p < 0.05$ - $p < 0.0001$). Vector biodistribution shows a dose response in brain regions. This study demonstrates a strong dose-response and greater than two-year gene expression durability in this novel gene therapy for SD with implications for improved survival and quality of life in a clinical setting.

625. Dual Site Administration of AAV Gene Therapy for Treatment of Feline GM1 Gangliosidosis

Amanda L. Gross[1], Courtney Garrett[1], Juan Rodriguez[1], Douglas R. Martin[1,2]

[1]Scott Ritchey Research Center, Auburn University College of Veterinary Medicine, Auburn, AL,[2]Department of Anatomy, Physiology and Pharmacology, Auburn University College of Veterinary Medicine, Auburn, AL

GM1 gangliosidosis is a fatal neurodegenerative disease caused by a deficiency of lysosomal β-galactosidase (βgal). There are currently two ongoing clinical trials for the treatment of GM1 gangliosidosis using adeno-associated viral (AAV) gene therapy administered either intravenously (IV) or in the cerebrospinal fluid (CSF) via the cisterna magna (CM). Preclinical trials of either administration route show that single site treatment significantly increases lifespans in a feline model of GM1 gangliosidosis. We hypothesize that deficiency in efficacy caused by either single site injection will be addressed by the additive effect of combinatorial administration. GM1 cats received $6.0x10^{13}$ vector genomes/kg of AAV9 split equally between IV and CM administration at approximately 2 months of age. Four animals will be followed to humane endpoint and four animals will be evaluated 16 weeks following treatment. Untreated GM1 animals survive approximately 8.0 ± 0.6 months while treated animals have a significant increase in average lifespan to 13.2 ± 2.8 months, with 3 animals in the long term cohort still alive at 16.4, 14.2 and 8.8 months of age (Figure 1A). Clinical assessments include neurological exams, CSF biomarkers, and 7T magnetic resonance imaging (MRI) and spectroscopy (MRS). Postmortem analysis included βgal and AAV distribution. Neurological abnormalities, which in untreated GM1 animals lead to an inability to stand and humane endpoint by 8 months of age, were attenuated in all treated animals (Figure 1B). Aspartate aminotransferase (AST) and lactate dehydrogenase (LDH), biomarkers of central nervous system damage when measured in the CSF, were both reduced in in treated animals in comparison to untreated animals. 7T magnetic resonance imaging (MRI) revealed a delay in progression of neurodegeneration and preservation of brain architecture. While animals are ongoing we are unable to determine if CM+IV administration of AAV is better than either single administration route however, the combined treatment did significantly improve length and quality of life in GM1 cats.

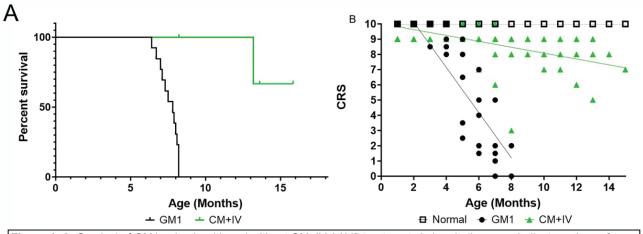


Figure 1. A. Survival of GM1 animals with and without CM+IV AAV9 treatment. A drop in the curve indicates a loss of an animal in the cohort. P=0.0018. **B.** Clinical rating score (CRS) of treated animals. A decrease in value designates progression of neurological deficits. A line of best fit is graphed for each cohort.

626. Developing an AAV-Based Gene Therapy for CSA Related Cockayne Syndrome

Aine Scholand[1], William Callahan[1], Cassandra Izzo[1], Robert King[2], Miguel Sena-Esteves[1], Ana Rita Batista[1]

[1]Neurology / Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Radiology, UMass Chan Medical School, Worcester, MA

Cockayne syndrome (CS) is an autosomal recessive, progressive developmental and neurodegenerative disease, primarily caused by mutations in the CSA/ERCC8 or CSB/ERCC6 gene. Mutations in CSA account for approximately 30% of cases, and it results in defects in transcription-coupled nucleotide excision repair and dysregulation of RNA Pol II mediated transcription. Patients with CS present cutaneous photosensitivity, severe growth failure, dwarfism, short life span and a progressive degeneration of the central nervous system, as well as eye abnormalities (congenital cataracts and retinal degeneration) and hearing loss. CSA is also involved in repair of

oxidative DNA damage, and mitochondrial metabolism, and contribute to the severe manifestations of growth failure and neurodegeneration seen in children.Currently there are no therapies for these patients, with the disease being managed symptomatically. We designed a new self-complementary AAV vector encoding human CSA under a CBA promoter. Initial in vitro experiments in CSA knock-out (KO) HEK293T cells, as well as in CS patient fibroblasts, show that restoration of CSA expression also restores cell survival in a killing assay using the chemotherapeutic drug Illudin S. We tested the therapeutic efficacy of the vector by neonatal ICV injection in CSA/XPA KO mice. This mouse model is a double CSA and XPA KO model, with a very aggressive phenotype, displaying very low body weight (< 5 g at weaning) and median survival of 22 days. We documented an increase in lifespan >8-fold, with the median survival for treated mice recorded at 189 days. Western blot and immunohistochemistry analysis show broad distribution of human CSA in the brain, as well in peripheral tissues. However, we also observed an increase in kidney and liver metabolites in treated animals, when compared to age-matched C57BL6/J controls, as well as presence of microbleeds in the brain, and an increase in autophagy markers in brain and liver. These observations are likely significant contributors to the limited lifespan of the model compared to WT mice. More experiments to investigate impact in metabolism of treatment of CSA/XPA KO mice are currently ongoing. The success of these AAV proof-of-concept experiments support transition to a clinical trial in CS patients.

627. Clinical Safety and Efficacy of EXG001-307 in SMA Type 1 Patients: A Next-Generation AAV-Based Gene Therapy

Zhenhua Wu[1], Chunjuan Song[1], Wenhui Li[2], Xiaojing Li[1], Xiaomei Zhu[2], Hui Li[2], Meijia Yang[1], Wenshu Yang[1], Rui Ji[1], Kuan Lu[1], Yanna Zhang[1], Haifeng Li[3], Lijun Wang[1], Guo-jie Ye[1], Qiang Shu[3], Yi Wang[2]

[1]Exegenesis Bio Inc., Spring House, PA,[2]Children's Hospital of Fudan University, Shanghai, China,[3]The Children's Hospital, Zhejiang University School of Medicine, Hangzhou, China

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disorder that leads to progressive muscular weakness and hypotonia. Gene therapy for SMA has been successfully developed, and the commercialized medicine Onasemnogene abeparvovec (Zolgensma), which was approved by FDA in 2019 as a one-time administration to SMA patients below the age of 2 years, demonstrated significant clinical benefits including prolonged survival and motor milestone achievement . However, current gene therapy faces various challenges including insufficient expression of the *SMN1* gene in the target organs/tissues and off-target organ toxicity such as liver failure. Thus, there is a need for improved gene therapies for SMA with tissue specific *SMN1* expression and reduced off-target toxicity. We developed an AAV-based gene therapy, EXG001-307, favoring neuronal transgene expression. Nonclinical studies have demonstrated that EXG001-307 improved the survival rate, further enhanced the motor function, and significantly reduced toxicity in the SMA mouse model. Moreover, our clinical trial demonstrated an excellent safety and efficacy profile for EXG001-307. The IND for EXG001-307 was approved by the National Medical Products Administration (NMPA) in China and an open-label, multicenter, single-arm, non-randomized, single-dose escalation clinical trial to evaluate the safety and efficacy of a single IV injection of EXG001-307 in patients with type 1 SMA is ongoing and patients enrollment for the Phase 1have been completed. Main inclusion criteria are: 1) SMA is diagnosed with bilateral allelic SMN1 mutations (deletions or point mutations) and 2 copies of the *SMN2* gene; 2) On the day of administration, the age of the subject shall not exceed the 180th day after birth; 3) The clinical history and physical signs are consistent with the symptoms of type 1 SMA. Six patients have been enrolled, and two doses have been studied, $1.1x10^{14}$ vg/kg and $1.5x10^{14}$ vg/kg. EXG001-307 were well tolerated in both groups, with the most common test article related adverse events (AEs) as fever, transient sinus tachycardia; mild elevation of aspartate aminotransferase (AST), troponin I, creatine kinase-MB (CK-MB), activated partial thromboplastin time (APTT) and prothrombin activity; and a reduction trend in platelet, lymphocyte and white blood cell counts. No occurrence of dose-limiting toxicity (DLT), or test article related >Grade 2 serious AEs (SAEs), or ≥ Grade 2 elevation of transaminases or cardiac enzymes in the trial has been reported. All patients achieved significant increase in CHOP-INTEND scores. All three patients in $1.1x10^{14}$ vg/kg group achieved head control in 3-6 months post dose, while all three patients in $1.5x10^{14}$ vg/kg group achieved head control in 1-2 months post dose. The first patient in $1.1x10^{14}$ vg/kg dose group has achieved sitting without assistance around 10 months post dose. In summary, while first-generation gene therapies like Zolgensma have shown substantial benefits for SMA patients, challenges persist in achieving sufficient *SMN1* gene expression in the target organs and avoiding off-target toxicity. Our AAV-based gene therapy, EXG001-307, tackles these issues by favoring neuronal transgene expression. Positive outcomes in our clinical trial, including patients reaching significant milestones with well-tolerated responses, highlight the potential of EXG001-307 as a promising next generation treatment for SMA. Ongoing research in this direction aims to advance EXG001-307, providing targeted SMN expression and minimizing off-target effects for improved outcomes in SMA patients.

628. SKG1201: Liver De-Targeting Vector for Spinal Muscular Atrophy Gene Therapy via Intrathecal Injection

Hui Liu, Carol Huang, Yanqun Shu, Qiu Yao, Xia Liu, Shihua Cao, Ruojie Wang, Yu Wu, Peilu Li, Yanjun Shen, Xiaorui Bian, Li Wang, Xinyi Wu, Qi Deng, Xing Ding, Yuwen Du, Hao Luo, Jay Hou

Skyline Therapeutics, Cambridge, MA

Spinal muscular atrophy (SMA), a devastating neuromuscular disease involving multiple organ dysfunction, is caused by homozygous deletion or mutation of the survival motor neuron (*SMN*)1 gene, resulting in reduced expression of SMN protein, which leads to degeneration of α motor neurons in the spinal cord and muscle atrophy. Gene replacement therapy, Onasemnogene abeparvovec (Zolgensma) demonstrated significant clinical benefits for type I SMA patients. However, due to its high systemic adeno-associated virus (AAV) dose, the adverse events were accompanied, such as significant liver and blood-related toxicity. Moreover, the adverse events observed after intrathecal injection (IT) of Zolgensma in Type II SMA patients in the recent STRONG trial were consistent with what noticed after its intravenous (IV) delivery. Hence, a new AAV gene therapy for

SMA with better safety and efficacy profiles is still in desperate need. Previously we developed SKG0201 for type I SMA patients. A single IV administration of SKG0201 successfully demonstrated an outstanding potency with no liver toxicity compared to the reference vector (scAAV9-CMVen/CB-hSMN1). Currently SKG0201 is in investigator-initiated trials (IIT) to validate its clinical benefits. Recently, we have innovatively developed another novel AAV-mediated gene replacement product (SKG1201) which meticulously comprises liver de-targeting capsid and packaged with a fully optimized SMN expression cassette. Single intracerebroventricular (ICV) delivery in SMNΔ7 mice at postnatal day 1 resulted in a significant improvement in the body weight and extension in the lifespan at less than 1/10 IV doses of SKG0201. Hence, relative to IV delivered SKG0201, IT delivered SKG1201 at a tenfold lower dose could potentially produce an equivalent effect in the brain and spinal cord of the animals. Additionally, head-to-head comparing to the reference vector, SKG1201 showed much better efficacy with the median survival time of ~3 months at a low dosage of 7.5E9 vg/mouse (5E12 vg/kg). Furthermore, SKG1201 consistently demonstrated higher SMN expression level in the spinal cord and much less distribution in the liver compared to the reference vector by IT delivery in adult FVB mice. Currently both nonclinical toxicology and biodistribution studies are being planned to evaluate its safety and biodistribution in cynomolgus monkeys. In summary, SKG1201, a new generation vector which exemplifies a synergistic effect of liver de-targeting and restoration of high expression in the spinal cord, is a prospective gene therapy product for IT delivery to type II/III SMA patients.

629. AAV-RNAi Mediated Lowering of *HTT* for the Treatment of Huntington's Disease

Johanna Adams[1], Brenda Richards[1], Catherine O'Riordan[1], Martin Goulet[1], Robert G. Jackson[2], John Bladon[2], Ye He[2], Zhengyu Luo[2], Christian Mueller[1], Shyam Ramachandran[1], Bradford Elmer[1]

[1]Genomic Medicine Unit, Sanofi, Waltham, MA,[2]IVWG, Sanofi, Cambridge, MA

Huntington's disease (HD) is a fatal, monogenic neurodegenerative disease that affects between 5-17 of every 100,000 persons. Expanded trinucleotide repeats in the huntingtin gene (*HTT*) confer a toxic gain-of-function that causes progressive motor, cognitive and neuropsychiatric impairment for which there is currently no disease modifying treatment. Partial suppression of HTT mRNA and protein in the brain has been demonstrated to be both safe and efficacious in animal models of HD, providing proof-of-concept for a HTT lowering therapeutic strategy that is agnostic to allele type. Artificial microRNAs (amiRNAs) act at the transcriptional level to reduce levels of target RNA, and when expressed from adeno-associated viral (AAV) vectors enable long-term target knockdown. Here, we designed novel amiRNAs targeting *HTT* mRNA and evaluated them *in vitro* and *in vivo* for target engagement and strand loading analysis. We also validated phenotypes in the BACHD rat model of HD for future efficacy studies. Using an updated miRNA screening process to ensure amiRNAs have a low propensity for off-targeting, we designed and screened amiRNA sequences for *in vitro* knockdown of endogenous, human *HTT* mRNA. These amiRNA candidates were ranked *in vitro* based on capacity to reduce *HTT* mRNA and small RNAseq strand processing data to inform selection of five HTT amiRNA sequences for *in vivo* studies. Intrastriatal injection of AAV-HTTamiRNA candidates was performed in HD model YAC128 mice which express full length human mutant HTT. Striatal tissue taken at six weeks post-dosing shows significant *HTT* mRNA knockdown leading to robust 53-63% knockdown of mutant HTT protein. Notably, most sequences have improved strand loading bias *in vivo* relative to *in vitro* assessments. We also validated robust behavioral phenotypes in the BACHD rat model of HD, finding a significant decrease in several open field metrics (male and female) and a robust decrease in rotarod fall latency (females) relative to wildtype. Importantly, intrastriatal administration of our AAV-HTTamiRNA candidates generates significant lowering of striatal HTT protein by 26-47%, proving viability for efficacy studies. Ultimately, four HTTamiRNA candidates show strong therapeutic potential for the treatment of Huntington's disease.

630. From Review to Readiness: CMT4J and the Road to Clinical Trials

Sydney Cooper[1], Keith Gottlieb[2], Souad Messahel[2], Rachel Thomas[2], Jocelyn Duff[3], Terry Pirovolakis[2], Michael Shy[4], Carsten G. Bonnemann[5], John Day[6], Susan Iannaccone[1]

[1]University of Texas Southwestern Medical Center, Dallas, TX,[2]Elpida Therapeutics, Encino, CA,[3]CureCMT4J/Talia Duff Foundation, Ipswich, MA,[4]University of Iowa, Iowa City, IA,[5]NINDS/NIH, Bethesda, MD,[6]Stanford Medicine, Palo Alto, CA

First described in 2007, Charcot-Marie-Tooth Disease Type 4J (CMT4J) is an autosomal recessive neurological disease caused by loss-of-function mutations in the FIG4 gene. It is characterized by progressive loss of motor and sensory neuronal function, with a wide spectrum of symptoms and varying severity. CMT4J is an ultra-rare, severe version of CMT found in less than 0.24% of patients. A review of over 50 published cases of CMT4J characterized the heterogeneity of symptom onset in this patient population. The majority of affected individuals exhibited symptoms before adulthood while a subset experienced adult-onset disease, and a smaller group had unspecified symptom onset. Earlier onset is associated with more severe disease progression, while later onset shows a slower progression, sometimes with symptoms that resemble Parkinsonism. Most common initial symptoms include muscle weakness, neuropathy, and gait difficulties. Progression of the disease can lead to respiratory impairment and dependence on a wheelchair. Treatment is currently limited to management of these symptoms. To address the unmet needs of CMT4J patients and facilitate future therapeutic interventions, the Bespoke Gene Therapy Consortium (BGTC) has selected CMT4J as a leading neurological program for its platform. Furthering this partnership, Elpida Therapeutics will conduct a prospective, observational natural history study aimed at characterizing CMT4J's clinical and genetic spectrum. In this study, 20 individuals will be recruited across four specialized medical centers. Over five years, annual evaluations will be conducted, encompassing a comprehensive array of assessments. These include clinical and neurological exams, laboratory tests to investigate potential biomarkers, CMT-specific outcome measures, neuropsychological tests, nerve conduction studies, muscle fat fraction as measured by MRI, pulmonary function tests, and scoliosis series

x-rays. These assessments will provide invaluable insights into the disease course, inform future therapeutic strategies, and enhance readiness for clinical trials in this rare neurodegenerative disorder.

631. Trial Readiness for First Gene Therapy Interventions in Cockayne Syndrome

Robert Thompson[1,2], Amanda Nagy[1], Nina Gold[2], Eric Macklin[1], Terry Flotte[3], Florian Eichler[1]

[1]Neurology, Massachusetts General Hospital, Boston, MA,[2]Genetics, Massachusetts General Hospital, Boston, MA,[3]University of Massachusetts Medical School, Worcester, MA

CockayneSyndrome (CS) is an autosomal recessive disorder caused by pathogenic variantsin ERCC8 and ERCC6, leading to defective nucleotide excision repair. Patientswith CS develop a progressive, variably severe presentation characterized bygrowth failure, microcephaly, global developmental delay, premature aging,photosensitivity, and multi-organ system pathology. No disease-modifyingtreatment is available. Pre-clinical studies on intracerebroventricularadministration of scAAV9-mediated hERCC8 gene replacement have prompted trialdesign for CS. The report of a 15-point CS severity score in the literature aswell as a medical record review provides a framework for quantifying diseaseseverity and estimation of the window for intervention. We performed a literature review in PubMed assessing papersrelated to CS published between 1992 and 2023. We identified 11 relevant articlesand applied the 15-point CS severity score to define the disease course andsteepest rate of decline after symptom onset. This previously published severity scaleassigns a score of 0 to 3 (0 indicating worst disease and 3 indicating nodisease) for head circumference, weight/height, neurosensorial symptoms,autonomy/motor development, and communication. Giventhe risk of insertional oncogenesis with gene therapy, we reviewed theliterature on whether defective nucleotide excision repair increases cancerrisk in CS. Next, we performed a retrospective chart review on 4 patientsfollowed at our center with ERCC8-related CS (3 living, 1 deceased). We thencalculated a severity score for each patient at 3 different time points. Weused these data to inform our proposed clinical trial design. The literature indicates that classic CS patients decline mostrapidly in the first 2 years of life and their symptoms typically plateauthereafter. Patients with growth failure at birth may have symptoms tooadvanced to warrant trial enrollment. Review of the literature suggests that,despite their defective nucleotide excision repair, patients with CS do nothave an increased risk of cancer (possibly related to arrested transcription). Asexpected severity scores for patients in our cohort declined with time, indicatingworsening disease burden. At last examination the living patients (ages 3-4)had severity scores of 5, 6, and 8, suggesting moderate to severe disease. Thedeceased patient (age 17) was neurologically assessed 3 months prior to deathand had a score of 1 indicating very severe disease. Basedon the existing literature, the optimal time of enrollment in a gene therapytrial would be under 2 years of age which is when patients typically experiencemilder symptoms. The natural history of our deceased 17-year-old patient parallelsreported natural history data in the literature. To exclude patients withgrowth failure at birth or severe motor and communication impairments, wepropose that a severity score of <5 be used as an exclusion criterion.Primary objectives would be safety and tolerability of scAAV9-ERCC8gene therapy, increased MFD free survival, and improved growth parameters.Primary endpoints would be the absence of dose limiting toxicities, time tooccurrence of major functional disabilities, duration of survival,stabilization of CS severity scores and growth parameters, and neurologicalassessments (e.g. brain MRs, developmental scales). Our secondary objectivewould be to assess non-neurological disease burden. Secondary endpoints wouldinclude standard assessments of disease in the pertinent organ systems. Recoveryof RNAsynthesis (decreased in CS) pre and post gene therapy would be an exploratoryendpoint. We will monitor oncogenic risk post gene therapy.

632. An iPSC Based Platform to Develop AAV Gene Therapies for ALS Using Specific and Robust Phenotypic Assays Covering Multiple Disease-Relevant Readouts

Isabel Onofre, Kimberly Riegman, Benoit Samson Couitre, Asha Das, Shushant Jain, Georgios Kosmidis

Ncardia, Leiden, Netherlands

The formation and accumulation of protein aggregates is a hallmark of many neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, frontotemporal dementia or amyotrophic lateral sclerosis (ALS). Misfolding and self-aggregation of proteins consequently lead to alterations in important neuronal functions and progressive neurodegeneration. Currently, available treatments partially ameliorate the symptomatology but do not modify the course of the disease. It is of major interest to develop therapies that can prevent aggregates formation and accumulation, thus, changing patient's prognosis. To enable fast and confident selection of effective therapies we developed specific and robust phenotypic assays to study protein mis-localization and aggregation with iPSC-derived *in vitro* models. In this study, we developed assays with human iPSC-derived motor neurons to study mis-localization and aggregation of TAR DNA-binding protein 43 (TDP-43), a recognized hallmark for ALS as well as multiple neurodegenerative diseases. In addition, mis-splicing of STMN2 and electrophysiological dysfunction were robustly quantified. Utilizing a AAV based gene therapy, we successfully achieve high transduction efficiencies in complex co-cultures of motor neurons and astrocytes, reduce TDP-43 aggregation. alleviate the electrophysiological deficits associated with the motor neurons derived from mutated iPSC lines. Use of a human, physiologically relevant in vitro system, allowed for not only validation of the therapeutic but also enable advancements of a subset of therapeutics with a higher degree of confidence.

633. Relevance of Sex as a Biological Variable in Assessing the Efficacy of a Gene Replacement Therapy for a Mouse Model of XLID98

Eve Racette[1], Melissa Mitchell[2], Peter Gagolewicz[1,3], R. David Andrew[1,3], Jagdeep S. Walia[1,2,3]

[1]Centre for Neuroscience Studies, Queen's University, Kingston, ON, Canada,[2]Department of Pediatrics, Queen's University, Kingston, ON, Canada,[3]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada

Five to ten percent of intellectual disability (ID) cases in males are linked to mutations of various genes located on the X chromosome, one of which is the Neurite Extension and Migration Factor gene (*NEXMIF*). The loss of NEXMIF causes X-Linked Intellectual Developmental Disorder 98 (XLID98), a syndrome characterised by intellectual disability, autism spectrum disorders and drug-resistant epilepsy. While the role of NEXMIF is poorly understood, studies have suggested its involvement in neurite outgrowth and orientation, in neuronal migration and in gene expression. Previous studies of the role of Nexmif in rodents have only been carried out in males, limiting the relevance of these models for the assessment of future gene therapy. Our hypothesis is that the loss of Nexmif causes a variety of neuronal phenotypes due to the alteration of neurodevelopmental processes, such as motility, neurite growth, synaptic formation, gene expression, etc. To verify this hypothesis, we assessed **a)** cognitive impairments using behavioural assays, **b)** hippocampal network excitability and short term facilitation using *ex vivo* local field potential recordings and **c)** gene expression alterations in the brain using MicroArrays. Similar to what is observed in XLID98 patients, loss of Nexmif is associated with sex-specific phenotypes in mice. Affected females are hyperactive in the Open Field test and their hippocampal network activity is decreased as compared to age-matched controls. Affected males are hypoactive in the Marble Burying test, indicative of a lack of interest for the external environment, have hyperactive hippocampal networks and display abnormal gene expression patterns. Interestingly, males also present with adult-onset obesity and frequent seizures. Our data suggests that, like in humans, XLID98 is associated with a variety of sex-specific phenotypes, further reinforcing the relevance of assessing both sexes in the development of gene replacement therapy for this disorder.

634. Development of Helper Dependent Adenovirus Vector (HdAd) Delivery Methods for Wide-Spread Transduction of the Cerebellum

Neda Mokhberian[1], Emre Kul[1], Uchechi Grace Okoroafor[1], Aberman Leah[1], Samuel M. Young[1,2]

[1]Department of Anatomy and Cell Biology, University of Iowa, Iowa City, IA,[2]Department of Otolaryngology, University of Iowa, Iowa City, IA

Helper-dependent Adenoviral vectors (HdAd) are non-toxic, high-capacity viral vectors for gene therapy approaches. They have tremendous potential to treat neurodevelopmental and neurodegenerative disorders that require expression of large transgenes or multiple transgene cassettes from a single vector. In particular, hereditary cerebellar ataxias are monogenic disorders which result in debilitating impairments in movement, cognition, behavior, and ultimately premature death are attractive candidates for HdAd gene therapy approaches. However, cerebellar intracranial injections of HdAd vectors result in limited restricted distribution of the viral vector to the site of injection. Therefore, optimizing delivery techniques to maximize vector distribution and achieve wide-spread transgene expression is an important step in developing viable HdAd gene therapy approaches for cerebellar disorders. Convection-enhanced delivery (CED) is a direct intracranial injection technique that facilitates broad distribution of macromolecules and viral vectors to broad areas within the brain. More importantly, this technique has had tremendous success in clinical trials with AAV vectors to treat human neurological disorders. Therefore, we set out to evaluate CED approaches with HdAd in a mouse cerebellum. We compared this CED to a traditional single bolus injection with a small glass needle. In addition, we optimized our injection protocols to maximize the number of transduced Purkinje cells. Our results demonstrate that these two different delivery techniques results in different transduction pattern and spread within the cerebellar tissue. Further results and implications of our findings will be discussed.

635. Engineering ARMMs with Engagers to Direct Biodistribution to Specific Neurons as a Therapeutic Strategy for Friedreich Ataxia

Wendy Zhao[1], Zahra Baghbantaraghdari[1], Brandon Gerhart[2], Mike Thomas[1], Shu-Lin Liu[1], Jennifer A. Badji[1], Kevin Le[1], Silvia Piccinotti[1], Qin Yu[1], Jill S. Napierala[3], Joseph F. Nabhan[1]

[1]Vesigen Therapeutics, Cambridge, MA,[2]Neurology, University of Texas Southwestern Medical Center, Dallas, TX,[3]Neurology, University of Texas Southwestern Medical Center, Cambridge, MA

The pathological insufficiency of Frataxin (FXN) in Friedreich's ataxia (FA) is caused by bi-allelic expansion of an intronic trinucleotide sequence, GAA, in the *FXN* gene. The expanded repeat region drives formation of heterochromatin and limits potentiation of transcription and access to the transcription start sequence. Consequently, FXN expression in patient cells is reduced by >70% compared to healthy non-carrier levels. FA is a multi-system disease that primarily manifests in sensory proprioceptive neurons and cardiomyocytes. Various strategies such as gene replacement with AAV or FXN protein or mRNA supplementation to treat the loss of proprioceptive functions in FA have encountered significant challenges. Vesigen has been developing a novel approach to potentially treat the major component of FA pathology. We explored the use of ARMMs (ARRDC1-Mediated Microvesicles) as a non-viral and proprioceptor-targeted delivery vehicle for Cas9/gRNA protein complexes to excise the pathogenic repeat expansion in *FXN*. We screened and identified a pair of gRNAs that can efficiently excise the GAA repeat containing region and demonstrated that ARMMs-mediated delivery of Cas9 and 2 gRNAs successfully excise the GAA repeat containing region in a) a cell line containing the non-pathogenic expansion and b) FA iPSC-derived neurons containing bi-allelic pathogenic expansions. Additionally, we developed a strategy to target ARMMs to the proprioceptive neurons. To assist the evaluation of targeted delivery of Cas9/gRNA by surface engineered ARMMs, we established an in vitro testing system and demonstrated targeted delivery using a proprioceptor-specific engager. In summary, our approach provides the foundational evidence for a disease-modifying strategy for FA that could potentially significantly improve the quality of life across the FA patient population.

636. Restoration of Normal Gene and Protein Expression in Mouse and Human Disease Models ofSCN2A Haploinsufficiency Using Zinc Finger Activators

Andrew Olin[1], Jennifer Hodges[1], Yolanda Santiago[1], Jason Eshleman[1], Luke Bogart[2], Jack Hsiao[2], Mary Gulino[2], Madison Riley[2], Stephanie Hromadka[2], Stephanie Petit[2], Patrick Dunn[1], Jisoo Lee[1], Daniel Chung[1], Giulia Cisbani[3], Chiara Melis[3], Finn Peters[3], Toufan Parman[1], Eloise Hudry[2], Katie Worringer[2], Gregory Davis[1], Amy Pooler[1], Bryan Zeitler[1]

[1]CNS, Sangamo Therapeutics, Inc., Richmond, CA,[2]Novartis, Cambridge, MA,[3]Evotec, Hamburg, Germany

Approximately three hundred babies are born each year in the USA with mutations causing SCN2A haploinsufficiency, a rare neurodevelopmental disorder for which no effective treatment exists. These patients suffer global developmental delays commonly associated with autism spectrum disorder (ASD) and intellectual disability (ID), and comorbidities with sleepimpairment and gastrointestinal disturbances. SCN2A haploinsufficiency is caused by de novo loss-of-function mutations in the SCN2A gene resulting in insufficient gene expression and abnormally low levels of its encoded protein, neuronal voltage-gated sodium channel Nav1.2. A potential therapeutic approach to treating genetic haploinsufficiency disorders involvestranscriptional upregulation of the healthy allele to restore normal protein levels. We utilized our zinc finger transcriptional activator (ZFA) platform to upregulate SCN2A expression in healthy and disease model neurons in vitro and in vivo in wildtype (WT) and heterozygous Scn2a+/- mice. In a proof-of-concept study, several hundred ZFAs targeting multiple transcription start sites were designed and screened to identify hot spots for gene activation.We identified several ZFAs targeting either the human SCN2A or mouse Scn2a promoter that upregulated the target gene up to two-fold with high specificity. Selective upregulation of target gene expression by ZFAs subsequently increased and restored Nav1.2 protein to normallevels in vitro in both Scn2a+/- mouse cortical neurons and engineered human embryonic stem cell (hESC)-derived neurons containing common patient mutations modeling SCN2A haploinsufficiency. In adult mice treated intravenously with an AAV. PHP.B ZFA, a 1.5-2-fold increase in Scn2a and Nav1.2 expression was confirmed in multiple brain regions using bulk RT-qPCR and immunohistochemistry (IHC). Upregulation of Scn2a levels by more than 2-fold was verified at the single-cell level via multiplexed in situ hybridization (ISH) and IHC. Corroborating the results in WT mice, Scn2a upregulation was also observed in the brains of Scn2a+/- mice treated with AAV9-ZFA four weeks following intracerebroventricular (ICV) administration.Taken together, our results demonstrate a proof-of-concept that targeted SCN2A upregulation by ZFAs increases gene expression in human and mouse models of SCN2A haploinsufficiency and restores Nav1.2 levels to normal in Scn2a+/- mouse cortical neurons, thereby providing support for the advancement of a ZF Activator approach to treat SCN2A haploinsufficiency.

637. Zinc Finger Mediated Repression and Replacement of MFN2 Leads to the Rescue of Cellular Disease Phenotype in CMT2A Patient-Derived Cells

Mohammad Samie, Luke Workley, Matthew Mendel, Mihika Jalan, Lee Jisoo, Patrick Dunn, Jason Eshleman, Hung Tran, Sarah Hinkley, Bryan Zeitler, Amy Pooler

Sangamo Therapeutics, Inc., Richmond, CA

Charcot-Marie-Tooth Type 2A (CMT2A) is a dominant negative disease caused by damage to motor and sensory neurons. Patients develop progressive muscle weakness and atrophy at an early age in the feet, ankles, and legs that ultimately leads to paralysis. CMT2A symptoms are a result of loss of function mutations in the Mitofusin 2 (MFN2) gene. MFN2 is a GTPase protein expressed on the outer mitochondria membrane and responsible for mitochondrial fusion, metabolism, and transport. Considering the critical role MFN2 protein plays in mitochondria homeostasis and function, biallelic repression of the mutated gene is predicted to reduce disease toxicity but would potentially result in a loss-of-function phenotype due to lack of a healthy protein. Indeed, complete loss of MFN2 in animal models is known to be lethal. Thus, in a proof-of-concept study, we developed a novel approach to repress both the mutant and healthy endogenous alleles, and to replace them with a functional/healthy cDNA of MFN2. The goal of this repress and replace strategy is to simultaneously rescue the disease phenotype and restore MFN2 protein function in cells. A library containing more than 400 engineered zinc finger repressors (ZFRs) was initially screened in human and rodent neuronal cell lines and the lead ZFRs were packaged into AAV and screened in human iPSC-derived GABAergic and rat primary cortical neurons *in vitro*. We identified ZFRs that repressed MFN2 mRNA and protein by more than 90% over a wide dose range with minimal detectable off-target activity as measured by global transcriptomics analysis, in both human and rat primary neurons. Lead ZFRs were cloned into a vector expressing a codon optimized MFN2 transgene. Vectors containing both the MFN2 transgene and the ZFR under a single neuronal promoter were tested for expression and activity of both MFN2 and ZFR in primary rat neurons and human iPSC-derived GABAergic neurons. We were able to observe a significant reduction of the endogenous MFN2 gene in both rat and human neurons, simultaneously we observed significant expression of MFN2 transgene from the MFN2 cDNA, replacing the endogenous MFN2 in the cells. To evaluate the feasibility of the repress and replace strategy, we evaluated the efficacy of the vector in patient derived cells. The vector restored wildtype MFN2 while concurrently knocking down endogenous mutant MFN2 in the cells which led to a rescue of the mitochondria depolarization phenotype observed in disease cells. Together, these data confirm that the repress and replace strategy could potentially provide a novel therapeutic approach for the treatment of CMT2A.

638. AAV Conjugated to Antibodies Against P75^{NTR}: A New Platform to Develop Pain Therapeutics Targeted to Nociceptive Sensory Neurons

Adina Buxbaum[1], Mathieu Desclaux[2], Jennie Lipinski[1], Sando Dickinson[1], Chun Wai Yip[2], Osama Chaudry[1], Robert Breese[1], Poulami Samai[2], Karole D'Orazio[2], Lynn Macdonald[1], Christos Kyratsous[2], Sven Moller-Tank[2], Nicole Alessandri-Haber[1]

[1]Neuroscience, Regeneron Pharmaceuticals, Tarrytown, NY,[2]Regeneron Genetic Medicines, Regeneron Pharmaceuticals, Tarrytown, NY

Chronic pain is a prevalent condition, affecting about 20% of the population, with a high unmet need for pain relieving drugs with high efficacy and low off-target side effects. Despite decades of preclinical research, standard of care remains opioids and non-steroidal anti-inflammatory drugs. Antibody-based delivery of therapeutic cargo to the cells underlying pain pathology may provide improved drug efficacy and reduced off-target effects. Cross functional, in-house efforts identified the neurotrophin receptor p75^{NTR} as a potential candidate for targeting the peripheral nervous system. We find that antibodies against p75^{NTR} internalize in sensory neurons in Dorsal Root Ganglia (DRG) and Trigeminal ganglia (TG), as well as the parasympathetic nervous system in the colon and sympathetic neurons. When anti-p75^{NTR} antibodies were conjugated to AAV particles and injected systemically, GFP expression driven by the viral episome was detected in 85-95% of the DRG neurons and GFP expression was enhanced 7-fold relative to undecorated AAV. Local, subcutaneous injections of anti-p75^{NTR} conjugated to AAV demonstrated the ability to travel retrogradely from the injection site to innervating sensory neurons; Injection of anti-p75^{NTR} -conjugated AAVs in the whisker pad transduced ipsilateral, but not contralateral TG neurons. Similarly, when anti-p75^{NTR} -conjugated AAVs were injected into the synovial fluid in the knee, ipsilateral lumbar DRG neurons exhibited GFP expression at similar levels to DRG neurons from animals that were injected systemically with up to 8x the viral load.The ability to non-invasively and specifically deliver AAVs or other therapeutic cargo to nociceptive sensory neurons has been a great unmet need in the pain field. The voltage-gated sodium channel Nav1.7 is one of the best validated pain targets; genetic and physiological evidence support its key role in pain processing. Therefore, we generated an shRNA targeting mouse Nav1.7 and packaged it in AAV9 with modified tropism. We demonstrate here that intrathecal delivery of AAV expressing an shRNA targeting mouse Nav1.7 effectively reduced thermal hyperalgesia in a preclinical animal model of inflammatory pain. Systemic or local subcutaneous injections of AAV conjugated to antibodies against p75^{NTR} could be transformative in the development of therapeutics for chronic pain, especially in the context of challenging targets such as Nav1.7.

639. Translating Lumbar Intrathecal RNAi-Based Gene Therapy for CMT1A Using CMT1A Mice, Human Cells, and Non-Human Primates

Lindsay M. Wallace[1], Merlin Thangaraj[1], Marina Stavrou[2], Brian Price[3], Gloria Zender[1], Bilal Bayazit[1], Noah Taylor[4], Kleopas A. Kleopa[5], Scott Q. Harper[6]

[1]Nationwide Children's Research Institute, Columbus, OH,[2]The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus,[3]Armatus Bio, Columbus, OH,[4]Nationwide Children's Hospital, Columbus, OH,[5]The Cyprus Institute of Neurology & Genetics, Nicosia, Cyprus,[6]Ohio State University & Nationwide Children's Hospital, Columbus, OH

Background: CMT1A is the most common inherited demyelinating peripheral neuropathy, affecting ~1.5 million people. It is caused by *PMP22* gene duplication, leading to over-expression of the PMP22 protein, myelin sheath destabilization in Schwann cells (SC), secondary axonal loss, and disability. We propose that normalizing PMP22 levels is the most direct route to CMT1A treatment. To do this, we developed an AAV9 vector expressing an engineered miRNA (scAAV9-U6-miR871) that reduces *PMP22* expression and improves neuropathy in a CMT1A mouse model following lumbar intrathecal (IT) delivery. This program received orphan drug and rare pediatric disease designations from FDA for the treatment of CMT1A, and we are now working to demonstrate safety and translatability by assessing potential off-target effects in human cells, as well as demonstrating biodistribution, target engagement and tolerability in *Macaca fascicularis*. Although lumbar IT delivery of AAV9 in NHPs was previously tested, to our knowledge no one has examined SC transduction using this route of administration in a large animal model. Methods: For off-target studies, we designed lentiviruses expressing U6-miR871 and a puromycin resistance gene, or puromycin alone. We transduced human neuron, hepatocyte, or SC cultures, collected RNA after puromycin selection, and performed RNA-seq to assess gene expression changes, followed by binding assays to empirically determine if miR871 and potential target sites on downregulated transcripts are real. In NHPs, we IT-delivered 2 doses of AAV9-U6-miR871 (6e13 or 1.2e14 vg/animal), or saline, and analyzed animals 6- or 12-weeks later. We performed clinical and neurological observations and collected longitudinal samples to assess hematology, serum, coagulation, chemistry, and urine parameters. Prior to study termination, animals underwent ophthalmic testing, electrocardiogram, nerve conduction studies, and testing for AAV9 neutralizing Abs, followed by histopathological and molecular analysis of necropsied tissues. Results: We found minimal transcript reductions in miR871-treated cells versus controls. Importantly, *PMP22* was significantly reduced by miR871 in each cell line, supporting target engagement in humans. Bioinformatics analyses do not support binding of downregulated transcripts by miR871, but we are empirically assessing target site interactions. These results support that our RNAi approach is on-target and safe. NHP studies support translatability of scAAV9-U6-miR871, with both doses well-tolerated, resulting in efficient transduction of PNS tissues even in distal nerves. Subclinical DRG inflammation was noted in some treated animals, but in-life observations showed no behavioral abnormalities, no clinical chemistry alternations, and no impairment of nerve conduction. Importantly, we also demonstrate PMP22 protein knockdown *in vivo*, supporting target engagement. Discussion: We provide the first detailed biodistribution analysis of an intrathecally-delivered AAV9 vector targeting Schwann

cells in the PNS of *Macaca fascicularis*, along with promising safety data *in vivo* and in human cell lines representing organs highly transduced by AAV9. Collectively, our data support biodistribution to peripheral nerves, target engagement, safety, tolerability, and the therapeutic potential of scAAV9-U6-miR871. We believe this study not only supports translation of CMT1A gene therapy, but also establishes a strategy to treat other demyelinating neuropathies.

640. Development of hfCas12Max-Based Gene Editing Therapy for Huntington's Disease

Dong Yang[1], Guoling Li[1], Xiaoqing Wu[1], Jin Zheng[1], Lecong Wang[1], Tong Li[1], Shaoran Wang[1], Xuan Yao[1], Alvin Luk[1], Hui Yang[1,2], Linyu Shi[1]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: Huntington's disease (HD), a lethal and inherited disease, is the most frequent autosomal dominant neurodegenerative condition caused by the CAG trinucleotide repeat expansion in the exon 1 of the huntingtin (*HTT*) gene, resulting in the production of mutant huntingtin (mHTT) protein with an elongated polyglutamine tract which leads to progressive disruption of neuronal physiology. This disorder is characterized by the progressive degeneration of neurons in the striatum and cerebral cortex, manifesting as chronic movement, psychiatric problems, and cognitive impairments that appear during mid-life and slowly progressive decline over two decades until death with no approved treatment. It was reported knockdown of *HTT* effectively inhibits the formation of mHTT protein. Here, we introduce a novel CRISPR-DNA gene-editing approach using high-fidelity Cas12Max (hfCas12Max), an engineered Cas12i variant demonstrating high editing efficiency and specificity, with guide RNA targeting *HTT* (g*HTT*) as a potential treatment for HD. **Methods:** We constructed a single recombinant adeno-associated viral (AAV) vector packaging for both hfCas12Max and g*HTT*. Subsequently, we performed bilateral intrastriatal injections of either AAV-hfCas12Max-g*HTT* or PBS to the striatum of 3-month-old zQ175 mice, a knock-in (KI) mouse model of HD. Four weeks after the injection, we evaluated the indel efficiency and mHTT protein levels in the striatum. We then checked for mHTT protein aggregation in the striatum four months after the injection and monitored any changes in motor function of the zQ175 KI mice monthly beginning one month after the injection. **Results:** In the zQ175 KI mice treated with AAV-hfCas12Max-g*HTT*, the indel ratio of *HTT* reached 67.5% ($p<0.001$) and the knockdown efficacy of mHTT protein was up to 72% ($p=0.0018$) in the striatum four weeks after the injection when compared to the zQ175 KI mice injected with PBS (Fig.1a). The mHTT protein aggregates in the striatum of zQ175 KI mice 4 months after injection the AAV-hfCas12Max-g*HTT* was decreased 80% when compared to those zQ175 KI mice treated with PBS. Notably, the levels were almost rescued to those observed in wild-type mice ($p<0.01$, Fig. 1b). Furthermore, zQ175 KI mice treated with AAV-hfCas12Max-g*HTT* showed shorter traverse times on the balance beam at 1-, 2-, 3-, and 4-month post-injection, indicating motor improvements (at 4-month; $p=0.0003$) when compared to the zQ175 KI mice treated with PBS at 4-month post-injection. Their agility approached that of wild-type mice, suggesting near-normalization of motor function in the beam test (Fig. 1c). **Conclusions:** Our findings provide a solid foundation of using AAV-hfCas12Max-g*HTT* gene-editing therapy approach to treat HD, which has demonstrated to reduce the mHTT protein level and improve the motor function in mouse model of HD.

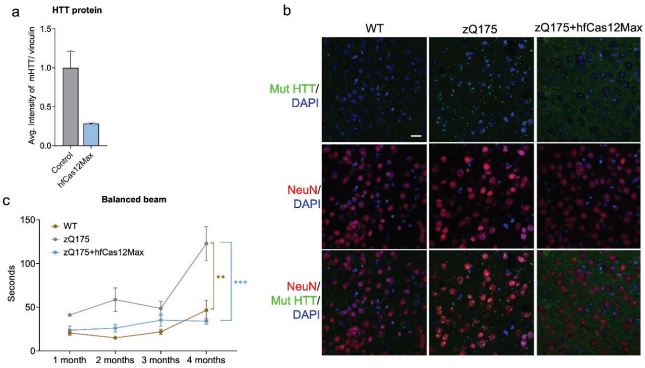


Figure 1. The treatment effect of AAV-hfCas12Max-g*HTT* in HD mice. (p<0.01, ***p<0.001)**

641. Understanding Drivers of Patient Acceptance Towards Invasive Gene Therapy Clinical Trials

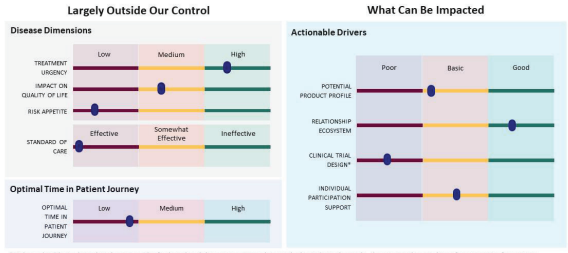
Carl Vandeloo[1], Olivier Baron[1], Alok Gangaramany[2], Christina Hanschke[2], Kerrie-Anne Ho[3], Hritika Parekh[4], Jesper Jørgensen[3]

[1]UCB Pharma, Anderlecht, Belgium,[2]Final Mile Consulting, New York, NY,[3]UCB Pharma, Slough, United Kingdom,[4]Final Mile Consulting, Mumbai, India

INTRODUCTION: The unique properties of gene therapies (GTs) present certain challenges in identifying patients willing to participate in GT clinical trials. Patients may lack trust and can be apprehensive about participating, particularly where the GT administration route is highly invasive. Concerns are heightened as GTs are new, highly technical, and levels of patient understanding can vary (efficacy, safety, durability of response). Patients express concerns that participation in GT trials may exclude them from taking part in future trials. This study aims to understand what drives patient GT acceptance, how it changes over time, and how key stakeholders, such as researchers and trial investigators, can support patient choice. **METHODS:** A literature review performed on 13 rare diseases and neurologic conditions, and insights from patient, caregiver, healthcare provider (HCP), and clinical research organization (CROs) interviews helped identify key drivers of patient GT acceptance. An acceptance framework was developed, and actionable drivers, and barriers to participation were identified. The acceptance framework comprised three elements: disease dimensions, optimal time in a patient journey, and actionable drivers (rated qualitatively low, medium, high). **RESULTS:** In total, 102 articles/conference proceedings were identified exploring behavioral aspects of each disease, caregiver preferences, clinical decision-making, patient-provider communication, trial acceptance, altruism, and GT acceptance; insights were gathered from 10 patients/caregivers, 20 HCPs, and one CRO. Research and stakeholder interviews identified trial design and individual participation support as key drivers of patient GT acceptance. The acceptance framework (**Figure 1**) showed that convergence of several elements makes patients more receptive to

GT trials. Key insights include: (1) Acceptance is a dynamic process that evolves over time. Patients and caregivers may be more receptive to GTs at certain points in the treatment journey, but not in others due to disease severity/prognosis, quality of life (QoL) impact, and standard of care (SoC) efficacy. Openness to GT trials is higher at certain points along the journey; (2) Acceptance is not binary. Agreeing to try GT does not negate hesitation; patients may still have questions and concerns. Even when people agree to take part, support is needed to ensure they feel confident to participate; (3) Acceptance is different from participation. Despite GT openness, several logistic and personal factors need to be addressed to support participation (patient demographics, trial knowledge, risk and benefit perceptions, personal motivations). It is important to have mechanisms in place to help participants overcome the practical barriers when GT trials are designed. Disease archetype (slowly/rapidly degenerative, early development disruption, adaptable) is a critical factor affecting GT acceptance, treatment urgency, QoL impact, and risk appetite. SoC perception can vary by disease; actionable drivers can be strengthened through engagement strategies. **CONCLUSIONS:** When developing GTs, it is critical to take account of three elements: disease dimensions, optimal time in a patient journey, and actionable drivers to increase the likelihood of patient participation in GT clinical trials.

Figure 1. Summary of GT Acceptance Framework



*Only applicable in clinical trial context. The further the sliders are positioned towards the right in the scale, the greater the window of opportunity for patient acceptance for a GT clinical trial.

642. A Zinc Finger Activator Platform to Restore Normal Gene & Protein Expression in Cellular Models of Dravet Syndrome

Jennifer L. Hodges[1], Andrew Olin[1], Yolanda Santiago[1], Dave Paschon[1], Irene Tan[1], Gillian Houlihan[1], Patrick Dunn[1], Jisoo Lee[1], Qi Yu[1], Annemarie Ledeboer[1], Giulia Cisbani[2], Chiara Melis[2], Finn Peters[2], Greg Davis[1], Amy Pooler[1], Bryan Zeitler[1]

[1]Sangamo Therapeutics, Inc., Richmond, CA,[2]Evotec, Hamburg, Germany

Dravet Syndrome (DS) is a severe neurodevelopmental disorder and epileptic encephalopathy characterized by intractable seizures, intellectual disability, motor deficits, and a 10% to 20% rate of premature death. Most DS cases are caused by *de novo* loss-of-function mutations in the *SCN1A* gene resulting in insufficient gene expression and abnormally low levels of its encoded protein, neuronal voltage-gated type I sodium channel, $Na_v1.1$. Genetic restoration of $Na_v1.1$ has been shown to reduce seizures and extend survival in various mouse models of DS. Our approach to restoring normal $Na_v1.1$ levels involves targeting a zinc finger protein fused to a novel transcriptional activator domain to the *SCN1A* promoter to drive transcriptional upregulation of the healthy allele. In contrast to other published transcriptional activator approaches that incorporate elements of bacterial or viral origin in the DNA-binding domain and/or effector domain, our zinc finger activator (ZFA) platform consists of human-derived components naturally expressed in the brain. In a proof-of-concept study, we identified several ZFAs targeting conserved regions of the *SCN1A* promoter that upregulated *SCN1A* gene expression by multiple-fold in human induced pluripotent stem cell (iPSC)-derived neurons and in mouse cortical and hippocampal neurons derived from wild-type (WT) and heterozygous $Scn1a^{+/-}$ mice modeling DS. Selective upregulation of target gene expression by ZFAs subsequently increased and restored $Na_v1.1$ to normal levels *in vitro* in disease-model $Scn1a^{+/-}$ mouse cortical neurons. Many ZFAs were shown by microarray analysis to be highly specific for activating the *SCN1A* gene with no detectable off target regulation in human iPSC-derived neurons. The functionality and safety of our ZFA approach was demonstrated *in vivo* in adult WT mice treated intravenously with an AAV.PHP.B-ZFA under control of the human Synapsin 1 promoter (hSyn1). No clinical or cellular signs of toxicity were observed, and significant upregulation of *Scn1a* mRNA levels was confirmed in single cells expressing the ZFA via multiplexed *in situ* hybridization and immunohistochemistry (ISH/IHC). Our results demonstrate proof-of-concept for our zinc finger transcriptional activator (ZFA) platform as a potential therapeutic approach to treating Dravet syndrome.

643. Low-Intensity Focused Ultrasound Targeting of AAV Serotypes to the Central Nervous System

Rachel M. Bailey[1], Ibrahim Youseff[2], Sydni Holmes[1], Irvin T. Garza[1], Rajiv Chopra[2], Bhavya R. Shah[2]

[1]Center for Alzheimer's and Neurodegenerative Diseases, UT Southwestern Medical Center, Dallas, TX,[2]Department of Radiology, UT Southwestern Medical Center, Dallas, TX

Recombinant adeno-associated virus (rAAV) vectors are routinely used for gene therapy due to their efficient gene transfer, safety profile, and long-term transgene expression in non-dividing cells, such as neurons. AAV9 is currently the most frequently used serotype for treating neurological disorders as it can be delivered in blood or cerebrospinal fluid (CSF) to broadly transduce the brain in a dose-dependent manner. Direct brain injections are effective for focal brain treatments but are an invasive procedure. With non-invasive systemic delivery, high vector doses are needed to achieve sufficient brain transduction in adults, which increases the risk of severe immune response. Use of transcranial low-intensity focused ultrasound (FUS) to temporarily open the blood brain barrier (BBB) can significantly reduce systemic vector doses and target rAAV vectors to select brain regions. To determine viral vectors amenable to FUS-mediated delivery, we assessed transduction of striatal neurons using a panel of naturally occurring and engineered AAV capsids in C57Bl/6 mice. Mice received an IV infusion of Definity microbubbles and underwent FUS-mediated opening of 3 targets in the striatum of the right hemisphere. BBB opening was confirmed using gadolinium-enhanced MRI and then mice were infused with $1x10^{12}$ vg/kg of an AAV vector. Three weeks after IV+FUS delivery, brain and liver transduction were assessed by measuring expression of the transcapsidated GFP reporter. Of the serotypes assessed, AAV9, AAV1, and AAV8 resulted in the highest brain transduction, while

AAV2, AAV4, and AAV5 resulted in little to no striatal transduction. These findings demonstrate that FUS-mediated delivery differentially enhances delivery of AAV serotypes to the brain. While IV+FUS delivery is promising for focal treatments, this limits the ability of AAV to efficiently transduce non-targeted brain regions. CSF delivery of AAV9 significantly reduces the amount of vector needed to achieve equivalent or greater brain transduction compared to systemic delivery and achieves high CNS transduction relative to the site of injection. Therefore, we hypothesized that transient disruption of the BBB can direct IT injected AAV to specific brain regions while also broadly transducing the hindbrain and spinal cord. To test our prediction that FUS-mediated opening of the BBB can enhance brain transduction of IT-injected AAV9, we performed a pilot study in C57BL/6 mice. Mice received an IT or IV injection of scAAV9/Cbh-GFP $4x10^{10}$ vg/mouse and FUS was used to unilaterally open the BBB in the hippocampus and the striatum in half of the IT treated mice and in all IV treated mice. Brains were assessed 3 weeks post-vector delivery for IHC staining with a GFP antibody. Tissues were also collected and processed for vector biodistribution. We found that compared to the FUS+IV route, FUS+IT resulted in 1) greater transduction of targeted brain region, 2) greater transduction of non-targeted hind brain regions, 3) greater transduction of spinal cord, and 4) decreased vector distribution to the liver. Compared to IT alone, FUS+IT delivery resulted in ~30% higher transgene expression in the hippocampus when FUS was applied. In an additional study, we found that IT+FUS delivery can also be used to target IT-injected AAV8 to the brain. Overall, these results support that IT+FUS delivery of AAV has the potential to provide greater treatment benefit with decreased risk of toxicity as compared to IT alone or IV+FUS delivery.

644. AAV Hamartin Gene Therapy in a Stochastic, Cranial Mouse Model of TSC1

Xandra O. Breakefield

Neurology, Massachusetts General Hospital / Harvard Medical School, Charlestown, MA

Tuberous sclerosis complex type 1 (Tsc1) is a dominantly inherited disease in which individuals are born with one defective allele for hamartin, with a somatic loss of the other allele, during development or later in life, leading to upregulation of cell growth in susceptible tissues. Ninety-five percent of affected individuals have brain involvement, including epilepsy, cognitive impairment, autism and/or sleep disorders. In the stochastic, cranial mouse model of Tsc1, loss of function of hamartin is induced at birth by injection of Cre recombinase encoded in an adeno-associated virus (AAV) vector into the cerebral ventricles at birth. This leads to: 1) proliferation of subventricular cells, 2) enlarged neurons, 3) elevated phosphorylation of ribosomal protein S6, 4) dysmyelination and 5) early death. Intravascular administration of an AAV9 vector encoding hamartin at postnatal day 21 led to significant ($p<0.05$) normalization of perturbations 1), 2) and 3), with a trend towards normalization of 4) by postnatal day 42, with extension of average lifespan from 50 days to over 150 days in 85% of animal receiving a dose of $5x10^{13}$1.5E13 vg/kg (n=11).

645. Clinical Dose Selection for ICV-Administered AAV9-NGLY1 Gene Replacement Therapy (GS-100)

Lei Zhu, William F. Mueller, Jennifer W. Cook, Alicia Newton, Selina S. Dwight, Brendan Beahm, Matt Wilsey, Becky Schweighardt

Grace Science LLC, Menlo Park, CA

GS-100 is an AAV9 gene replacement therapy for the treatment of N-glycanase 1 (NGLY1) Deficiency, an ultra-rare, debilitating recessive disorder due to loss-of-function mutations in the NGLY1 gene. Patients suffer from global developmental delay, intellectual disability, hyperkinetic movement disorder, elevated liver enzymes, (hypo)alacrima, and peripheral neuropathy. NGLY1 is a cytosolic enzyme that cleaves glycans from proteins as part of the degradation pathway. NGLY1 specifically cleaves between a GlcNAc residue and an asparagine residue on N-glycosylated proteins targeted for degradation. In NGLY1's absence, GNA (GlcNAc-Asn) accumulates as a biomarker of NGLY1 Deficiency. Increased levels of GNA can be detected in the CSF and plasma of NGLY1 Deficiency patients.We assessed the efficacy, safety, and biodistribution of intracerebroventricular (ICV) administration of GS-100 in a *Ngly1*$^{-/-}$ rat model that exhibits phenotypes similar to those found in patients. ICV administration of GS-100 to *Ngly1*$^{-/-}$ rats was associated with dose-dependent vector biodistribution and hNGLY1 mRNA expression, reduction in GNA biomarker levels, motor function improvement, and improvement in DRG disease-related pathology. Locomotor function in GS-100 treated rats, as assessed by rotarod, reached levels near wild-type following GS-100 treatment. In addition to the observed phenotypic improvements, GS-100 prevented disease progression over time in the *Ngly1*$^{-/-}$ rats. GNA reduction in CNS tissues and CSF significantly and inversely correlated with functional improvements in *Ngly1*$^{-/-}$ rats across all pharmacology studies. Additionally, the significant correlation between GNA levels in CNS tissues and CSF suggests that assessing CSF GNA levels in the clinic will provide a way to assess the activity of GS-100 in CNS target tissue. Safety and biodistribution of GS-100 were assessed in wild-type rats and NHPs. Based on these safety studies and the pharmacology studies in the *Ngly1*$^{-/-}$ rat model, the clinical starting dose of 4e14 vg/individual was selected as a dose anticipated to be both safe and offer the prospect of direct benefit to NGLY1 Deficiency patients. ICV administration at or near the clinical starting dose was not associated with any mortality, clinical observations, body weight changes, or changes in clinical pathology parameters that were considered adverse. Additionally, there were no negative treatment-related effects on functional observational battery testing or nerve conduction velocity (NCV). Widespread dose-dependent biodistribution of GS-100 vector DNA was detected in the brain, spinal cord, and multiple peripheral tissues in rats and NHPs.The GS-100 IND application has been approved by the FDA to support a first-in-human (FIH) open-label, dose-escalation Phase 1/2/3 study (National Clinical Trial identifier [NCT] 0619953) to assess the safety and efficacy of ICV-administered GS-100 in subjects with NGLY1 Deficiency. The co-primary outcomes in the pivotal part of the clinical trial will be a change from baseline in cerebrospinal fluid GNA biomarker and improvement or stabilization of motor function.

646. Astrocyte to Neuron Reprogramming for Neural Repair: The Impact of Promoters, Cellular and Regional Heterogeneity on Astrocyte Targeting

Jack W. Hickmott, Gajeni Prabaharan, Tom Enbar, Ricky Siu, Varanan Vejeyathaas, Cindi M. Morshead
Surgery, Donnelly Centre, University of Toronto, Toronto, ON, Canada

Neurogenesis in the adult brain is rare and restricted to specific neurogenic regions (*e.g.* the periventricular lining in the forebrain and the dentate gyrus in the hippocampus). Consequently, when neurons are lost due to brain injuries such as stroke, the result can be life long cognitive and physical impairment, depending on the location of the insult. Currently strategies to treat stroke seek to minimize neuron loss during the injury. There are no approved therapies for stroke recovery, with the exception of rehabilitation to promote neuroplasticity, which has limited success, highlighting the need for novel therapeutics. One promising avenue is direct cellular reprogramming to replace lost neurons. Astrocytes are abundant GFAP expressing cells that are activated following brain injury, making them a promising target for astrocyte to neuron (AtN) conversion. The approach involves the transduction of neural cells with adeno associated viruses (AAV) that will deliver proneural factors expressed from the astrocyte-specific, short human GFAP (shGFAP) promoter, resulting in astrocyte specific reprogramming. A number of considerations for optimizing AtN conversion include: (1) Astrocyte heterogeneity: astrocytes are a heterogenous cell population and the efficacy of AtN conversion within the distinct pools has not been explored, (2) Regional heterogeneity: the degree of AAV transduction varies across brain regions, and (3) Promoter promiscuity: the shGFAP promoter may not retain astrocyte specificity throughout the brain. Here we investigate the fidelity of the shGFAP promoter in driving astrocyte versus neuron specific gene transcription and compare it to the performance of an alternative astrocyte promoter from the human *S100B* gene. First, AAV5 expressing Cre from the shGFAP promoter (AAV5-shGFAP-Cre) was injected into two regions of the cortex (medial prefrontal cortex or motor cortex) of adult Ai14 (CAG-Lox-Stop-Lox-tdTomato) reporter mice. As predicted, astrocytes expressed tdTomato at 21 days post transduction. Surprisingly, we observed tdTomato+ neurons within the brain with significantly more tdTomato+NeuN+ cells in the medial prefrontal cortex compared to the motor cortex (52% and 11% of transduced cells, respectively; $p = 0.0105$). We next asked if *Gfap* is transcribed in NeuN+ cells using RNAScope for *Gfap* mRNA. Interestingly, *Gfap* mRNA was detected in NeuN+ cells in a regionally distinct manner (51% vs 42% of NeuN+ cells in the medial prefrontal and motor cortex, respectively, $p = 0.039$), revealing regional heterogeneity of *Gfap* RNA within neurons, which may account for off-target expression from the shGFAP promoter. A transcription factor binding site analysis of the shGFAP promoter revealed binding sites for 13 transcription factors modestly expressed in neurons, which is consistent with the shGFAP promoter driven expression in neurons. Given the shGFAP promoter driven transgene expression from AAV in neurons, we tested an alternative *S100B* derived astrocyte promoter. AAV5-S100B-EmGFP or AAV5-GFAP-EmGFP were injected into the motor cortex of C57Bl/6 mice. Interestingly, using this direct EmGFP reporter system (i.e. expression is not Cre dependent) we observed high specificity for the GFAP+ subpopulation of astrocytes with both promoters (55% vs 56% EmGFP+GFAP+ cells; shGFAP vs S100B promoters, respectively) and rare off-target expression in NeuN+ neurons (2% vs 3% EmGFP+NeuN+ cells; shGFAP and S100B promoters, respectively). These findings suggest that astrocyte specificity may be dependent on the Cre-Lox based reporter system. Together, these findings highlight neural cell heterogeneity as an important consideration when developing AtN reprogramming strategies for neural repair.

Wednesday Posters: Ophthalmic and Auditory Diseases

647. Preclinical Development of a Gene Augmentation Therapy for PRPF31-Associated Retinitis Pigmentosa

Lindsay Wong[1], Bin Wang[2], Ingrid Pardo[3], Steven Tabor[1], Ankur Thomas[2], Edward R. Guilmette[2], Zhongxiao Wang[1], Vic Kostrubsky[3], Ashley Mackey[1], Meihua Ju[1], Junghae Suh[2], Kip Connor[1], Devin S. McDougald[2]
[1]Ophthalmology Research Unit, Biogen, Cambridge, MA,[2]Gene Therapy Accelerator Unit, Biogen, Cambridge, MA,[3]Nonclinical Safety, Biogen, Cambridge, MA

Retinitis pigmentosa (RP) is a collection of inherited retinal dystrophies characterized by peripheral-to-central visual decline coinciding with the degeneration of outer retinal cell types. Autosomal dominant retinitis pigmentosa (adRP) affects approximately 20-30% of all RP patients, and mutations in the *Prpf31* gene account for the second leading cause of disease presentation. *Prpf31* encodes a ubiquitous factor in spliceosome assembly, and reduction in wild-type protein expression impacts alternative splicing and culminates in a retinal-specific phenotype. Gene replacement is an attractive approach to arrest retinal cell degeneration and visual loss for inherited blinding diseases. Here we describe the preclinical development of an AAV-mediated gene therapy for PRPF31-associated retinitis pigmentosa. AAV reporter vectors were screened in the context of mouse retina, human RPE cells, and nonhuman primate retina to provide a matrixed assessment of capsid and cargo performance across translational models. Following selection of optimal vector components, we assessed preclinical safety and proof of biology in the *Prpf31*$^{+/-}$ mouse model. Subretinal administration increased PRPF31 protein expression in target cell types including photoreceptors and retinal pigment epithelial cells (RPE). Moreover, gene transfer rescued RPE phagocytic deficits in this model to near wild-type function. Finally, we demonstrated the safety and expression profile of a lead vector candidate following subretinal administration in nonhuman primate.

648. Preclinical Development of a Combined Gene Silencing and Replacement Vector for Autosomal Dominant Retinitis Pigmentosa

Shabnam Majidi[1], Leslie Ramos[1], Lindsay Wong[2], Edward R. Guilmette[1], Steven Tabor[2], Bin Wang[1], Ishaq Viringipurampeer[2], Nicolas Lonfat[2], Venkata R. M. Chavali[2], Junghae Suh[1], Kip Connor[2], Devin S. McDougald[1]

[1]Gene Therapy Accelerator Unit, Biogen, Cambridge, MA,[2]Ophthalmology Research Unit, Biogen, Cambridge, MA

Retinitis pigmentosa (RP) is a collection of inherited retinal dystrophies characterized by a peripheral-to-central loss of vision mediated by photoreceptor degeneration. Mutations in rhodopsin (RHO) account for approximately twenty-five percent of autosomal dominant forms of RP where currently there is no effective treatment to halt visual decline. Rhodopsin is a G-protein coupled receptor specific to rod photoreceptors responsible for initiating the phototransduction cascade. Dominant RHO mutants exert toxic gain-of-function effects that disrupt important molecular processes culminating in activation of cell death signaling pathways. Here we describe a gene therapy strategy designed to halt rod loss and restore RHO function irrespective of the patient-specific mutation. We developed a vector that silences endogenous RHO transcripts via targeted expression of an artificial microRNA (amiR) thereby diminishing the presence of gain-of-function protein variants. Moreover, this approach simultaneously rescues wild-type rhodopsin function through rod-specific augmentation of an amiR-resistant RHO coding sequence. We evaluated several cargo configurations using an in vitro expression and target engagement assay. Lead cargo designs were packaged into various capsids and assessed in preclinical systems following subretinal administration. Nonhuman primate data reveals distinct effects upon retinal function and structure dependent on cargo, capsid, and dose selection. Collectively, these findings provide important considerations for lead vector selection, application in preclinical disease models of RHO-associated retinitis pigmentosa, and guidance towards optimal dose selection for clinical translation.

649. rAAV-Mediated *PEX1* Retinal Gene Augmentation Results in Long-Term Improvements in Retinal Function and Structure in a Mouse Model of Mild Zellweger Spectrum Disorder

Catherine Argyriou[1], Samy Omri[1], Erminia Di Pietro[1], Junwei Sun[2], Devin S. McDougald[2], Joseph G. Hacia[3], Jean Bennett[2], Nancy E. Braverman[1]

[1]Research Institute of the McGill University Health Centre, Montreal, QC, Canada,[2]University of Pennsylvania, Philadelphia, PA,[3]University of Southern California, Los Angeles, CA

Zellwegerspectrum disorders (ZSDs) are caused by autosomal recessive pathogenic variantsin any of 13 *PEX* genes required for peroxisome assembly andfunction. Most cases are due to *PEX1* deficiency, which causesprogressive retinopathy leading to blindness. We previously reported aproof-of-concept trial for AAV8.CMV.*HsPEX1*.HA retinal gene augmentationtherapy using our mouse model homozygous for PEX1-G844D, the murine equivalentof a common hypomorphic human ZSD allele [1]. Untreated mice display slowretinal degeneration that mimics the milder forms of ZSD. Subsequently, wereported the preliminary effects of *PEX1* gene delivery onretinal pigment epithelium (RPE) health in these mice, using a re-designedclinically-translatable vector (AAV8.CAG.*HsPEX1*) [2]. Wereport here for the first time the results of our long-term preclinicalefficacy trial using an optimized vector design and dose, and expanded andoptimized structural and functional outcome measures. 1.2x10^9 vg AAV8.CAG.*HsPEX1* was administered bysubretinal injection to 1-month-old homozygous PEX1-G844D and wt littermatemice. Control conditions included no injection, vehicle, and AAV8.CAG.eGFP. 2,4, and 6 mos post gene delivery, photopic and scotopic functional vision wasassessed by optomotor reflex, measuring spatial acuity and contrastsensitivity. Functional vision in *HsPEX1*-treated G844D mice wasnearly normalized across all measures starting at 2 mos, durable to theexperimental endpoint. Similarly, full-field flash electroretinogram (ffERG)(scotopic, photopic, c-wave) was significantly improved starting at 2 mos postgene delivery, and at 6 mos was over 5-fold that of untreated mice. Photopicand c-wave ffERG response improved over baseline, while the decline in scotopicresponse was attenuated. Retinal structure was also improved starting at 2 mosin *HsPEX1*-treated G844D mice, and at the 6-mo endpoint: (i) coneouter segment length and density were restored to near wt levels, (ii) outernuclear layer thickness was completely preserved at the ventral pole and over7-fold improved at the dorsal pole, (iii) RPE cell density was doubled andgeographic atrophy reduced by 90%, (iv) subretinal macrophage infiltration wasreduced by 30%, and (v) gliosis was strongly reduced. Finally, we measuredperoxisome metabolite levels in neural retina and RPE by LC-MS/MS. C26:0 lysophosphatidylcholinelevels (whose catabolism is impaired and thus accumulates in G844D mice) werereduced by 60-70% in tissues from treated relative to untreated mutants. Incontrast, ether phospholipid levels, whose biosynthesis is impaired and arethus reduced in G844D mice, were unchanged by treatment. These data demonstrate the potential of retinal gene augmentation therapy topreserve retinal health and visual functions in milder forms of ZSD. Theseindividuals are now detected at birth through newborn screening across theUnited States. Overall, our studies support continued development of *PEX1* retinalgene therapy and the transition to clinical trial enabling studies. 1. Argyriou C. *et al. AAV-mediated PEX1 gene augmentationimproves visual function in the PEX1-Gly844Asp mouse model for mild Zellwegerspectrum disorder.* Mol Ther Methods Clin Dev, 2021. 23: p 225-240. 2. Argyriou C. *et al. rAAV-Mediated PEX1 Gene DeliveryCauses Dose-Dependent Improvement in RPE Cell Function and Structure in a MouseModel of Mild Zellweger Spectrum Disorder*. Mol Ther 30(4S1): p 144. (In:Complete listing of the accepted abstracts for presentation at ASGCT's 25thAnnual Meeting, May 16-May 19, 2022)

650. A Human iPSC-Derived RPE Culture System for the Functional Assessment of AAV Vectors Expressing Anti-TNF Alpha Antibodies

Chris Tully, Jessica Gumerson, Mi Shi, Ryan Kruse, Olivier F. Danos, Ye Liu, Brendan Lilley, Kwi Hye Kim

REGENXBIO Inc., Rockville, MD

Uveitis comprises a heterogenous group of inflammatory intraocular diseases that can result in vision loss. Non-infectious uveitis (NIU)

of the posterior segment of the eye can be difficult to treat, requiring long-term systemic immunosuppression associated with significant side effects. Tumor necrosis factor-alpha (TNFα) is a proinflammatory cytokine that has been implicated in NIU. To reduce local inflammation in eyes with NIU, we developed AAV vectors expressing anti-human TNFα antibodies in IgG, Fab and scFv formats. In vivo, the scFv showed significantly improved expression levels over IgG and Fab, generally by about three-fold. However, due to species incompatibility of human anti-TNFα antibodies against mouse TNFα, testing efficacy of the transgene product in mouse models of NIU posed a challenge. Thus, there was a need to develop a human in vitro model where the expression of antibodies and functional and biochemical outcome can be measured. Retinal pigmented epithelial cells (RPEs) function as a barrier and homeostasis regulator of the retina and may be disrupted in response to inflammation. Here, we cultured and maintained human induced pluripotent stem cell (iPSC) derived RPEs in porous membrane inserts for up to 60 days, allowing the cells to develop characteristic transepithelial electric resistance (TER) and observe changes induced by the addition hTNFα. Treatment of RPEs with low concentration of hTNFα reduced TER over time with dose dependency demonstrating that TER can be used as the primary outcome. Downstream effects of TNFα on released cytokines and proinflammatory markers were also characterized by analyzing collected media post exposure to TNFα using multiplexed Luminex panels. Once RPE culture conditions and readout measurements were established, RPEs were transduced with AAVs expressing anti-human TNFα antibodies. Efficacy of TNF-α inhibition of each transgene product was measured by monitoring TER and cytokine release. Up to 100% of TER was preserved at a multiplicity of infection (MOI) of 3e5 GC/cell IgG and scFv formats when treated with 0.8ng/ul hTNFα compared to 50% TER preservation in non-transduced cells. This study provides evidence that iPSC-derived RPE cultured in inserts has the potential to be utilized as a platform in human-specific ocular gene therapy development for TNFα-mediated inflammation.

651. Local Delivery of Vectorized Anti-TNF-Alpha Antibodies as a Treatment for Non-Infectious Uveitis

Mi Shi, Jessica Gumerson, Ryan Kruse, Chris Tully, Leo Kwok, Wei-Hua Lee, Justin Glenn, Kwi Hye Kim, Michele Fiscella, Olivier F. Danos, Ye Liu, Joseph T. Bruder, Brendan Lilley

REGENXBIO INC., Rockville, MD

Non-infectious uveitis (NIU) of the posterior segment of the eye requires a long-term systemic immunosuppression treatment, which is associated with significant side effects. We hypothesize that local inhibition of the proinflammatory molecule, TNF-α, is sufficient to suppress ocular inflammation, and thus, a single dose of adeno-associated virus (AAV) mediated delivery of TNF-α inhibitor has the potential to reduce disease severity and the treatment burden for NIU. Multiple formats of TNF-α inhibitors (IgG, Fab, and single chain antibody fragment variables (scFv)) were vectorized and transgene product (TP) expression was tested in 293T cells. Secreted inhibitors demonstrated robust anti-TNF-α activity in multiple cell-based assays (L929, HEK-blue and human iPSC-derived RPE cells). The affinity and binding kinetics of the recombinant inhibitors with TNF-α was measured using a Bio-layer Interferometry (BLI) assay. Furthermore, we demonstrated *in vivo* activity of AAV vectors expressing a subset of the TNF-α inhibitors in mouse ocular inflammation disease models (Experimental Autoimmune Uveitis (EAU) and an AAV-human-TNF-α overexpression target engagement model). A lead AAV vector expressing anti-hTNFa-scFv was evaluated in a study in non-human primates (NHP) and demonstrated dose-dependent transgenic protein expression in NHP ocular fluids and tissues and was well tolerated. These data together indicate that local AAV-mediated inhibition of TNF-α limits inflammation associated with uveitis and support further pre-clinical development of a vectorized therapeutic approach to the treatment of non-infectious uveitis.

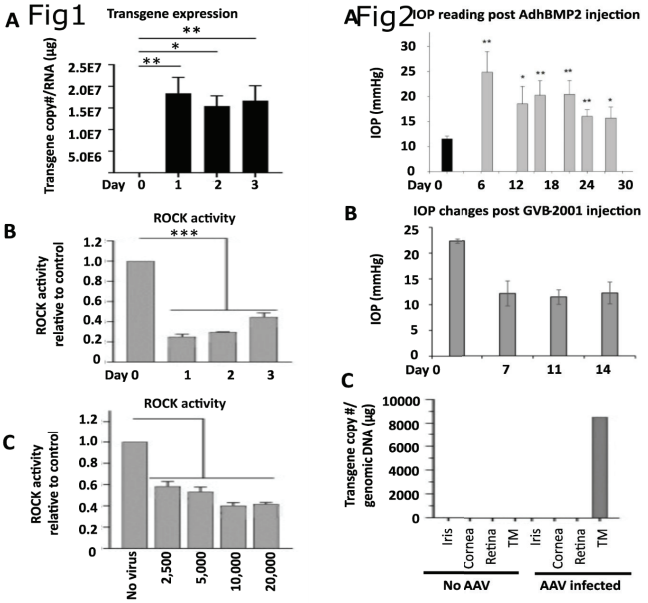
652. A New Strategy of AAV-Based Gene Therapy to Treat Glaucoma and Ocular Hypertension

Carola J. Maturana, Ling Wu, Zulian Yao, Wei Wang, Bo Liang, Laura Hong

Iview Therapeutics Inc., Cranbury, NJ

Purpose: Glaucoma stands as a prominent contributor to irreversible blindness, presenting a formidable challenge for healthcare providers. Elevated intraocular pressure (IOP) is recognized as a significant risk factor in the development of glaucoma. While conventional treatments have demonstrated efficacy in reducing IOP, patient adherence to these interventions remains suboptimal. Gene therapy emerges as a promising avenue to tackle compliance issues, instilling renewed optimism for patients. The objective of this study was to create a new strategy of adeno-associated virus (AAV)-based gene therapy to mitigate IOP elevation by inhibiting the activity of Rho kinase (ROCK) in the human trabecular meshwork (HTM). The HTM plays a crucial role in keeping the resistance to the outflow of aqueous humor, a pivotal element in maintaining a physiologically balanced IOP. **Methods**: An AAV vector, named GVB-2001, was specifically engineered to express a transgene to inhibit the activity of Rho kinase (ROCK) in the trabecular meshwork. Research-grade GVB-2001 was manufactured for proof-of-concept studies. In the in vitro part, ROCK activity, measured by ELISA, served as a metric to assess the efficacy of viral transduction in primary culture of human trabecular meshwork (HTM) cells. Various time points post-infection and multiple Multiplicity of Infection (MOI) values were systematically tested. For the in vivo segment, an ocular hypertensive rat model was induced by infecting rat eyes with Ad. hBMP2. This model was employed to investigate whether intracameral injection of GVB-2001 could effectively reduce IOP. Rats were euthanized two weeks post-AAV injection, and quantitative polymerase chain reaction (qPCR) was conducted to examine the biodistribution of GVB-2001 within ocular tissues. **Results**: The transgene exhibited robust expression in HTM cells following viral infection, as illustrated in Figure 1A. Furthermore, AAV infection demonstrated a noteworthy and consistent reduction in ROCK activity within HTM cells across various time points and MOI levels, as depicted in Figures 1B and 1C. In the rat model, the IOP readings showed an increase following the injection of Ad. hBMP2, as illustrated in Figure 2A. Subsequently, a significant reduction in IOP was observed 7 days post-AAV injection, and this effect was sustained through day 14, as depicted in Figure 2B. Biodistribution analysis confirmed the exclusive detection of GVB-2001 in the trabecular meshwork (TM)

of rat eyes, with no presence in the iris, retina, or cornea, as shown in Figure 2C. Ongoing investigations include dose-escalating efficacy studies and toxicity assessments. **Conclusions**: GVB-2001 was a robust gene therapy for glaucoma treatment, demonstrating its capacity to lower intraocular pressure (IOP) by effectively inhibiting Rho kinase (ROCK) activity in the trabecular meshwork. The proof-of-concept animal study lends substantial support to the viability of a gene therapy strategy, showcasing the potential for achieving a stable and enduring reduction in IOP with a singular intracameral injection. Fig.1 *In vitro* characterization of GVB-2001. Fig.2 IOP was measured by TonoLab in rat model.



653. Innovative RNA-End Joining Technology Enables Full-Length ABCA4 Expression in Mouse Retinal Photoreceptors

Lukas C. Bachmann, Kathryn Hilde, Binh Chu, Theresa Slaiwa, Haunani E. Bautista, Ashley Renteria, Robert Cano, Allan Kaspar, Brian K. Kaspar

Insmed Gene Therapy, LLC, San Diego, CA

Stargardt Disease is the most prevalent form of inherited childhood blindness, affecting 1:9000 individuals in the United States. Stargardt disease is caused by mutations in the Abca4 gene encoding a large (256kDa) transmembrane protein that clears retinoids from photoreceptors. Loss of Abca4 leads to accumulation of toxic byproducts that cause degeneration of the photoreceptors and apposing retinal pigment epithelium. Despite a well characterized disease mechanism, treatment for Stargardt patients is restricted to symptom management, as there is currently no cure or method for stalling progressive vision loss. Monogenic inherited retinal diseases (IRDs) such as Stargardt disease are in principle particularly suitable for gene replacement therapy, both because there is loss of function of a single gene and because of the relative accessibility and "immune privilege" of the affected retinal tissue. In vivo mouse studies of Stargardt disease have established that Abca4 gene replacement can be an effective treatment, however a major challenge in gene therapy has been the ~5-kilobase cargo size limitation of AAV and thus an inability to replace large genes efficiently and safely such as Abca4. Insmed's RNA-end joining (REJ) technology hopes to address the challenges related to AAV-based gene therapy by delivering a gene of interest as fragments in two (or more) individual AAVs and subsequently reconstituting the full-length coding sequence on the mRNA level in the target cell. REJ is therefore uniquely positioned to potentially address the challenges of conventional gene therapy approaches, and in particular advance the use of AAV strategies in large gene related ocular diseases such as Stargardt disease. We have developed and characterized a therapeutic REJ-AAV-Abca4 gene replacement vector formulation that expresses Abca4 protein from two AAV8 vectors. Each construct drives expression of the N- or C-terminal half of the protein by the human GRK1 promoter. The two fragments join and elements designed to inhibit fragment expression function to block translation of truncated protein from either single AAV. Following subretinal injection in the Stargardt Disease mouse model of the two AAVs in a 1:1 ratio (~1E10 vg per fragment), Abca4 protein expression is restored to physiological or even supraphysiological levels. Abca4 is specifically expressed in photoreceptors and predominantly localizes to the photoreceptor outer segment, showing the intended biodistribution. Specific ddPCR to each construct shows a 1:1 ratio of genomic transduction in the photoreceptor cells and RT-ddPCR confirms expression and RNA-joining. REJ-junction specific in situ hybridization demonstrates widespread distribution within the target cells. Taken together, these results support further study of Abca4 gene therapy for Stargardt Disease.

654. A Novel Mutation-Independent CRISPR-Based Gene Therapy to Treat Autosomal Dominant Retinitis Pigmentosa

Antonio Casini[1], Ambra Sarracino[1], Laura Pezzè[1], Maddalena Bosetti[1], Matteo Bello[1,2], Sally Bertolini[1], Kalina Badowska[1], Matteo Ciciani[2], Federica Esposito[3], Alberto Auricchio[3], Anna Cereseto[2]

[1]Alia Therapeutics, Trento, Italy,[2]University of Trento, Trento, Italy,[3]TIGEM, Naples, Italy

More than 200 RHO mutations causing autosomal dominant retinitis pigmentosa, a common form of severe retinal degeneration, have been identified to-date. In the absence of effective gene replacement approaches, this high genetic heterogeneity in patients makes the development of a personalized gene editing therapeutic approach challenging. In the attempt to generate an inclusive treatment option, we developed a mutation-independent CRISPR-based strategy to selectively abrogate the expression of the mutant form of RHO. Our strategy exploits a combination of two sgRNAs to introduce large edits in the mutated RHO allele by targeting respectively RHO intronic regions (bi-allelic cut) and a high-frequency non-pathogenic single nucleotide polymorphism (SNP) within the RHO gene (allele-specific editing). Exquisitely selective CRISPR editing tools are required to discriminate single nucleotide differences among genomic targets in order to selectively edit the desired SNP allele. We sourced novel CRISPR systems from natural human microbiome reservoirs exploiting a proprietary discovery platform for rapid hit to lead screening including highly efficient in silico PAM prediction, with immediate advantages for human therapeutics applications. Screening through

a range of different and novel PAM motives and smaller editors, we were able to identify candidates with superior target editing accuracy and optimal size range for all-in-one AAV in vivo delivery to overcome cargo limitation of current systems. By leveraging on our in silico discovery pipeline, we streamlined the identification of several candidates to edit the SNP locus, which were then tested side-by-side and ranked for their activity and allele specificity. After the identification of suitable intron-targeting guides, we identified nucleases which were able to consistently produce the desired large edits in the RHO locus, outperforming the SpCas9 benchmark. We validated the genome-wide specificity of the approach and demonstrated the ability to selectively downregulate mutated RHO at the mRNA and protein level in minigene-expressing cells. Thanks to the reduced protein size (<1100aa), the top candidate nuclease and both its sgRNAs were included into an optimized all-in-one AAV vector to evaluate the editing efficacy in a humanized mouse model bearing the human RHO P23H mutant allele. Optimizations of the candidate nuclease through directed evolution platforms were implemented to maximize allele discrimination, which is a critical attribute for clinical applications in autosomal dominant disorders. Our uniquely tailored approach based on the identification of compact, highly active and specific fully novel CRISPR nucleases from metagenomic samples offers a first-in-class mutation-independent and allele-specific targeting strategy for RHO-dependent adRP, which could be potentially extended to many other autosomal dominant conditions.

655. Preclinical Safety and Efficacy of Intein-Based Dual-AAV Gene Therapy for Stargardt Disease

Miquel Vila-Perelló, Marianna Di Scala, Izarbe Aisa, Victor Abad, Gerard Caelles, Julian Esteve Rudd, Silvia Frutos, David Favre

SpliceBio, Barcelona, Spain

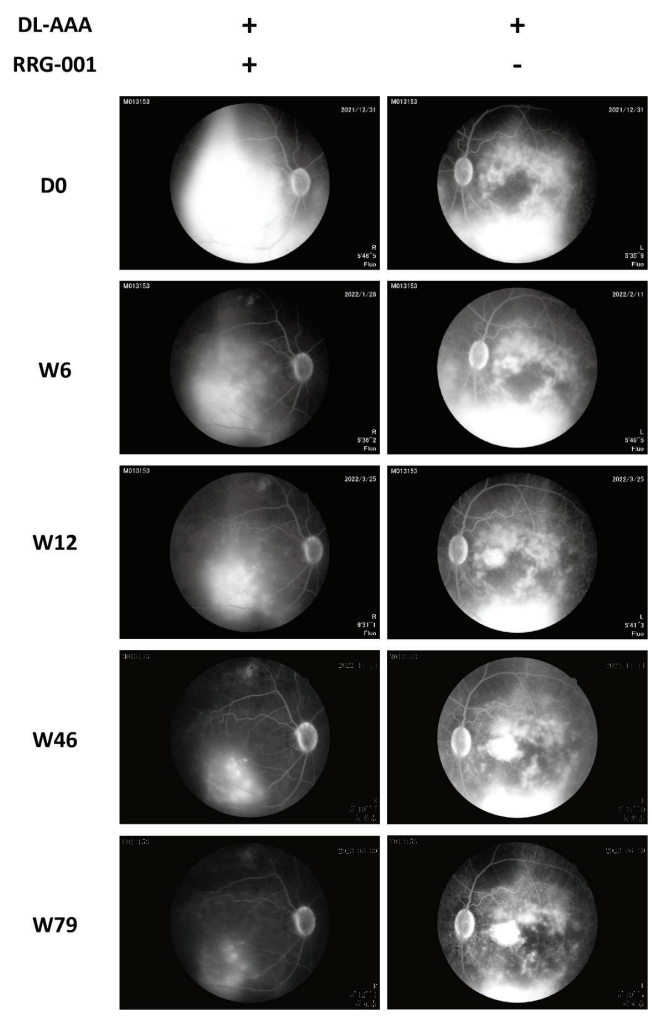
Purpose: There is currently no approved therapy for Stargardt disease (STGD1), an autosomal recessive inherited retinal dystrophy caused by mutations in *ABCA4*. AAV-mediated retinal gene therapy is safe and effective in humans, but a single AAV cannot deliver large genes such as *ABCA4*. Here we report the preclinical *in vitro* and *in vivo* characterization of a novel dual-AAV8 (SB007) encoding proprietary engineered split-inteins for the reconstitution of functional human ABCA4 protein (hABCA4) via protein trans-splicing (PTS). **Methods:** SB007 consists of two AAV8 expressing the N- and C-regions of the hABCA4 fused to Cfa-based split-inteins under the photoreceptor-specific promoter hGRK1. Expression (measured by WB), subcellular localization (IF), post-translational modifications (MS) and ATPase activity were tested *in vitro*. Subretinal injection of SB007 in Abca4$^{-/-}$ mouse and Abca4$^{ex42-/-}$ rat models, Göttingen mini-pigs (MP) and Cynomolgus macaques (NHP) were assessed longitudinally (FA, SD-OCT, ERG) and at necropsy by histology, hABCA4 expression in total retina (WB, CE), *in situ* mRNA or protein expression (RNAscope, IF) and accumulation of the bisretinoid A2E in total eyecup (LC/MS/MS), a pharmacodynamic marker (PD) of STGD1 in rodent models. **Results:** SB007 led to full-length high-level reconstitution of functional hABCA4 *in vitro.* Physiological levels of hABCA4 were achieved *in vivo* in Abca4$^{-/-}$ mice after sub-retinal injection of SB007 at 5E9-1E10 vg/eye. Overall, A2E accumulation was reduced by 30% (mouse) and 50% (rat) over control saline-injected groups, consistent with a 100% PD effect in the transduction area. A dose dependent A2E response was observed starting at 3E8 vg/eye and plateauing at 1 to 2E9 vg/eye in mice and rats, respectively. In MP and NHP, *in situ* analyses confirmed high-level hABCA4 expression in photoreceptors, including the cone rich NHP fovea. Histology safety findings were sporadically observed at SB007 doses ≥ 3E11vg/eye, mostly minimal/mild, without imaging or functional deficit (SD-OCT, ERG). **Conclusions:** The intein-based dual-AAV SB007 achieved high-level hABCA4 functional reconstitution *in vitro* and *in vivo* with dose-dependent reductions in A2E levels in two animal models of STGD1. These preliminary results support the safety profile and therapeutic potential of SB007 for Stargardt patients. Non-clinical IND-enabling studies are ongoing to support Ph1/2 clinical trial initiation.

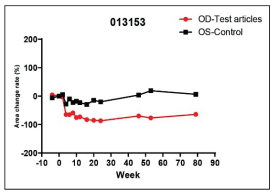
656. RRG001: Innovative Anti-VEGF AAV Gene Therapy for Retinal Disease

Qingqiao Tan, Xinxing Liu, Xi Cao, Jie Huang, Qiuyu Chen, Kaiyu Gao, Rui Zhou

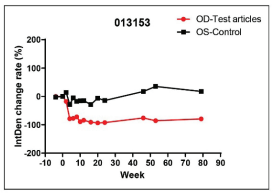
Refreshgene Therapeutics, Shanghai, China

Purpose: Sustained VEGF inhibition via Adeno-Associated Virus (AAV) delivery is promising for wet age-related macular degeneration treatment. We optimized a novel anti-VEGF drug to achieve efficacy at lower dose with better local accumulation in eyes. While laser-induced CNV model recovery spontaneously and shortly, DL-AAA induced persistent retinal neovascularization is an effective model for evaluating long-lasting therapeutic efficiency. This study is to evaluate anti-VEGF AAV gene therapy drug RRG-001 on DL-AAA induced persistent retinal neovascularization (PRNV) NHP model. Methods: Cynomolgus macaque monkey were applied for PRNV NHP modeling by DL-AAA intravitreal injection in both eyes. RRG-001 was subretinal injected into right eyes and vehicles were injected into left eyes 4 months after DL-AAA injection. IOP and OCT were performed before, after and 2 weeks after RRG-001 injection. FFA were performed before and 2,4,6,8,10,12,16,20,24,46,53 and 79 weeks after RRG-001 injection. The FFA data was analyzed by Image J to measure fluorescence area and intensity. All NHP experiments were finished under the support of Kunming Biomed International (KBI). And all animal procedures were approved in advance by the Institutional Animal Care and Use Committee of KBI. Results: In PRVN modeling, 2 monkeys were intravitreal injected with DL-AAA in both eyes. Four months later, RRG-001 or vehicle was subretinal injected into right or left eye respectively. In 013153# monkey which eye leakage maintained stable under vehicle treatment, RRG-001 treatment significantly reduced leakage and maintained inhibition to our last FFA examination at 79 weeks. In 050065# monkey which eye leakage became larger over time and grow to 4 folds larger at week 12 under vehicle treatment, RRG-001 treatment inhibited leakage growth to our last FFA examination at 79 weeks. Meanwhile, we did not find any RRG-001 drug-relative adverse reaction in DL-AAA treatment eye or contralateral eye. All monkeys' IOP before, after and 2 weeks after RRG-001 injection was at normal range. Conclusions: Our RRG-001 is a safe, well-tolerated and long-lasting drug for neovascularization inhibition in PRNV monkey model.

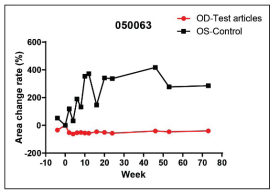




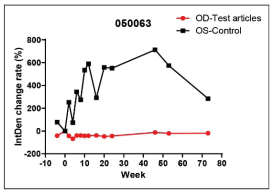
Area



Gray value



Area



Gray value

657. Efficacy and Safety of a Novel RPGR^{ORF15} Gene Therapy (FT-002) for the Treatment of RPGR-Associated XLRP

Zhong-Dong Shi[1], Hongbin Xu[1], Chao Ren[2]

[1]Frontera Therapeutics, Bedford, MA,[2]Frontera Therapeutics, Shanghai, China

Background: X-linked retinitis pigmentosa (XLRP) is a rare inherited retinal disease manifesting as impaired night vision and peripheral vision loss that progresses to legal blindness. One of the most common causes of XLRP is pathogenic variants in the retinitis pigmentosa guanosine triphosphatase regulator (RPGR) gene. Treatments for RPGR-XLRP are currently not available. FT-002 is a novel AAV5 gene therapy vector expressing codon-optimized hRPGR^{ORF15} under the control of a GRK1 promoter to rescue the functional/structural loss of the photoreceptor cells and improve visual function of XLRP patients via subretinal administration. **Results:** *In vitro*, FT-002 can efficiently transduce CHO-K1 cells and express polyglutamylated hRPGR^{ORF15} protein in a dose-dependent manner. *In vivo*, the efficacy of FT-002 was evaluated in 2-month-old male *Rpgr*-KO mice which is a slow progress model of XLRP. A one-dose, bilateral subretinal injection of FT-002 led to a significant rescue of the thickness of the retinal outer nuclear layer (ONL) on Day 270 post-dosing and a dose-dependent increase of ERG response at different flash intensities on Day 240 post-dosing. Persistent expression of hRPGR^{ORF15} protein (up to Day 270 post-injection), and time-dependent progressive rescue of rhodopsin mislocalization in the inner segment of the photoreceptor layer were observed, which correlated well with vector tissue distribution of FT-002 in the ONL of the retina. A GLP-compliant safety study with a 13-week recovery period in non-human primates confirmed that FT-002 was well tolerated with a favorable safety profile, supporting the translation of this AAV-based gene therapy into human patients. **Conclusion:** Together, the comprehensive preclinical assessment demonstrates that subretinal delivery of FT-002 led to durable hRPGR^{ORF15} expression in the retina and provided phenotypic and functional rescues of XLRP in the mouse model. The data supports the initiation of Ph I/II clinical study for the treatment of RPGR-associated XLRP patients with FT-002.

658. Mutation-Independent Genome Editing Approaches for Treatment of Stargardt Disease

Federica Fioretto[1,2], Eugenio Pugni[1], Erika Tenderini[1], Marida Sansonetti[1], Annarita Casciello[1], Maria Cascone[1], Francesca Razzano[1], Nadia Mazzaro[1], Mariangela Lupo[1], Elena Marrocco[1], Xabier Bujanda Cundin[1], Gennaro Gambardella[1], Ivana Trapani[1,2]

[1]TIGEM, Pozzuoli, Italy,[2]Advanced Biomedical Sciences, Università degli Studi di Napoli Federico II, Napoli, Italy

Genome editing via the CRISPR-Cas9 system is an exciting field of biomedical research which is gaining increasing interest for treatment of several diseases. However, classical genome editing approaches still have limitations, including: i. sequence-specificity, that limits its applicability to diseases with high allelic heterogeneity; ii. inability of the currently available techniques to achieve efficient precise integration of a corrective DNA template in post-mitotic cells. These limitations have thus far hindered development of effective gene editing

approaches for several inherited retinal diseases, including the most common form of inherited macular degeneration, Stargardt disease (STGD1), which is due to more than 1200 different mutations in the large retinal-specific *ABCA4* gene. Therefore, in this project, we aim to develop novel adeno-associated viral (AAV) vector-based genome editing strategies for integration of large templates in *ABCA4*, to allow correction of multiple mutations with a single therapeutic DNA template. To achieve integration of the template we planned to explore different repair pathways which are active in post-mitotic cells like photoreceptors, the target cells for STGD1 treatment. Accordingly, we have developed multiple candidate sets of AAV-Abca4 vectors for targeting either the murine or the human *ABCA4* gene. We side-by-side compared the different approaches *in vitro*, in cell lines, and found that they effectively mediate template integration at the endogenous locus, resulting in Abca4 expression. Subretinal delivery of the AAV-based approaches in the retina of *Abca4-/-* mice results in targeted DNA template integration, followed by productive reconstitution of *Abca4* expression in transduced photoreceptors. Further evaluation of the therapeutic efficacy of the approaches in *Abca4-/-* mice is ongoing.

659. A Novel AAV Capsid Mediated RS1 Gene Therapy Could Rescue the b-wave Amplitude of XLRS Animal Model to Wild Type Mice Level

Sisi Ma[1], Xing Wei[2], Haoliang Cui[1], Shan Zhang[1], Duanyang Wang[1], Cheng Wang[1], Ruifang Sui[2]

[1]Beijing InnoVec Biotherapeutics Inc., Beijing, China,[2]Peking Union Medical College Hospital, Beijing, China

X-linked retinoschisis (XLRS) is a neurodevelopmental retinal abnormality caused by mutations in the gene encoding the protein retinoschisin (RS1). It affects 1:5,000 to 1:25,000 males worldwide and is one of the most common causes of vision loss from macular degeneration in young men. An absent or mutated RS1 protein in the retina of patients with XLRS leads to abnormalities in the normal laminar structure of the retina, resulting in impaired visual acuity and increased propensity to retinal detachment. Currently, no approved treatments are available for XLRS and a replacement gene therapy could provide a promising strategy. We developed a novel AAV serotype AAV.IVT18, which could efficiently transfect photoreceptor and bipolar cells. At the same time, we also developed a new RS1 animal model, which carries R213W missense mutation the same as clinical patients. The $RS1^{R213W}$ mouse model had typical cysts in the retina, and the b-wave amplitude of scotopic ERG was greatly impaired, representing the similar phenotype of clinical patients. AAV.IVT18 mediated expression of RS1 could recover the degree of retinoschisis in the model animals significantly. Surprisingly, The ERG b-wave amplitudes of the treated groups were significantly increased than those of control group from 8 weeks post injection, and recovered to WT mouse level by 16 weeks of injection (Figure 1). The b/a ratio also recovered to the normal level close to that of WT mice, indicating that retinal signal transduction and visual function of mice were significantly restored. The preclinical data indicates that the gene therapy of AAV.IVT18 mediated RS1 expression might restore visual function of XLRS patients in future clinical study.

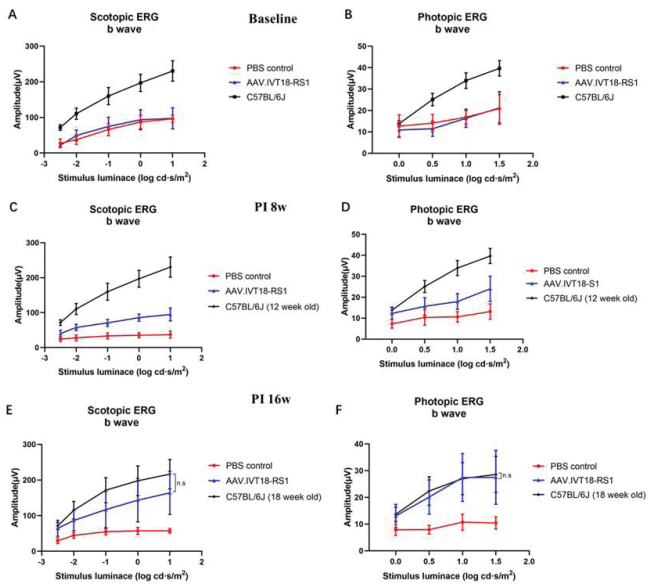


Figure 1. The ERG b-wave amplitudes of the mice.
Scotopic ERG b wave (A) and Photopic ERG b wave (B) of baseline.
Scotopic ERG b wave (C) and Photopic ERG b wave (D) of 8w post injection.
Scotopic ERG b wave (E) and Photopic ERG b wave (F) of 16w post injection.

660. ARMMs as Non-Viral Vehicles for the Delivery of Genome Editors to Treat Inherited Retinal Diseases

Jinling Yang, Wendy Zhao, Yashodhan Chinchore, Zahra Baghbantaraghdari, Jennifer A. Badji, Qiyu Wang, Chou-Wei Chang, Adam Miller, Qin Yu, Joseph F. Nabhan

Vesigen Therapeutics, Cambridge, MA

Inherited retinal diseases (IRD) are caused by monogenic mutations that lead to the decline in central vision and visual acuity. Most IRDs have no treatment options. Stargardt disease (STGD1) and Usher syndrome type II (USH2) are two of the most common autosomal recessive IRDs, which are caused by mutations in ABCA4 gene or USH2A gene. Due to the causal effects of genetic mutations in IRDs, genome editing has emerged as an attractive therapeutic strategy. However, sub-optimal packaging and delivery of gene editing components presents a major challenge to effectively correct the pathological mutations. Here, using our non-viral platform of engineered extracellular vesicles called ARRDC1mediated microvesicles (ARMMs), we demonstrated the potential utility of ARMMs in two genome editing strategies: base editing for STGD1 and CRISPR/Cas9 based exon-skipping for USH2. ARMMs were generated by packaging the adenine base editor with guide RNA (gRNA) either targeting the wild type sequence or a common ABCA4 mutation for STGD1. Western blotting and qPCR results showed that both genome editors and gRNAs were effectively packaged in ARMMs. Genome editing efficiency was assessed by Sanger and Next-generation sequencing in various ARMMs-treated cell lines, including an engineered cell line harboring the targeted pathological ABCA4 mutation. We were able to achieve robust A-to-G conversion at the ABCA4 mutation site directly or at a surrogate site in wild-type ABCA4. Furthermore, using ARMMs packaged with genome editors

and gRNA targeting the murine Abca4 sequence, we demonstrated in vivo editing of mAbca4 in ocular tissue following subretinal injection. For USH2A exon 13 skipping, we screened pairs of gRNAs for efficient excision of the genomic region harboring exon 13. ARMMs loaded with Cas9 and 2 gRNAs were generated and evaluated for functional delivery of RNP complexes. Efficient USH2A exon 13 excision was achieved with multiple combinations of gRNAs. Collectively, our data demonstrate functional delivery of therapeutic genome editors via ARMMs in various models and provide the foundation for further evaluation of ARMMs-mediated genome editing as an emerging strategy to treat IRDs.

661. Corneal Intrastromal AAV8 Delivery of Human SLC4A11 Gene Rescues Corneal Edema in *Slc4a11*$^{-/-}$ Mouse Model of Congenital Hereditary Endothelial Dystrophy

Wenlin Zhang[1], Doug Chung[2], Matthew L. Hirsch[3], Anthony J. Aldave[1]

[1]Stein Eye Institute, UCLA, Los Angeles, CA,[2]Stein Eye Institute, UCLA, Rancho Palos Verdes, CA,[3]University of North Carolina, Chapel Hill, NC

Purpose: Congenital Hereditary Endothelial Dystrophy (CHED) is caused by biallelic mutations in *SLC4A11*. The *Slc4a11*$^{-/-}$ mouse model recapitulates the clinical phenotype of CHED, characterized as progressive corneal edema and corneal endothelial dysfunction. We evaluated the efficacy of intrastromal delivery of a recombinant AAV8 vector encoding human SLC4A11 cDNA under transcriptional control of human elongation factor-1 alpha promoter - rAAV8-EF1α-hSLC4A11. **Methods:** A single corneal intrastromal injection of 3 µL rAAV8-EF1α-hSLC4A11 vector was performed in one eye of *Slc4a11*$^{-/-}$ mice at 4 weeks of age. Three dosages 10e9 (n = 13), 10e8 (n = 10), 10e7 vg/eye (n = 10) were tested. For each mouse, the eye receiving the AAV injection was randomized, and the contralateral eye served as the control. Anterior segment OCT was performed in both eyes before injection to measure baseline central corneal thickness (CCT, mean ± SEM) and bi-weekly after injection until post-injection week 14 (e.g. W14). Slit-lamp examination of the anterior ocular segment and retinal OCT were also performed bi-weekly post-injection to monitor for evidence of toxicity and host immune response. Mixed-effects analysis with post-hoc multiple comparisons was used to compare CCT values between AAV-injected eyes and control eyes at different post-injection time points. **Results:** AAV-injected eyes showed a sustained reduction of CCT from baseline in a dose-dependent manner, whereas untreated contralateral eyes showed a progressive increase in CCT. For the 10e9 vg/eye group, AAV-injected eyes showed a 66.2 µm reduction in CCT compared to control eyes at W14 (p = 0.002). The CCT (µm) at baseline, W6 and W14 were 161.1 ± 3.3, 143.4 ± 7.1 (p = 0.048), and 171.5 ± 10.9 (p = 0.41) in AAV-injected eyes and 160.2 ± 2.7, 176.2 ± 5.1 (p = 0.011), and 237.7 ± 11.1 (p < 0.0001) in control eyes. For the 10e8 vg/eye group, AAV-injected eyes showed a 37.8 µm reduction in CCT compared to control eyes at W10 (p = 0.0007). The CCT (µm) at baseline, W6, and W10 were 162.5 ± 3.2, 148.5 ± 5.6 (p = 0.021), and 170.9 ± 5.3 (p = 0.26) in AAV-injected eyes, and 166.7 ± 5.2, 169.2 ± 5.5 (p = 0.72) and 208.6 ± 6.3 (p = 0.0012) in control eyes. For the 10e7 vg/eye group, AAV-injected eyes showed a 43.0 µm reduction in CCT compared to control eyes at W8 (p = 0.02). The CCT (µm) at baseline, W6, and W8 were 152.8 ± 6.1, 152.3 ± 5.1 (p = 0.93) and 160.3 ± 5.7 µm (p = 0.31) in AAV-injected eyes, and 154.7 ± 3.4, 175.4 ± 7.4 (p = 0.012) and 203.3 ± 7.8 µm (p = 0.0001) in control eyes. Corneal subepithelial deposits were observed in a dosage-dependent manner in AAV-injected eyes. No evidence of anterior chamber inflammation was observed. Vitreous inflammation was observed in 2 out of 32 eyes injected with AAV vector. **Conclusions:** Intrastromal delivery of rAAV8-EF1a-hSLC4A11 effectively rescues corneal edema in *Slc4a11*$^{-/-}$ mice, indicating a functional recovery of corneal endothelial pump function. Corneal subepithelial deposits and vitreous inflammation may be associated with the immune responses to the rAAV8 vector.

662. The Effect of Antioxidant MitoQ on Cell Viability in Human Corneal Endothelial Cells Harboring *SLC4A11* Mutations Modelling Corneal Endothelial Dystrophies

Doug D. Chung[1], Saloni Gupta[1], Anthony J. Aldave[2]

[1]Ophthalmology, Stein Eye Institute, UCLA, Los Angeles, CA,[2]Stein Eye Institute/UCLA, Los Angeles, CA

Purpose: Solute carrier family 4 member 11 (*SLC4A11*) mutations are associated congenital hereditary endothelial dystrophy (CHED) and Fuchs endothelial corneal dystrophy type 4 (FECD4). These mutations are hypothesized to diminish oxidative stress protection in human corneal endothelial cells (hCEnC), ultimately leading to the loss of hCEnC viability and/or function. Therefore, we sought to determine the impact of MitoQ, a mitochondria-targeted antioxidant, on the viability of hCEnC-based models of CHED and FECD4 during oxidative stress to investigate the therapeutic potential of MitoQ. **Methods:** Cell-based models of CHED and FECD4 were generated in a hCEnC line, hCEnC-21T. Briefly, *SLC4A11* wildtype (*SLC4A11*^{WT}) and mutant (*SLC4A11*^{MU}) hCEnC lines were created to express either *SLC4A11* variant 2 (V2) or variant 3 (V3) by stable transduction of *SLC4A11*$^{-/-}$ hCEnC-21T with lentiviruses containing either *SLC4A11*^{WT}, an empty vector, or one of the following *SLC4A11* mutations: V2 mutants c.374G>A (CHED), c.1813C>T (CHED), c.2263C>T (CHED), or c.2224G>A (FECD4); V3 mutant homologs c.326G>A (CHED), c.1765C>T (CHED), c.2215C>T (CHED), or c.2176G>A (FECD4). Dose range assays were performed to determine the maximum non-toxic dose of MitoQ in hCEnC. Cell viability was measured by exposing MitoQ-treated and untreated cells to oxidative stress agent tBH followed by XTT assays and spectrophotometry to measure cell densities. **Results:** After 72 hours of media supplementation with MitoQ concentrations ranging from 0 to 0.25 µM, *SLC4A11* empty, V2^{WT} and V3^{WT} hCEnC exposed to ≤ 0.01 µM MitoQ retained over 90% of the cell density observed in untreated hCEnC. Media supplementation with 0.01 µM MitoQ led to increased viability in *SLC4A11* empty hCEnC, compared to MitoQ-free media, after exposure to 25-50 µM tBH. Each of the CHED-associated hCEnC lines demonstrated increased cell viability when treated with 0.01 µM MitoQ, compared to untreated, following exposure to 25 -50 µM tBH. The FECD4-associated V2^{MU} c.2224G>A and V3^{MU} c.2176G>A hCEnC lines exhibited no significant increase in viability when treated with 0.01 µM MitoQ, compared to untreated, following tBH exposure. **Conclusions:** Media supplementation with the antioxidant MitoQ has beneficial effects on cell viability in hCEnC harboring CHED-associated *SLC4A11* mutations following exposure to tBH-induced oxidative stress.

663. Biodistribution of ARMMs as Non-Viral Vehicles for Delivery of Therapeutic Payloads by Subretinal Administration in Minipigs and Non-Human Primates

Yashodhan Chinchore, Jinling Yang, Adam Miller, Pearl Moharil, Qin Yu, Joseph Nabhan
Vesigen Therapeutics, Cambridge, MA

Therapeutic delivery of genetic medicines to the back of the eye has been a challenge in treatment of inherited retinal diseases. Non-viral delivery offers many potential advantages over conventional viral delivery systems, especially for genome editing applications. We developed a novel non-viral platform that co-opts a class of extracellular vesicles called ARRDC1-mediated microvesicles (ARMMs) for delivery of therapeutic payloads. Using this platform, we previously demonstrated delivery of fluorescent proteins and nuclear genome modifiers in murine ocular tissue via subretinal injection, a clinically approved route-of-administration. However, the biodistribution characteristics of ARMMs in higher mammals is not known. We assessed biodistribution of ARMMs loaded with fluorescent proteins in Gottingen Minipigs and African Green Monkeys via subretinal injection. Eyes were harvested and assessed for colocalization of fluorescent proteins with cell type-specific markers for rod and cone photoreceptors and the Retinal Pigmented Epithelium (RPE) as a proxy for ARMMs uptake in these cell types of interest. Consistent with murine uptake data, we observed robust localization of ARMMs in the RPE in both minipigs and monkeys. In the neural retina, we observed a time-dependent increase in the localization of ARMMs with photoreceptors. Both rod and cone photoreceptors, including cones of the macular region in the monkey retina, showed ARMMs localization. Together, these data strongly suggest that ARMMs are non-viral vehicles that enable delivery of payloads to RPE and photoreceptors, two important cell types for many human retinal diseases.

Wednesday Posters: Musculo-Skeletal Diseases

664. Insights for Gene Therapy from Molecular Evolution - Why Has Dystrophin Remained the Same Size for 600 Million Years?

Hansell H. Stedman[1], Christopher D. Greer[2], Andrew Mead[2], Yafeng Song[2], Rachel Sweeney[2], Derek Stefanik[2], Leon Morales[2], Coral Kasden[2], Benjamin Kozyak[3]
[1]University of Pennsylvania Medical Center, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, PA,[3]University of Pennsylvania School of Medicine, Philadelphia, PA

Duchenne muscular dystrophy (DMD) has been regarded as a "Holy Grail" for gene therapy because of the high global incidence and extreme burden of the lethal monogenic disease. At almost 3 million basepairs, the enormous size of the causative X-linked DMD/dystrophin gene presents an exceptional target for novel mutations, with a majority represented by large, multi-exon frameshifting deletions and duplications. Designing effective gene therapies for DMD requires reconciling a mismatch between the largest gene (DMD) and smallest vector genome (AAV). In doing so, we mustconfront the limits of our current knowledge about the correlation of structure with function for the protein dystrophin. A central challenge reflects the nature of dystrophin's discovery: the protein accumulates to such low abundance that a positional cloning approach was required. Although this groundbreaking paradigm provided detailed information about the gene product's primary structure, it offered only the vaguest hint about its tertiary structure and function. Forty years later, less than 5% of dystrophin's largest domain, the rod, has been subjected to protein crystallography - the rest is "terra incognita". Here we use comparative phylogenomics to reconstruct a remote evolutionary history of dystrophin, its related and associated proteins, and other critical muscle proteins to develop a detailed model of the functional constraints on ancestral dystrophin genes. We find that unlike the promiscuous length of the 10-fold larger protein titin, dystrophin has been fixed in size at ~400 kD since its inception before the phylogenetic split between radially and bilaterally symmetrical organisms such as jelly fish (Cnidaria-Radiata) and most familiar land animal species (Bilateria). Even minor deviations from this optimal length, by deletion or duplication, produce Becker muscular dystrophy (BMD), associated with site-specific fragmentation of the internally truncated protein in muscle. This raises two related questions of relevance to gene therapy for DMD: (1) Why has dystrophin remained the same size for over 600 million years? (2) In which protein ancestral to dystrophin did the repetitive, spectrin-like triple helical region of the rod expand to its current size in Dp427, the 4 primary isoform of dystrophin in modern day human and cnidarian muscle? We propose testable answers to these questions on the basis of bioinformatic analysis, illustrated using novel software developed to aid visualization of the relationships between gene and protein structure across inferred branchpoints in remote phylogenesis. In related abstracts we present the implications of this derived evolutionary history, informing rational design of novel AAV transgenes with the potential to confer significantly improved functionality over conventionally miniaturized dystrophins.

665. A Central Challenge of Gene Therapy for Duchenne Muscular Dystrophy - Rational Design of Truncated, AAV-Compatible Rod Domains to Preserve Tensile Strength

Hansell H. Stedman[1], Coral Kasden[2], Leon Morales[2], Benjamin Kozyak[3]
[1]University of Pennsylvania Medical Center, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, PA,[3]University of Pennsylvania School of Medicine, Philadelphia, PA

Our reconstructed evolutionary history of dystrophin (presented elsewhere) suggests that the rod domain has been static in length since the protein's emergence about 600 million years ago. All prior expansion occurred in the context of an ancestral protein resembling modern-day microtubule-actin crosslinking factor (MACF) and, based on the gene structures, was driven by an intron-dependent process. Converging lines of evidence suggest that dystrophin coopted the rod domain from its ancestral MACF, and in doing so locked in a functional constraint tailored to the emerging protein's relocation from the cytoplasm to the cortical cytoskeleton and cell membrane. In this

scenario, the functional constraint is tensile strength, not length *per se*. The strength is conferred by the overlap between the interhelical loops of adjacent spectrin-like triple helical repeats, in which the interconnecting C-A helix has been shortened relative to that in the remote common ancestor. This is best visualized by comparing the X-ray crystal structures for triple helical repeats derived from a spectrin and a spectroplakin, e.g. 3EDV versus 5J1I, respectively. In spectrins and alpha-actinins, individual triple helical repeats have no mechanism for force transmission along the long axis except through the C-A helix, with cooperative unfolding of isolated triple helices at relatively low tensile loads. In contrast, the inter-loop hydrogen bonds between adjacent repeats of MACF and dystrophin has the potential to vastly increase tensile strength if the triple helix is simultaneously loaded across all three helices, as would be expected *in vivo*. The behavior of the helix bundle would be strongly impacted by the site and directionality of the applied load, like the tightening versus untying of a shoelace depending on whether one pulls on the loop or the free ends. If evolution has primarily selected for strength, not length, in force transmission between the sarcomere and the sarcolemma, this opens the possibility that novel transgenes could prioritize strength in small, AAV-compatible mimetics for dystrophin and utrophin. This suggested a series of testable hypotheses centered on the rational design of recombinant proteins in which the triple helices are cut and re-annealed across a transverse section at the three-dimensional midpoint. The transecting plane is placed where the highly conserved tryptophan residues in the A and C helices exhibit pi system polarization in the hydrophobic core. We used this approach to engineer a novel class of recombinant, miniaturized dystrophins and utrophins, denoted nanotrophin-#D and #U, where # denotes the number of remaining triple helical repeats downsized from > 20 in the native full-length proteins too big for AAV. Here we present the structural basis for the design of four preferred nanotrophins: nantrophin-4U, -4D, -5U and 5D. Of these four, the latter two are supported in part by X-ray crystal structures reported for the first spectrin-like repeats of utrophin and dystrophin. The positions selected for splicing the poorly conserved B helices were derived by the combined application of Hidden Markov Modelling and hypothetical protein rendering by AlphaFold. The transgenes resulting from this rational design approach have been synthesized and rigorously compared to conventional microutrophin and microdystrophin in the context of systemically delivered AAV vectors, with compelling results as reported in detail in a co-submitted abstract.

666. Exon Skipping for the Second Calponin Homology Domain of Dystrophin Using AAV. U7snRNA - *In Vitro* & Intramuscular Studies Using a Novel Murine Model of Duchenne Muscular Dystrophy

Alex Brinkman, Daniel Lesman, Ding Li, Dhanarajan Rajakumar, Atre Chaitrali, Yacidzohara Rodriguez, Camila Almeida, Nicolas Wein

Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH

Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting disease, caused by mutations in DMD gene leading to absence of dystrophin. 5-6% of DMD patients have mutations in exons 6-8 encoding for the second calponin homology domain of dystrophin. To test the efficiency of a novel vectorized exon skipping (VES) for patients with these mutations, we developed a multiple VES constructs that was screened *in vitro* in HEK293 and patient fibroblasts differentiated into myotubes to select our best VES candidates that mediate exon 6-8 skipping. In addition, we tested our lead candidates in a new humanized hDMD mdx mouse called hDMDm7 that was generated using CRISPR/Cas9. This mouse model does not express murine dystrophin and additionally contains a nonsense mutation in exon 7 (hDMDm7) resulting in no human dystrophin expression. Intramuscular (I.M.) injections using selected VES constructs were performed for hDMDm7 mice at 6 and 7 weeks of age. Exon skipping was evaluated by RT-PCR, dystrophin expression by WB and IF, and muscle force using electrophysiology. This mouse model presents a reduction of muscle integrity with centronucleation (>90% at 3 and 6 months of age) using H&E staining. Little to no dystrophin expression was determined by western blot, and the average percent drop over 10 consecutive eccentric contractions was 59% in hDMDm7 vs 9% in hDMD. Four lead VES constructs were selected and evaluated in the hDMDm7. One VES construct was tested effective in muscle force measurement at 3 months of post I.M. injection, which brought the percent drop of eccentric contraction back to 40%. This exon skipping strategy was able to restore muscle functions following I.M. treatment for hDMDm7 mouse model. This VES for exons 6-8 could be beneficial for 5-6% of DMD patients.

667. Alternative Delivery of Adeno-Associated Virus 9 for the Treatment of Duchenne Muscular Dystrophy to Target CSF and Muscles- GFP Biodistribution Study in WT Mice

Cintia Gomez[1], Archana Iyer[1], Alex Brinkman[1], Florence Roussel[1], Ding Li[1], Daniel Lesman[1], Cassandra Dennys-River[1], Hannah Huang[1], Vicky McGovern[2], Arthur Burghes[2], Shibi B. Likhite[1], Dave Arnold[3], Kathrin Meyer[1], Nicolas Wein[1]

[1]Center for Gene Therapy, Abigail Wexner Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]The Ohio State University, Columbus, OH,[3]University of Missouri, Columbia, MO

Duchenne muscular dystrophy (DMD) is a progressive muscle-wasting disease, caused by mutations in *DMD* gene leading to absence of dystrophin. Additionally, around one-third of patients present cognitive impairments. Several therapeutic approaches including exon-skipping have used efficient systemic delivery of adeno-associated virus (AAV). However, the high dose of vector needed to treat adult patients represents a risk, especially for the liver. We have investigated an alternative administration route using cerebrospinal fluid (CSF) delivery to avoid potential liver toxicity and improve brain transduction. Newborn C57BL/6 mice were intracerebroventricular (ICV) or face vein (FV) injected with 1E+11vg scAAV9.CBA.GFP to study virus biodistribution. After one and three months (mo) post-injection (pi), GFP expression on fresh organs was evaluated using fluorescent stereomicroscope. GFP mRNA and protein expression were assessed by RT-PCR and western blot (WB). Vector DNA

biodistribution was performed by ddPCR. At 1 mo and 3 mo pi, similar levels of GFP expression were observed in all muscles for CSF and systemic groups. However, a higher expression was detected in the brain after ICV injection and liver was less transduced by ICV injection compared to FV, which was confirmed by RT-PCR and WB. To conclude, CSF and systemic delivery allowed similar levels of muscle transduction by AAV9, although the brain transduction after ICV injection was superior and liver was less transduced, supporting the use of CSF delivery for the simultaneous distribution of AAV9 to muscles and CNS.

668. *In Vivo* Treatment of Duchenne Muscular Dystrophy Using Lentiviral Vectors Containing the Mini-Dystrophin Gene

Xiaoyu Wang[1], Yanghui Zhu[1], Taiqing Liu[1], Lingyan Zhou[1], Yunhai Fu[1], Jinhua Zhao[1], Yinqi Li[1], Yeteng Zheng[1], Xiaodong Yang[1], Xiangjie Di[1], Yang Yang[2], Zhi-Yao He[1]

[1]West China Hospital, Sichuan University, Chengdu, China,[2]Sichuan University, Chengdu, China

Duchenne muscular dystrophy is an incurable X-linked recessive genetic disorder caused by mutations in the dystrophin gene. Numerous researchers were working to restore truncated dystrophin using viral vectors, but their clinical application was hindered by the limited capacity for packaging and immunogenicity of these vectors. In this study, we developed four lentiviral vectors containing sequence-optimized and truncated dystrophin genes, which were regulated by muscle-specific promoters. They stably expressed mini-dystrophin *in vitro*. To evaluate the impact of the treatment *in vivo*, we administered the lentiviral vectors to neonatal mice. Treated mice showed an improvement in the expression of modified dystrophin, which was also restored in its distribution. Simultaneously, the treatment mice demonstrated a recovery of pulling strength and a reduction in the quantity of mononuclear cells. The therapeutic effect lasted for duration of 3-6 months *in vivo*. Additionally, we conducted an initial analysis on the insertion sites of the lentiviral vectors. To summarize, this study provided preliminary evidence for the feasibility and safety of using lentiviral vectors with mini-dystrophin for DMD gene therapy, and introduced a novel strategy for restoring truncated dystrophin.

669. Therapeutic Expression of Large Dystrophins and a CRISPR Base-Editor for Duchenne Muscular Dystrophy by Scarless RNA Trans-Splicing

Ryan H. Hsu[1], Claire E. Williams[1], Geraldine Maier[2], Miriam Gullo[2], Karen Lettieri[2], Cindy Alvarez[2], Kip J. Hermann[2], Lukas C. Bachmann[3], Samuel L. Pfaff[1]

[1]Salk Institute & UC San Diego, La Jolla, CA,[2]Salk Institute, La Jolla, CA,[3]Insmed Inc., San Diego, CA

Adeno associated viruses (AAVs) have proven to be safe and efficacious vectors for delivering genetic therapies. However, one major limitation that remains is its packaging capacity of ~4.7 kilobases (kb), restricting the scope of both gene-editing (e.g. CRISPR-Cas9 base editors) and gene-replacement therapies. To overcome this boundary, we engineered a novel high-efficiency RNA trans-splicing technology to express functional levels of any gene of interest by dual or triple AAV delivery, termed RNA end-joining (see abstract: *Optimization of RNA End Joining (REJ) to safely and efficiently express large proteins*, Bachmann et al.). In REJ, a coding sequence of interest is split into multiple viral genomes, each containing an exon. Each exon is flanked by synthetic engineered intron sequences and is packaged individually into AAV. These constructs are delivered to target cells *in vivo*, where the expressed RNAs undergo a trans-splicing reaction mediated by the endogenous spliceosome, resulting in high levels of scar-free mRNA that is translated into full-length protein. Duchenne muscular dystrophy (DMD) is a devastating monogenic disease characterized by progressive muscle degeneration, weakness, and eventual fatal cardiomyopathy. Despite clinical advancements in gene-replacement using micro-dystrophins designed to fit within AAV, their limited efficacy is becoming increasingly evident, underscoring the need for larger, more functional dystrophins. Toward this goal, research efforts have included engineering alternative CRISPR editors (<4.7 kb) or intein-based protein joining, which necessitates potentially antigenic foreign peptides and a scar which may disrupt protein function. In this work, we use REJ to develop dual- and triple-vector AAV therapeutic candidates for the delivery of a CRISPR Adenine Base Editor (dual, 6 kb), mini-dystrophin (dual, 7 kb), or full-length dystrophin (triple, 11 kb) and characterize their efficacy in DMD model mice. By intramuscular treatment, all delivery strategies result in functional levels of dystrophin expression with significant phenotypic improvements. We observe recovery in immunohistochemical markers of disease, such as centro-nucleation, morphology, and fibrosis. Transcriptomic signatures of disease are improved and *in situ* contractile force measurements demonstrate significant increases in muscle strength. In the gene-editing approach, up to 40% of muscle fibers exhibit restored dystrophin expression. Current work includes intravenous systemic treatment using myotropic AAV with comparable positive outcomes. These findings collectively validate REJ as a broadly applicable technology and identify several promising clinical candidates for further investigation in the pursuit of a treatment for Duchenne muscular dystrophy in patients.

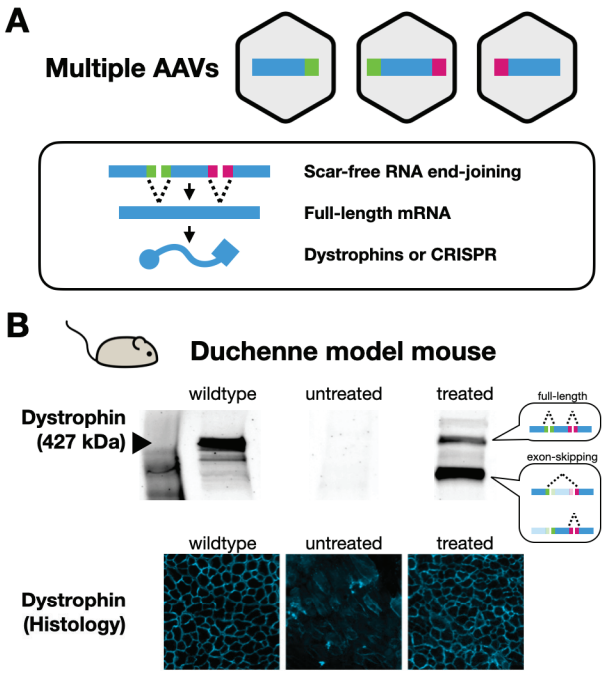


Figure Legend: (A) Large therapeutic genes (blue) are split, flanked by REJ modules (green, purple), and packaged into individual AAVs. Once delivered *in vivo*, the transcribed RNAs undergo trans-splicing and produce full-length mRNA, resulting in robust protein expression. (B) Triple AAVs encoding full-length dystrophin were delivered to DMD model mice by intramuscular injection. 4 weeks later, full-length dystrophin was detected along with exon-skipping products.

670. Disease Exacerbation in 3D MYOtissues Derived From Duchenne Muscular Dystrophy iPSC Reveals Muscle Strength Loss and Enables Gene Therapy Screening

Laura Palmieri[1,2], Louna Pili[1,2], Abbass Jaber[1,2], Melissa Moula[1], Ai Vu Hong[1,2], Riyad El-Khoury[1,2,3], Guy Brochier[3,4], Anne Bigot[5], David Israeli[1,2], Isabelle Richard[1,2], Sonia Albini[1,2]

[1]GENETHON, Evry, France,[2]Université Paris-Saclay, Univ Evry, Integrare Research Unit UMR_S951, Evry, France,[3]Institut de Myologie, Paris, France,[4]AP-HP, Centre de Référence de Pathologie Neuromusculaire Nord/Est/Ile de France, Paris, France,[5]Sorbonne Université, Inserm, Institut de Myologie, Centre de Recherche en Myologie, Paris, France

Duchenne muscular dystrophy (DMD) is a lethal muscle-wasting disease caused by the absence of Dystrophin, a protein essential to preserve muscle integrity continuously challenged by contractions. Gene therapy utilizing adeno-associated virus (AAV) to deliver truncated forms of dystrophin (µDys) is currently the most promising therapeutic approach. However, the therapeutic outcome in treated patients has not been as successful as anticipated by animal studies, underscoring the need for improved and high-throughput models for fast and accurate prediction of human response. Among the in vitro cellular models, organoid-like structures are becoming an appealing resource for disease modeling and replacement of animal models. Here, we describe the generation of MYOtissues, an in vitro 3D muscle platform based on direct myogenic conversion of human induced pluripotent stem cells (iPSC) and inclusion of fibroblasts to ensure proper muscle structure. MYOtissues derived from DMD-iPSC including DMD fibroblasts, show exacerbated pathogenic hallmarks such as fibrosis and muscle force loss, across the different DMD iPSC cell lines employed, independently from the genetic background of the DMD fibroblasts used. As proof of the suitability of our system for gene therapy screening, we showed that AAV9-mediated µDys gene transfer in DMD-MYOtissues improved muscle resistance and recovered, although only partially, membrane stability with consequent reduction of the inflammatory environment. Our data, obtained in three control and DMD cell lines including isogenic controls, were supported by transcriptomic analysis which demonstrated an amelioration of mechano-stability and inflammatory hallmarks but also reveals that only a partial correction of the deregulated pathways in DMD was achieved. This study highlights the suitability of our human in vitro system for gene therapy screening and supports the need for developing more efficient gene therapy strategies for DMD patients.

671. Systemic Delivery of Circular RNA Encoding Delta17-48 Dystrophin for Treatment of Duchenne Muscular Dystrophy

Luke Rhym, Tatiana Fontelonga, Deirdre Boyer, Doug Pecze, Corey Ciullo, Emily Fitzgerald, Sharmistha Kundu, Justin Lee, Allie Lundberg, Elena Minissale, Shobu Odate, Kristen Ott, Raya Shu, Trent Stevens, Rahul Vungutur, Robert A. Wesselhoeft, Yessica Wiryawan, Junghoon Yang, Nelson Chau, Tracy Dimezzo, Allen Horhota, Tom Barnes, Robert Mabry, Isin Dalkilic

Orna Therapeutics, Watertown, MA

Duchenne muscular dystrophy (DMD) is a severe degenerative muscle disorder caused by mutations in the *DMD* gene that results in the loss of dystrophin protein. Full-length dystrophin protein replacement therapy for patients has been infeasible using existing technologies due to the large open reading frame (11kb) of the dystrophin gene. Becker muscular dystrophy is a milder version of the disease where patients express truncated versions of the dystrophin protein, suggesting that significantly truncated, yet still functional, protein placement approaches could be utilized to slow DMD disease progression in patients. Current protein replacement gene therapies utilize adeno-associated virus (AAV) delivery systems; however, these are limited by their immunogenicity, which precludes their potential for repeat dosing, and their relatively small packaging capacity. As result, the focus of AAV-based systems has primarily been on extremely truncated versions of the dystrophin protein (micro-dystrophin, ~1/3 full length protein). At Orna Therapeutics, we are developing a novel, synthetic circular RNA technology (oRNA®), that can be delivered systemically to musculature via our lipid nanoparticle (LNP) platform, which shows durable expression, as well as multi-dosing capability. Moreover, our platform allows for significantly larger protein coding capacity, enabling therapeutic approaches that utilize much larger dystrophin variants than are not feasible with current AAV-based approaches. We have taken multiple dystrophin variants, including micro dystrophin,

Becker patient variants, and full-length dystrophin, cloned into our oRNA vectors and showed expression from oRNA *in vitro* in human skeletal muscle cells. For *in vivo* studies, we have focused on a Δ17-48 dystrophin variant which has been described in a mild Becker patient in the 1990s. The coding sequence for this variant (~5.9kb) is considerably larger than micro dystrophins utilized in AAV platforms. One of the important features of oRNA technology is that protein expression in cells is driven by a sequence-based internal ribosome entry site (IRES) element in a cap-independent manner. To identify IRES sequences that can drive high protein expression in muscle cells, we screened >3000 IRES sequences extracted from viral genomes using our proprietary FoRCE platform in primary human skeletal muscle myotubes. A subset of IRESs that showed high muscle activity *in vitro* were selected to generate dystrophin expressing oRNAs and tested *in vitro* and *in vivo* in the mdx model. Our lead muscle IRES sequence has shown ~10x improvement in protein expression in diaphragms from IV dosed mdx mice. Through further optimization of the coding sequence and LNP composition, we have demonstrated expression of Δ17-48 dystrophin protein at ~3% of WT levels in diaphragms of mdx animals dosed IV with a single dose of 5 mg/kg. This data represents the first evidence of expression of the Δ17-48 variant from a systemically delivered gene therapy platform *in vivo* and demonstrates the potential of oRNA® technology as a scalable, durable protein replacement platform with a large cargo capacity that can provide a differentiated therapy for DMD patients.

672. Exon Reframing by Prime Editors Restores Dystrophin Expression and Function in DMD Patient Cardiomyocytes

Michelle O'Connor[1], Sascha Hernandez[1], David Waterman[1], Serge Kyrychenko[1], Naqi Haider[2], Vivian Choi[1], John Stiller[1], Katya Kosheleva[3], Matthew Malloy[1], Andrew V. Anzalone[4], Jeremy Duffield[1]

[1]Prime Medicine, Cambridge, MA,[2]Prime Medicine, Arlington, MA,[3]Prime Medicine, Somerville, MA,[4]Broad Institute, Cambridge, MA

Duchenne muscular dystrophy (DMD) is a progressive degenerative muscle disease caused by mutations in the *DMD* gene which encodes dystrophin, a large intracellular protein that tethers the cytoskeleton to the extracellular matrix. Most disease-causing *DMD* mutations are large deletions that disrupt the reading frame, causing premature termination of translation and subsequent loss of dystrophin expression. Most current therapeutic options for patients, including exon-skipping antisense oligonucleotides (ASOs), require repeated administration. The long-term clinical benefit of other therapeutic approaches, such as the recently approved micro-dystrophin gene therapy Elevidys, is yet to be fully determined. Therefore, there is a significant unmet medical need for a one-time curative treatment for DMD. Restoration of functional dystrophin expression through Prime Editing of the endogenous *DMD* gene represents a potential curative therapeutic strategy. Our approach leverages Prime Editors to reframe an exon adjacent to the pathologic deletion through targeted nucleotide insertions or deletions, thereby restoring the correct reading frame, which results in expression of a nearly full-length dystrophin protein from the endogenous gene. Using this exon reframing strategy, we demonstrated restoration of dystrophin protein expression in patient iPSC-derived cardiomyocytes with three predominant DMD genotypes: exon 45 deletion, exon 46-50 deletion, and exon 51-55 deletion. Compared to the untreated patient cardiomyocytes, those corrected with the Prime Editor exhibited normalization of Ca2+ handling and contraction frequency, as well as elimination of arrhythmogenic activity. These data highlight the potential of exon reframing by Prime Editors as a therapeutic strategy for a large number of DMD patients with unmet medical need.

673. Cardioprotective Effects of AAV9-Micro-Utrophin Gene Therapy in Novel Hit-and-Run Acute Injury Model in the Muscular Dystrophy *Mdx* Mouse

Coral M. Kasden[1], Maria P. Corzo[1], Christopher D. Greer[1], Rufranshell Reyes[1], Tanvi Singh[1], Alaine Castillo[1], Benjamin Kozyak[2], Hansell H. Stedman[3]

[1]University of Pennsylvania, Philadelphia, PA,[2]University of Pennsylvania School of Medicine, Philadelphia, PA,[3]University of Pennsylvania Medical Center, Philadelphia, PA

Duchenne muscular dystrophy (DMD) is the most common childhood-onset muscle wasting disease worldwide, caused by genetic deficiency of dystrophin and resulting in premature death due to cardiorespiratory failure. Ongoing DMD gene therapy clinical trials delivering functional miniaturized dystrophin substitutes with Adeno-associated virus (AAV) are promising. However, issues continue to emerge with durability and lack of central tolerance to dystrophin epitopes in a majority of DMD patients. An alternative gene replacement approach is to deliver miniaturized utrophin (uUtro), the evolutionarily and functionally related protein to dystrophin that confers immunological safety and therapeutic protection against disease progression in skeletal muscle in DMD mouse and dog models. With skeletal muscle restoration, voluntary activity increases, placing greater strain on the heart. It remains untested whether AAV-uUtro is protective in the dystrophic heart during bouts of greater cardiac strain. **Methods**. Because cardiac dysfunction in mdx mice does not recapitulate end-stage cardiomyopathy in DMD patients, methods to pharmacologically accelerate cardiac injury with isoproterenol (ISO) have been utilized. However, mdx mice show variable susceptibility to cardiac injury by ISO. We've developed a novel cardiac injury method termed "double-hit" in which we challenge with ISO while simultaneously block the parasympathetic branch. We measured cardiac troponin I (cTnI) and Evans blue dye (EBD) uptake into damaged cardiac myofibers. We then developed the "hit and run," a novel method coupling the double-hit to a treadmill run to assess cardiovascular performance following a cardiac stressor. Serial cTnI levels were measured at baseline, after the double-hit, and after the run. Total run distance was recorded at baseline, following the double-hit, and 24 hours post-challenge. Following the hit and run challenge, a subset of mice were run daily for 2 weeks to assess the impact of daily activity on cardiac fibrosis and remodeling following a cardiac stressor. *Mdx* neonates were injected with AAV-uUtro and aged to 5 months or 10 months, with untreated littermate *mdx* and WT controls. **Results**. Following the double-hit, *mdx* mice had elevated cTnI levels (*mdx* 4.92 ng/mL v WT 0.28 ng/mL, p<.0001) and large percentage of EBD by area (mdx 4.68% v WT 0.35%, p=.0039). uUtro-treated *mdx* mice had significant reductions in both cTnI levels (1.21 ng/mL, p=0.0362) and %EBD (0.32%, p=.0002).

Surprisingly, in the hit-and-run assessment, the run exacerbated cTnI levels from post-double-hit levels in *mdx* mice in both age groups (10 mo mdx 4.15 to 9.18 ng/mL), while WT and uUtro-treated groups experienced a significant reduction in cTnI levels (10 mo final cTnI WT 0.50 ng/mL p=.0036, uUtro 1.5 ng/mL, p=.0083). *Mdx* mice ran fewer meters (10 mo 172.9 m) than both WT (10 mo 225.2 m, p=.0006) and uUtro-treated mice (10 mo 210.8 m, p=.013), and *mdx* mice dropped significant distance compared to baseline performance (% change *mdx* -16.6%) while both WT (-2.1%, p=.0208) and uUtro (+0.18%, p=.0334) maintained. uUtro-treated mice also had less cardiac fibrosis by area than *mdx* in an expression-dependent manner (uUtro 2.24% p=.0214, mdx 5.1%, WT 1.4% p<.0001). **Conclusions**. We developed a novel catecholamine-exercise stress test in the mdx mouse. AAV-uUtro protects *mdx*mice against acute cardiac injury and improves cardiovascular exercise performance following a cardiac stressor. AAV-uUtro's cardioprotective efficacy permits daily physical activity to match the improved skeletal muscle capacity in the treated *mdx* mouse out to the human equivalent of 40 years.

674. Combinatorial Antisense Oligonucleotide Gene Therapy Enhances Muscle Regeneration and Dystrophin Restoration for Duchenne Muscular Dystrophy

James S. Novak, Young Jae Moon, Ravi Hindupur, Iteoluwakishi H. Gamu, Jyoti K. Jaiswal

Center for Genetic Medicine Research, Children's National Hospital, Washington, DC

Low level dystrophin restoration is a major challenge towards realizing the full potential of the phosphorodiamidate morpholino oligomer (PMO)-based antisense oligonucleotide (AO) gene therapy for Duchenne Muscular Dystrophy (DMD). Our work has identified macrophages and myoblast fusion are required for PMO delivery to nascent muscle fibers. Poor myogenesis and excessive fibrosis induced by high TGFβ activity in DMD muscle contribute to poor efficacy of dystrophin PMO therapy. Using the severe DMD model (D2-*mdx*) that exhibits greater TGFβ activity, increased muscle degeneration, and poor myogenesis as compared to the milder B10-*mdx* model, we find significantly poor PMO-mediated dystrophin restoration in the severe D2-*mdx* muscle. To tackle poor dystrophin restoration by dystrophin PMO monotherapy, here we describe the use of a combinatorial PMO therapy that simultaneously inhibits macrophage TGFβ production and enhances myofiber dystrophin production. We developed a TGFβ inhibiting AO that significantly reduced TGFβ-mediated SMAD3 signaling in vivo. Use of this PMO allowed enhanced *in vivo* muscle regeneration following spontaneous or induced D2-*mdx* muscle injury. This was associated with reduced accumulation of fibroadipogenic progenitors and a notable increase in muscle regenerative capacity. Combinatorial use of this TGFβ-targeting PMO with dystrophin exon skipping PMO led to a significant increase in the extent of dystrophin expressing myofibers even after an acute single dose of PMOs. Chronic use of this combinatorial PMO therapy not only recapitulated these beneficial effects, but also reduced muscle degeneration and allowed greater improvement of muscle functional measures as compared to dystrophin exon skipping PMO monotherapy. Thus, our findings offer the first PMO combinatorial gene therapy for DMD that demonstrates the potential to tackle challenges associated with poor efficacy of PMO-mediated gene therapy for DMD, while simultaneously improving muscle restoration and reducing muscle loss.

675. Ridsipam's Safety and Efficacy in a Patient with Co-Existing Spinal Muscular Atrophy with Sickle Cell Disease

Momen Almomen, Shiakhah Aldossary, Walaa Albdulqader, Toqa Alhamrani, Nora Bastaki

King Fahd Specialist Hospital- Dammmam, Dammam, Saudi Arabia

Sickle cell disease (SCD) is a common genetically inherited autosomal recessive disease that is prevalent in certain ethnicities, including the Mediterranean region and African Americans. It is characterized by recurrent painful crises, reduced blood count, and recurrent infection. Spinal Muscular Atrophy (SMA) is a genetic autosomal recessive disease with a carrier frequency of one in 3000 in Saudi Arabia. Despite the relatively high carrier frequency of both diseases globally, patients with co-existing SMA and SCD have never been reported in the past. In this article, we present one patient with a dual diagnosis of SMA and SCD who received two different treatments, namely, Risdiplam and Hydroxurea. To our knowledge, this is the first ever to report this unique disease combination case in the literature and shed light on the safety and response to SMA-disease modifying therapy in SCD.

Wednesday Posters: Base Editing and Prime Editing

676. Designing a Base Editing Gene Therapy for Collagen VI-Related Dystrophy Informed by Mosaic Patient Phenotype

Gregory H. Boyek, Veronique Bolduc, Astrid Brull, Riley McCarty, A. Reghan Foley, Carsten G. Bonnemann

Neuromuscular and Neurogenetic Disorders of Childhood Section, NINDS/NIH, Bethesda, MD

Collagen VI-related dystrophies (COL6-RDs) are a group of genetic muscle disease existing on a spectrum of severity that ranges from the severe Ullrich congenital muscular dystrophy (UCMD) to the milder Bethlem muscular dystrophy. Patients with UCMD exhibit congenital onset weakness and hypotonia that prevents or causes the loss of ambulation and progressive respiratory insufficiency. Patients with Bethlem muscular dystrophy exhibit proximal weakness and contractures but remain ambulatory into adulthood, and intermediate phenotypes also exist. We have previously described a recurrent, de novo deep intronic variant in intron 11 of *COL6A1* (c.930+189C>T) that creates an in-frame pseudoexon insertion with a dominant negative mechanism of action. We then found this *COL6A1* pathogenic variant to be one of the most commonly identified causative variants in the *COL6* genes. This intron 11 variant in full heterozygosity results in a severe phenotype with minimal early symptoms that rapidly progress to a severe Ullrich congenital muscular dystrophy phenotype. Here, we describe our findings in a patient presenting with a milder

Bethlem muscular dystrophy phenotype in whom the intron 11 variant was present as somatic mosaic. The mosaic patient retained not only independent ambulation but running, ascending and descending stairs at age nine. Quantifying the degree of mosaicism showed that approximately 40% of patient cells are positive for c.930+189C>T. This provides us the rationale for a base-editing approach for correcting the intron 11 mutation, as correction of the mutation in just a proportion of cells comparable to a somatic mosaicism would be expected to be disease modifying. We have designed several base editor guide sequences based on predictive machine learning algorithms and are evaluating them in cultured patient fibroblasts. Our technique uses an easily translatable single-AAV vector with the potential to permanently ameliorate the severe Ullrich muscular dystrophy symptoms to a milder phenotype.

677. Engineered Viral-Like Particles-Delivered Base Editor Ribonucleoprotein Complex Confers Efficient Base Editing in hiPSC and hiPSC-Derived Cardiomyocytes

Yuan Zhou, Chen Zhang, Renzhi Han

Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN

CRISPR/Cas9 gene editing in human induced pluripotent stem cells (hiPSCs) has provided an exciting platform for disease modeling and translational research of various human diseases. However, the low editing efficiency poses a challenge for hiPSC engineering. In this study, we utilized engineered virus-like particles (eVLPs) to deliver base editor ribonucleoproteins (RNPs) for precise base editing in hiPSCs. Our findings demonstrate that eVLPs achieved a dosage-dependent increase in editing efficiency in hiPSCs, with peak efficiency ranging from 60% to 99% at various genomic loci. The eVLPs-delivered base editing had minimal impact on the pluripotency or differentiation capacity of hiPSCs. Furthermore, we demonstrated efficient base editing in a Duchenne muscular dystrophy (DMD) hiPSC cell line carrying a large deletion spanning exon 48 through 54. The eVLPs targeting the splice acceptor of exon 55 resulted in the efficient conversion of AG to GG (85.69% ± 0.58%) and enabled exon 55 skipping. The editing efficiency was maintained even after ten passages (87.20% ± 0.41%). In addition, eVLPs delivery rescued dystrophin expression in DMD hiPSC-derived cardiomyocytes, as examined by RT-PCR, Western blot, and immunofluorescence staining. These results highlight the great promise of eVLPs delivery of genome editor RNPs to facilitate hiPSC engineering.

678. First Correction by Prime Editing of Mutations in the *RYR1* Gene

Kelly Godbout[1,2], Joël Rousseau[2], Jacques P. Tremblay[1,2]

[1]Molecular Medecine, Laval University, Québec, QC, Canada,[2]Neurosciences, Centre de recherche du CHU de Québec, Quebec, QC, Canada

BACKGROUND AND AIMS: Our aim is to develop a gene therapy treatment for myopathies caused by mutations in the *RYR1* gene. This gene codes for a calcium channel named Ryanodine receptor 1 in muscle fibers. Its failure causes muscle weakness in patients, which degenerates into motor disabilities. Currently, there are no effective treatments for those diseases. We are therefore trying to develop a gene therapy by Prime editing to correct the mutations and restore the healthy sequence of the gene. Those disorders are mainly caused by the mutation of a single nucleotide. For our treatment, Prime editing, which allows the modification of a precise nucleotide in the DNA, will be used. We are actually achieving the correction of the T4709M in the *RYR1* gene. We are also working on correcting the G3191R mutations. Additionally, we are developing a treatment introducing a protective mutation in the *RYR1* gene. **METHODS:** Derived from the CRISPR/Cas9 technology, Prime editing is composed of a Cas9 fused to a reverse transcriptase and a prime editing guide (peg). We first built the required components for Prime editing by cloning. Several versions of the peg were constructed to optimize the efficiency of Prime editing. The treatment has been tested in several cell types (HEK293T, patient fibroblasts, myoblasts, and C2C12). Through manual cloning, we also generated, by Prime editing from normal myoblasts, a myoblast cell line carrying the T4709M mutation in the *RYR1* gene at the homozygous state. We have succeeded in producing the prime editor mRNA by *in vitro transcription* and in delivering it to myoblasts by electroporation, which allowed us to increase Prime editing's effectiveness. **RESULTS:** In HEK293T cells, we achieved 35% editing for the T4709M mutation and 55% for the G3191R mutation by plasmid DNA delivery. In the T4709M mutant myoblast cell line, we corrected the mutation at 59% after three doses of the treatment when the Prime editing components were delivered as RNAs. It is to be noted that people who are heterozygous for the T4709M mutation are healthy. We have, therefore, reached our *in vitro* goal, which was to exceed 50% of correction. We also successfully introduced the protective mutation in the *RYR1* gene at 33% in myoblasts by delivering the Prime editing components as RNAs. **CONCLUSIONS:** These results are the first demonstration in the scientific community that it is possible to correct mutations in the *RYR1* gene. This project paves the way for treating RYR1-related myopathies by gene therapy. These breakthroughs will significantly impact the treatment of other hereditary diseases. Indeed, this method can be applied to other genetic mutations, and in each case, only the pegRNA would need to be modified!

679. Systematic Off-Target Analyses of Base-Editing Therapeutic Leads Show Reduced Off-Target Editing, Reduced Bystander Editing, and Increased On-Target Editing by Hybrid Guide RNAs

Dominique L. Brooks[1], Madelynn Whittaker[1], Aidan Quigley[1], Ishaan Jindal[1], Rebecca Ahrens-Nicklas[2], Kiran Musunuru[1], William Peranteau[2], Xiao Wang[1]

[1]Cardiovascular Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[2]Children's Hospital of Philadelphia, Philadelphia, PA

Phenylketonuria (PKU) and hereditary tyrosinemia type 1 (HT1) are autosomal recessive disorders linked to the phenylalanine hydroxylase (*PAH*) gene and fumarylacetoacetate hydrolase (*FAH*) gene, respectively. Potential therapeutic strategies to ameliorate disease include corrective editing of pathogenic variants in the *PAH* gene and, as a variant-agnostic approach, inactivation of the 4-hydroxyphenylpyruvate dioxygenase (*HPD*) gene, a modifier of

HT1, via adenine base editing. Here we evaluated the off-target editing profiles of therapeutic lead guide RNAs (gRNAs) that when combined with adenine base editors correct recurrent *PAH* variants or disrupt the *HPD* gene in human hepatocytes. To mitigate off-target mutagenesis, we systematically screened hybrid gRNAs with DNA nucleotide substitutions. Comprehensive and variant-aware specificity profiling of these hybrid gRNAs with OligoNucleotide Enrichment and sequencing (ONE-seq) identified certain hybrid gRNAs that dramatically reduced off-target editing and reduced bystander editing, while actually increasing on-target editing either *in vitro* or *in vivo*, e.g., in a humanized PKU mouse model treated with lipid nanoparticles (LNPs) with adenine base editor mRNA and a standard versus hybrid gRNA. These studies highlight the remarkable utility of hybrid gRNAs to improve the safety and efficacy of base-editing therapies. Moreover, they recommend a novel strategy for de-risking of LNP editing therapies: rather than the usual approach of assessing a standard gRNA by techniques such as ONE-seq, compiling a large list of nominated candidate genomic sites, and extensively analyzing the candidate sites for off-target editing in treated cells—instead using variant-aware ONE-seq to screen hybrid gRNAs and identify a hybrid gRNA that has preserved or improved on-target editing while reducing the number of nominated candidate off-target sites requiring further evaluation to a few or even zero.

680. Base Editor-Mediated Correction of a CSF1R Mutation That Causes ALSP

Jasna Metovic[1], Yedda Li[1], Christiano R. R. Alves[1,2], David Rufino-Ramos[1], Leillani L. Ha[1], Natalie Golovanov[1], Yi Gong[1], Benjamin P. Kleinstiver[2,3], Florian Eichler[1]

[1]Department of Neurology, Massachusetts General Hospital, Harvard Medical School, Boston, MA,[2]Center for Genomic Medicine, Massachusetts General Hospital, Boston, MA,[3]Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, MA

Adult Onset Leukodystrophy with Neuroaxonal Spheroids and Pigmented Glia (ALSP) is a rare, neurodegenerative, autosomal dominant disease caused by loss-of-function mutations in the colony-stimulating factor 1 receptor (*CSF1R*) gene located at 5q32 chromosome. CSF1R is a tyrosine kinase receptor, essential for regulating the proliferation and activation of microglial cells. There are currently no disease-modifying therapies, and supportive treatment is used to control some of the ALSP symptoms. CRISPR-based engineered base editor (BE) technologies have proven to be effective at correcting pathogenic point mutations by precisely editing DNA sequences without intentionally inducing DNA double strand breaks. Cytosine base editors (CBE) enable the efficient conversion of C to T in human cells when programmed with a guide RNA (gRNA) designed to target a specific locus. Here we explored the use of CBEs to correct a recurrent *CSF1R* c.2381 T>C mutation that causes a non-synonymous amino acid substitution, I794T, that leads to loss of CSF1R function. We designed various gRNAs that would position the CBE edit window over top of the CSF1R mutation and explored various established and recently engineered CBE constructs. Using a patient-derived fibroblast cell line bearing the *CSF1R*-I794T mutation, we performed nucleofections (Lonza) with plasmids encoding the CBEs and gRNAs to identify optimal CBE-gRNA pairs capable of efficient and specific mutation correction. The fibroblasts maintained normal morphology post-nucleofection. Importantly, we observed approximately 90% *CSF1R*-I794T correction with an optimized CBE-gRNA pair (using TadA-CBEd), substantially exceeding correction levels of 70% observed with a more conventional BE4max CBE construct. Most editing events included only the intended C>T substitution to correct I794T, with minimal unwanted bystander editing of other nearby cytosine bases. We anticipate that the genome editing tools hold great potential for precise gene correction in ALSP patients and will eventually allow for targeted treatment of this currently incurable and fatal disease. We are currently evaluating the genome-wide specificity of this approach, to gain insight into the long-term safety of these therapies *in vivo*. Continued development of this efficient and safe editing approach should benefit ALSP patients, with hope for favorable clinical outcomes for disease control, prevention, and cure.

681. Unconstrained Mitochondrial DNA Editing with Evolved TALE Proteins

Santiago R. Castillo[1], Karl J. Clark[1], Stephen C. Ekker[2]

[1]Mayo Clinic, Rochester, MN,[2]University of Texas at Austin, Austin, TX

The human mitochondrial genome is a 16,569 bp long, multicopy, circular double-stranded DNA (dsDNA) molecule that encodes 37 genes essential for cellular energy metabolism. Pathogenic mitochondrial DNA (mtDNA) variants are prevalent in ~1 in 8,000 people and are causal in incurable metabolic disorders. Current mtDNA editing strategies rely on all-protein systems, such as transcription activator-like effector (TALE)-based technologies. In general, these technologies are composed of a TALE, which acts as a programmable DNA-binding protein, fused to an effector domain. In particular, dsDNA deaminase A (DddA)-derived cytosine base editors (DdCBEs) consist of TALE-$DddA_{tox}$ fusions. Given its preference for dsDNA, $DddA_{tox}$, the deaminase domain of DddA, is split into two inactive halves to avoid toxicity. Thus, a DdCBE monomer incorporates either the N- or C-terminus of split $DddA_{tox}$ downstream of a TALE. In the context of DdCBE pairs, binding of their respective DNA-binding domains to adjacent target sequences enables the reassembly of functional $DddA_{tox}$, followed by targeted cytidine deamination. This process leads to programmed C•G-to-T•A conversions in mtDNA. Despite their robustness, the versatility of canonical DdCBEs is thought to be limited by the requirement of a thymine immediately upstream of their respective TALE target sequences. Following this conventionally accepted double 5'-T constraint (one per DdCBE monomer), about 12% of the human mitochondrial genome is theorized to remain inaccessible to canonical DdCBEs. Here, aiming to elucidate the relevance of the 5'-T constraint in the context of DdCBE-mediated mitochondrial base editing, and enable access to 100% of the editable motifs in the human mitochondrial genome, we generated DdCBEs containing TALE proteins engineered to bypass the 5'-T constraints, herein referred to as αDdCBEs. First, seeking to characterize the activity and specificity profiles of αDdCBEs relative to DdCBEs, we compared pairs of each type of editor in both 5'-T-compliant and 5'-T-noncompliant formats across multiple mtDNA loci accessible to both types of editors. Unexpectedly, we observed that DdCBEs are active even when they are 5'-T-noncompliant. Nonetheless, as hypothesized, 5'-T-noncompliant αDdCBEs tend to be significantly more active than 5'-T-noncompliant DdCBEs, reaching the same levels

of activity as 5'-T-compliant DdCBEs. Subsequently, we compared αDdCBEs and DdCBEs at *MT-TC* and *MT-TL1*, two mitochondrial loci considered to be inaccessible to DdCBEs. Remarkably, DdCBEs were as active as αDdCBEs at *MT-TC*, reaching editing efficiencies of up to 50% in HEK293T cells 3 days post-transfection. Nevertheless, at *MT-TL1*, αDdCBEs were more active than DdCBEs. Finally, seeking to optimize a base editing strategy for the installation of the pathogenic m.3242G>A variant in *MT-TL1*, we screened an array of αDdCBEs at this locus. Notably, all editors in this approach contained DddA11, an engineered $DddA_{tox}$ variant known to be highly active. Interestingly, we observed that sliding either one or both arms of an αDdCBE pair even just a couple of nucleotides led to dramatically distinct editing profiles. This screening approach resulted in the installation of the m.3242G>A pathogenic variant at about 50% editing efficiency with low bystander editing in HEK293T cells. In summary, αDdCBEs are generally better editors than DdCBEs, successfully bypassing the 5'-T constraint, which rather than being an absolute barrier for DdCBE-mediated mtDNA editing, acts as a limiting factor in most target loci. These observations support unconstrained mitochondrial base editing as a strategy for disease modeling and potentially gene therapy applications.

682. *In Vivo* Gene Editing Treatment of Alpha1-Antitrypsin Deficiency

Zi Jun Emma Wang

YolTech Therapeutics, Shanghai, China

Alpha-1 antitrypsin deficiency (AATD) is a rare genetic disorder that causes liver disease in children and adults, as well as lung disease in adults, and is mainly manifested as serum α1-antitrypsin (AAT) deficiency. AATD is most commonly caused by Z mutation, which results in the substitution of glutamic acid with lysine (Glu342Lys) and produce mutant proteins that are prone to misfolding and aggregation. To address lung and liver disease associated with AATD, gene editing drugs have focused on correcting Z mutation. YOLT-202 is an in vivo gene-editing therapeutic agent that is designed to treat AATD by increasing the concentration of AAT in serum. It is based on adenine base editor (ABE) system and comprises a lipid nanoparticle encapsulating messenger RNA for ABE and a single guide RNA targeting Glu342Lys of *SERPINA1*. Delivery of YOLT-202 into HepG2 cells resulted in 60% editing efficiency at position A7 at a dose of 30ng,which can correct Glu342Lys mutation. AATD Mouse serum ELISA data indicated a significant increase in serum AAT to 25 mg/mL at 7 days after a single dose (0.5 mg per kilogram and 2 mg per kilogram). There was no significant increase in alanine aminotransferase (alt) and aspartate aminotransferase (ast) at day 1,3,7 after infusion, indicating that YOLT-202 did not cause significant liver injury. Taken together, our findings suggest that YOLT-202, which can correct the Z mutation and restore the expression level of AAT in serum, can be effectively applied to the treatment of AATD.

683. Rescue of AAT-Z Mutant Protein Aggregation in PiZ Mice After Treatment with an LNP Delivered RNA Editing Oligonucleotide Targeting the E342k Mutation

Melissa Pink

Pharmacology, Korro Bio, Cambridge, MA

Alpha-1 antitrypsin (AATD) is a rare genetic disorder that results in abnormal protein aggregate formation within hepatocytes. The most prevalent mutation in humans for AATD is the Glu342Lys (also known as the E342K or Z) point mutation. Korro Bio is using an RNA editing approach to correct the E342K (G>A) mutation to target the Z allele. RNA editing is a natural physiological process that occurs in cells where a specific single base edit is mediated by an enzyme called Adenosine Deaminase Acting on RNA ("ADAR"). Korro Bio's RNA editing approach involves co-opting this endogenous editing system via a proprietary engineered oligonucleotide to introduce precise edits to RNA. To study the pharmacological activity of Korro Bio's LNP delivered oligonucleotide *in vitro* and *in vivo*, the PiZ transgenic mouse model of AATD liver disease was used. The livers of these mice have significant Z protein aggregation and secrete the mutant human Z-AAT protein into circulation. Invitro data demonstrated that an LNP delivered oligonucleotide can edit *in vitro* hepatocytes derived from PiZ mice. *In vivo,* sub chronic dosing resulted in editing of the E342K mutation that led to newly repaired M-AAT protein. The function of this protein was assessed in a neutrophil elastase (NE) inhibition assay. Data demonstrated that elastase inhibition correlated with the percent editing observed in the livers of the mice. These data support utilizing RNA editing to correct the mutated protein to achieve functioning AAT protein levels in PiZ mice and the potential to translate to similar results in AATD patients.

684. Novel and Efficient Base Editors Engineered to Comprehensively Target the Human Genome

Cristina N. Butterfield, Cindy J. Castelle, Kartik Lakshmi Rallapalli, Steve J. Flammer, Morayma Temoche-Diaz, Jin Wen Tan, Liliana Gonzalez-Osorio, Chia-Yao Lee, Michael L. Spelman, Emily C. Suter, Jared Muysson, Lisa M. Alexander, Daniela S. A. Goltsman, Christopher T. Brown, Brian C. Thomas

Metagenomi, Inc., Emeryville, CA

Base editors can knock out genes and correct pathogenic mutations with single nucleotide precision, making them promising tools for treating human diseases. Base editing systems consist of a programmable nickase fused to either a cytosine (CBE) or adenine deaminase (ABE) to catalyze site-specific nucleotide transitions. However, current systems cannot adequately address many disease indications due to efficiency and targeting limitations. Here, we used modular engineering to develop a flexible toolbox of efficient base editing systems from novel metagenomics-derived components. Base editor targeting ability was expanded by replacing the PAM interacting domain with variants that have different sequence preferences. Compared with first generation SpCas9 base editors, this platform has improved ABE human genome targeting potential from 18% to

95%, and CBE targeting potential from 15% to 53%. We demonstrated that this system can be used to efficiently knock out several genes for therapeutically-relevant editing of liver cells, and validated the system in vivo using mRNA and LNP delivery. Furthermore, we achieved efficient multiplexed gene editing and protein knockdown in primary T cells for cell therapy ex vivo applications, without impacting cell viability. By leveraging an expansive metagenomics database and rational and evolutionary protein engineering, we have developed ABEs and CBEs that significantly improve human genome coverage over SpCas9-based BEs, are highly efficient with minimal toxicity, and have demonstrated success with LNP delivery in vivo. This suite of highly active base editors has the potential to expand the scope of diseases that can be addressed, and accelerate the development of next generation genetic medicines.

685. Prospects and Challenges of CRISPR-Based Gene Editing of Tuberous Sclerosis Complex 2 Gene in Human Mesenchymal Stem Cells

Abdallah Salemdawod[1,2], Brandon Cooper[3], Yajie Liang[1], Piotr Walczak[1], Jaroslaw Maciaczyk[2], Miroslaw Janowski[1]

[1]Diagnostic Radiology and Nuclear Medicine, University of Maryland School of Medicine, Baltimore, MD,[2]Diagnostic of Neurosurgery, University Hospital Bonn, Bonn, Germany,[3]University of Maryland Greenebaum Comprehensive Cancer Center, University of Maryland School of Medicine, Baltimore, MD

Introduction: Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disorder that results from a mutation in either the TSC1 or TSC2 gene and is characterized by the presence of vascular hamartomatous tumors in various organs. While epilepsy is the most challenging symptom and our long-term interest, an increasing involvement of TSC2 mutation in cancerous tumors is being unfolded. Moreover, the aberrant morphogenic signals originating from mesenchymal progenitors with TSC2 mutations underscore their significant involvement in the pathomechanism of TSC. This emphasizes the suitability of mesenchymal stem cells (MSC) as an initial target for genome editing interventions. Therefore, we sought to test the ability of novel CRISPR-based gene editing tools to introduce a very frequent point mutation in the TSC2 gene to human MSCs (hMSCs), as a basis for a future attempt to correct it through genome editing. **Methods:** We targeted a frequently reported point mutation affecting the TSC2 gene (1864C>T (p. Arg622Trp)) in hMSCs *in vitro*. The guide RNA sequences were designed using AI-based tools with a high predicted editing efficiency of 98% and a low off-target editing yield of 1 potential unwanted SNP edit. Lipofectamine was used for co-transfection of gRNA with the base editor in the form of ribonucleoprotein (RNP) or mRNA. The editing efficiency was quantified using Sanger sequencing. **Results:** RNP scored an impressive 47% editing efficiency, while the performance of mRNA was three times lower but still satisfactory at 15%. In both cases, each on-target edit was consistently accompanied by one off-target edit within the site. **(Figure 1)**. **Conclusions:** Relatively high, double-digit efficacy of CRISPR-based editing of the TSC2 gene in hMSCs is feasible *in vitro* using clinically applicable strategies such as transfection. One unexpected and unpredicted off-target edit was observed within the PAM site but with very high consistency. Whole genome analysis is needed to detect all off-target edits. Furthermore, AI prediction of editing efficiency and off-target yield appears to be based on the genomic structure but does not consider the characteristics of the targeted cell population. In summary, CRISPR-based gene editing is a promising strategy for the treatment of tuberous sclerosis.

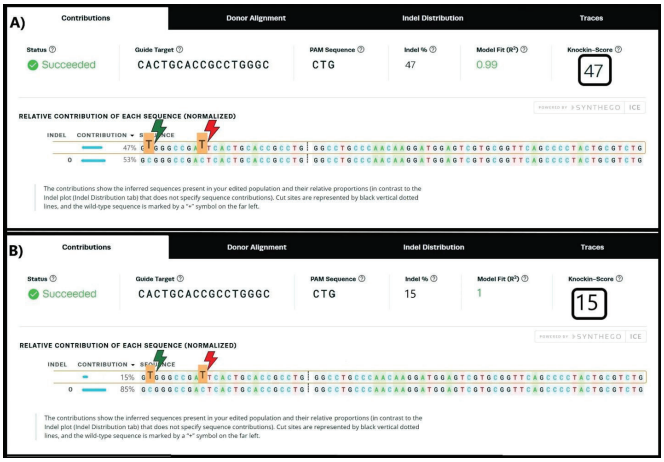


Figure1: A) ICE analysis of the sequenced amplicons form gene-edited MSCs using Cas9-RNP, showing an editing efficiency of 47% of C>T (Orange) for both on-target (green spark) and off-target (red spark) regions within the PAM-site. **B)** ICE analysis of the sequenced amplicons form gene-edited MSCs using Cas9-mRNA, showing a lower editing efficiency of 15% of C>T (Orange) for both on- target (green spark) and off-target (red spark) regions within the PAM-site.

686. Quantifying Prime Editing Kinetics - Timelapse Fluorescence Data and Model Inference

Nathalie Gabriele Schaeffler[1], Julian Geilenkeuser[2], Dong-Jiunn Jeffery Truong[2], Gil Westmeyer[2], Joachim Rädler[1]

[1]Faculty of Physics, Ludwig-Maximilians-Universität München, Munich, Germany,[2]Institute for Synthetic Biomedicine, Helmholtz Zentrum München, Oberschleißheim, Germany

Prime Editing (PE) enables a precise “search and replace” of gene elements to cure many gene and cell based diseases [1]. While current research focuses predominantly on enhancing its efficacy, the kinetics of PE remain underexplored. We study the kinetics of pDNA and mRNA based delivery of PE protein and pegRNA. As a reporter system, we use HEK293T cells, which constitutively express a blue-shifted mGreenLantern (bs-mGL) protein. Two proximal base edits on a single pegRNA are sufficient to revert it to the original mGreenLantern (mGL) sequence. Transfection of the PE system induces these edits, restoring the expression of the original mGL protein. Using automated single cell timelapse imaging (LISCA [2]), we record the time-to-edit and the onset distributions (see Fig. 1A) and model this edit process mathematically using the Julia programming language. We split the PE process into three steps: the delivery of the PE components, the expression of these and the editing process itself (see Fig. 1 B-D).

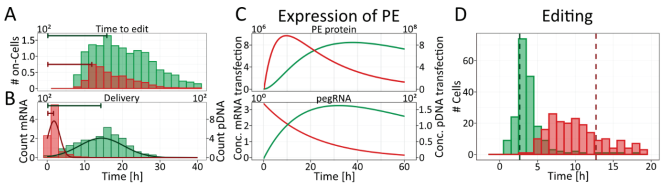


Fig 1: Graphical representation of PE kinetics (A) Time to edit: From transfection to onset of mGL fluorescence for pDNA(green) and mRNA(red) (B) Time from transfection till release of pDNA(green) and mRNA(red) based delivery (C) Concentration-Time courses inferred from the model for PE protein and pegRNA starting from either pDNA(green) or mRNA(red). (D) Editing time: Time between detected expression of PE protein and detected edit for different pegRNAs ($mean_{short}$ = 2.64h, $mean_{long}$ = 12.7h).

In order to estimate the parameters of the mathematical model, we performed additional, independent experiments. For the first step, delivery with mRNA or pDNA, we transfect the cells directly with mGL encoded in pDNA and mRNA and record a mean delivery delay of 14.5h (pDNA) and 1.4h (mRNA). For the second step, the expression of the PE protein and pegRNA, we simulate these with kinetic rate equations. The parameters for this were measured through experiments as explained in the literature [3]. These simulations show a slower onset for pDNA than mRNA, but higher end concentrations. Lastly, for measuring the third step, i.e. the editing time from expressed PE Protein to completed edit, we again use HEK293T cells with bs-mGL. In this iteration, a P2A-mScarlet3 region is added to the PE2 plasmid following a plasmid design from David Liu [4]. We now image the expression of both, PE2 via mScarlet and the edit via mGL, per cell and can therefore determine the time between mScarlet and mGL onset for pegRNAs. These pegRNAs target the same edit (bsmGL to mGL), but differ in edit length inside the Reverse Transcriptase template ($edit_{short}$ = 6bp, $edit_{long}$ = 21bp) . The mean editing time is 2.64h and 12.7h respectively. In summary, using experiments and modelling, we are able to extract the kinetics of single cell prime editing events. The time-to-edit consists of the delivery time, the protein expression and the editing time itself, which is influenced by the pegRNA design and can range from 2h for short edits up to several times that for longer edits. This matches with prior findings on DNA-strand replacement that showed how length and mismatches affect the duration of this process [5]. Our work shows that kinetic data combined with mathematical modelling can help to optimise pegRNAs and PE efficacy in silico, which may guide experimental screens in the future. [1] https://doi.org/10.1038/s41576-022-00541-1 [2] https://doi.org/10.3791/62025 [3] https://doi.org/10.1016/j.nano.2019.102077 [4] https://doi.org/10.1038/s41586-019-1711-4 [5] https://doi.org/10.1021/jacs.0c03105

687. Highly Efficient Base Editing of Homozygous and Heterozygous COL7A1 Variants in RDEB Patients' Fibroblasts Using ABE8e mRNA System Delivered via Novel LNP Formulations

Joanna Jackow-Malinowska[1], Ina Guri[1], Imogen R. Brooks[1], Yara Alrokh[1], Aidin Kazamizadeh[1], Carina Graham[1], Stephen L. Hart[2], John McGrath[1]

[1]King's College London, London, United Kingdom,[2]UCL Great Ormond Street Institute of Child Health, London, United Kingdom

Introduction and aim: Adenine base editors (ABEs) present an attractive approach to gene editing in the inherited blistering disease, recessive dystrophic epidermolysis bullosa (RDEB), which results from pathogenic variants in *COL7A1*. We previously showed that ABE8e, a new variant of ABE, confers remarkable efficiency (94.6%) and a favourable safety profile in correcting variants and restoring collagen 7 (C7) expression when electroporated into RDEB fibroblasts. With a goal to develop topical base editing creams, we now explore use of lipid nanoparticle (LNP)-encapsulated ABE8e mRNA system to edit two distinct variants in RDEB fibroblasts. **Methods:** Primary patient fibroblasts harboring a homozygous G>A mutation (exon 42) or a heterozygous C>T mutation (exon 54) were gene edited using ABE8e mRNA, delivered via lipofectamine or six different LNPs. To assess the gene editing efficiency, Sanger Sequencing and NGS methods were employed. Cell viability was evaluated via an LDH cytotoxicity assay, while functional restoration of C7 expression was assessed via western blotting of cell lysate and supernatant. Finally, topical delivery was tested on normal human skin equivalents (HSEs). **Results:** We identified an optimal ratio of 1:7 for sgRNA:ABE8e, at which editing efficiency increases with ABE8e dosage, with 400ng being sufficient for 90% bi-allelic editing. Formulations containing DTDTMA (C14) and DHDTMA (C16) LNPs demonstrated ~90% and ~80% editing, with and without the peptide, respectively, as well as reduced cytotoxicity compared to lipofectamine. Depending on the nature of the mutation, the edit corresponded to an increase in C7 expression in supernatant only or cell lysate as well, whereby C14 LNPs enabled two-fold C7 restoration. Finally, our HSEs demonstrated viability of topical delivery of lipofectamine-encapsulated GFP mRNA. **Conclusions:** We demonstrated the potential value of base editing as a gene therapy for RDEB as well as of these LNPs for clinical translation of the therapy. Nonetheless, further safety and functionality tests are to be carried out.

688. Innovative Transformer Base Editor (tBE)-Mediated Undetected Off-Target Base Editing and Its Potential Clinical Applications for Unmet Medical Needs

Lijie Wang[1], Peixue Li[1], Lang Zhang[1], Yichuan Wang[1], Zhuoan Cheng[1], Yan Zhang[1], Runni Guo[1], Hongxia Ma[1], Kewei Lan[1], Huiming Zheng[1], Di Zhu[1], Jinyan Zuo[1], Yuchen Zhang[1], Jing He[1], Qing Su[1], Xiaodun Mou[1], Jia Chen[2]

[1]CorrectSequence Therapeutics Co., Ltd, Shanghai, China,[2]ShanghaiTech University, Shanghai, China

The fusion of CRISPR-Cas9 with cytidine deaminases leads to base editors (BEs) capable of programmable C-to-T editing without DNA double strand break (DSB), which has potential in clinical applications but suffers from off-target (OT) mutations. Here, we used a cleavable deoxycytidine deaminase inhibitor (dCDI) domain to construct a transformer BE (tBE) system. After the colocalization of paired hsgRNA and msgRNA on genomic DNA, the assembled TEV protease cleaves the mA3dCDI off and mA3CDA1 is activated, which induces efficient and precise base editing at the on-target site. The tBE system could induce efficient editing with no detected genome-wide and transcriptome-wide OT mutations and avoid cell apoptosis and chromosomal abnormalities triggered by DNA DSB. It holds great potential in therapeutic applications, including previously undruggable targets due to its highly efficient on-target editing and undetected off-target mutations. Hematopoietic stem cells (HSCs) and T cells can

be edited by tBE *ex vivo* for the treatment of hematological diseases. Here we show that tBE could edit *HBG1/2* promoter region in the CD34+HSCs to trigger the reactivation of fetal hemoglobin (HbF) to treat transfusion-dependent β-thalassemia (TDT) patients. Due to the safety advantages of tBE, it can edit the most potent target site to trigger the highest HbF expression when compared with other CRISPR-based gene editing therapies. And tBE could edit multiple sites simultaneously without causing off-target mutations or chromosomal translocation in T cells to enhance its efficacy or reduce its immunogenicity for the next generation Chimeric antigen receptor T-cell (CAR-T) therapies development. Besides, we have demonstrated LNP and AAV delivery of tBE to disrupt *PCSK9 in vivo* in mice via introducing a premature stop codon, indicating that tBE could flexibly adapt to various *in vivo* delivery vehicles for liver-targeted gene editing therapy, such as hypercholesterolemia. Furthermore, tBE could precisely introducing several nonsense mutations simultaneously to silence Hepatitis B viral genes, enabling sustained reduction of the three major Hepatitis B viral (HBV) markers, HBsAg, HBeAg and HBV DNA *in vitro*. The above data all demonstrate the great potential of tBE for the treatment of various disease including blood disorders, metabolic diseases, and infectious diseases. tBE could be a powerful tool in developing novel therapeutic approaches for unmet medical needs.

689. Prime Editing for Correction of GATA2 Deficiency in Human CD34+ Hematopoietic Stem Cells

Jonas H. Wolff[1], Thomas W. Skov[1], Sofie R. Dorset[1], Anne Louise S. Revenfeld[2], Didde Haslund[2], Mette Holm[3,4], Trine H. Mogensen[1,5], Bjarne K. Møller[2], Rasmus O. Bak[1], Jacob G. Mikkelsen[1]

[1]Department of Biomedicine, Aarhus University, Aarhus, Denmark,[2]Department of Clinical Immunology, Aarhus University Hospital, Aarhus, Denmark,[3]Department of Paediatrics and Adolescent Medicine, Aarhus University Hospital, Aarhus, Denmark,[4]Department of Clinical Medicine, Aarhus University Hospital, Aarhus, Denmark,[5]Department of Infectious Diseases, Aarhus University Hospital, Aarhus, Denmark

Ex vivo gene editing of autologous human hematopoietic stem cells is being developed as a treatment for inborn errors of immunity (IEIs). However, the current gene editing platform relying on Cas9-directed DNA cleavage and the use of rAAV6 as a donor for homology-directed repair is challenged by indel formation, off-target DNA cleavage, and toxicity in hematopoietic stem cells. Thus, there is a need for improved genome editing strategies in hematopoietic stem cells that do not require delivery of a DNA donor template and act without generating DNA double-strand breaks (DSB). Here, we present work using the DSB-independent prime editing technology to correct a patient-derived 7 bp deletion in the *GATA2* gene causing GATA2 deficiency, which can cause immunodeficiency, bone marrow failure, and increased risk of leukemia. We screened several prime editing guide RNAs (pegRNAs) as well as nicking sgRNAs (ngRNAs) to efficiently correct the 7 bp deletion, and we found that editing efficiencies were highly dependent on the ngRNA, resulting in prime editing rates in model K562 cell lines that varied between 20% and 80%. We then aimed at establishing efficient prime editing in CD34+ hematopoietic stem and progenitor cells (HSPCs). Initial attempts using *in vitro*-transcribed PEmax mRNA in combination with synthetic pegRNAs and ngRNAs resulted in low editing efficacies. However, using enhanced pegRNAs (epegRNAs) as well as optimizing nucleofection conditions, mRNA structure and epegRNA:ngRNA ratios, we were able to achieve efficient prime editing of up to 60% of *GATA2* alleles in CD34+ HSPCs from healthy donors as well as in patient-derived HSPCs. Furthermore, we investigated the fitness of the CD34+ HSPCs following editing using either prime editors or conventional HDR-based gene editing. While prime editing generally resulted in less editing than conventional HDR-based gene editing, prime-edited HSPCs showed superior viability and proliferation. Furthermore, as opposed to RNP nucleofection and AAV transduction, which led to a 2-fold decrease in colony forming units (CFUs) compared to mock-treated HSPCs, prime-edited HSPCs showed no significant decrease in CFU potential. We are currently investigating the potential of prime-edited patient-derived CD34+ HSPCs to engraft in immunodeficient mice. We are also investigating off-target editing as well as potential adverse on-target genomic alterations using long-read sequencing. Together, our preliminary results indicate an increased fitness compared to traditional CRISPR-based gene editing approaches, supporting the further exploration of prime editing to treat IEIs.

690. Induction of HbF Using Improved CRISPR-Cas12a Base Editor

Stefano Stella[1], Haixia Wan[2], Alex Perez[1]

[1]Ensoma, Copenahgen, Denmark,[2]Ensoma, København N, Denmark

Base editing is a minimalist gene editing system that can induce precise base conversions within the target DNA. It includes a catalytically impaired Cas protein and a DNA-modifying enzyme. The most widely used base editors contain a nickase variant of Cas9 that binds and cleaves one strand of the target DNA and this nicking activity is necessary for efficient base editing. CRISPR-Cas12a is a type V CRISPR system containing a single DNA cleavage site where Cas12a undergoes protein rearrangement to sequentially bring each strand of the DNA target into the catalytic pocket to be cleaved. Thus, generating a highly efficient nickase enzyme with Cas12a scaffold can be challenging. However, given its multiplex capability and high specificity, Cas12a stands out as an appealing scaffold for developing a safe and easy-to-deliver gene editing system. At ENSOMA, using a structurally guided approach, we have generated and screened thousands of Cas12a variants to develop an Engenious™ base editing platform that induces high level of base editing, maintaining the multiplex ability and the high specificity of the Cas12a scaffold without cutting any strand of the DNA target. We have used our base editing approach to induce high level of fetal hemoglobin production.

691. Chemically Controlled Base Editors for Precise Gene Editing *In Vivo*

Xue Gao

University of Pennsylvania, Philadelphia, PA

DNA base editors use deaminases fused to a programmable DNA-binding protein for targeted nucleotide conversion. However, base editors often cause a broad range of off-targets, including the most widely used TadA deaminases. Here, we present two chemically controlled methods that utilize chemically induced dimerization (CID)

to control the catalytic activity of the deoxyadenosine deaminases. Our results show high on-target editing activity upon small molecule induction while maintaining low background activity without induction. Importantly, the chemically controlled base editing systems have reduced genomic and transcriptomic off-target effects. When delivered via dual adeno-associated virus vectors, we also efficiently convert a single A•T base pair to a G•C base pair on the *PCSK9* gene in mouse liver, demonstrating in vivo CID-controlled DNA base editing. Thus, our systems demonstrate precise control of base editing, which will have broad implications for basic research and in vivo therapeutic applications.

692. Developing a Novel Adenine Base Editor Tool for *In Vivo* Alpha-1 Antitrypsin Deficiency Gene Correction

Jenny Gao
RNA Therapeutics Institute, Worcester, MA

Background: Alpha-1 antitrypsin deficiency (AATD) is most commonly caused by a G-to-A mutation in the *SERPINA1* gene (PI*Z mutation), which encodes AAT protein, an abundant serum serine proteinase inhibitor. AAT is synthesized by hepatocytes and delivered via serum to the lung, where it neutralizes neutrophil elastase. Patients with AATD are at an increased risk of liver disease due to mutant protein aggregation and lung emphysema due to elastase overactivity. Adenine base editor (ABE) consists of an adenine deaminase (TadA) conjugated to a Cas9 nickase, mediating A to G base editing within a defined editing window. While ABEs based on *Streptococcus pyogenes* Cas9 (SpCas9) have been used to correct the PI*Z mutation, they were too large for efficient therapeutic delivery via a single adeno-associated virus (AAV) due to the SpCas9 protein size. Using a compact, evolved Cas9 nickase derived from *Neisseria meningitidis* (eNme2-C) that can be packaged using a single AAV, we assessed its editing efficiency at the PI*Z site. Not only was there high editing efficiency, but bystander editing rates can be decreased depending on the TadA deaminase used. **Methods:** The HEK293T PI*Z reporter cell line was generated by transduction of HEK293T cells with lentivirus carrying a plasmid encoding for the Z-AAT coding sequence followed by puromycin selection. To test the editing efficiency of ABEs in editing the AAT site, we transfected the PI*Z reporter cell line with plasmids and quantified editing efficiency by targeted amplicon deep-sequencing. The transgene was packaged into AAV8 and injected into PI*Z mice via tail vein at 9 x 10^{11} vg. Animals were sacrificed eight weeks after treatment. Five liver biopsies were taken per animal and the PI*Z site was sequenced using by targeted amplicon deep-sequencing. Two lobes were taken per animal to perform PAS-D staining. Sex-, age-, and weight- matched PI*Z mice were used as controls. **Results:** An all-in-one eNme2-C ABE8e/guide 2 plasmid achieved approximately 20% on target editing in HEK293T PI*Z cells, with bystander editing rates reaching as high as 12.83% depending on the adenine position (n=3). To minimize bystander editing, we used ABE9e, which has a narrower editing window than its predecessor, ABE8e. Indeed, eNme2-C ABE9e/guide 2 displayed lower bystander edits compared to ABE8e while maintaining high on-target editing. Its on-target editing rate averaged 21.08% while the highest bystander rate was 9.06% (n=3). An all-in-one eNme2-C ABE9e/guide 2 AAV was administered to PI*Z mice using AAV8 and showed approximately 30% editing efficiency after 8 weeks. PAS-D staining revealed a decrease in PAS-D^+ area and increase in PAS-D^- cells in treated mice compared to control mice. **Conclusions:** We demonstrated the feasibility of an eNme2-C based ABE in single AAV to edit the PI*Z site. ABE9e showed high on-target edits with low bystander edits. Moreover, it was able to decrease PAS-D^+ globules in PI*Z mice. Future work would focus on assessing the therapeutic threshold needed to prevent lung emphysema development.

693. *In Vivo* Dual AAV Split Prime Editing Mediated Correction of Pathogenic *Ppp2r5d* Mutations in Mouse Brain

Isaac J. Villegas, Rency Dhaduk, David L. Cameron, Julian Halmai, Kyle D. Fink
Department of Neurology, University of California Davis Health Systems, Sacramento, CA

PPP2 syndrome type R5D or Jordan's syndrome is a rare monogenic form of intellectual disability that arises from *de novo* missense mutations that occur within protein phosphatase 2, regulatory subunit B' delta (*PPP2R5D*, OMIM 601646). *PPP2R5D* encodes an isoform of the regulatory B subunit, B56δ, for the protein phosphatase 2A (PP2A) holoenzyme that is enriched within the brain. As an essential serine/threonine phosphatase, PP2A plays a significant role in regulating phosphorylation across many cell signaling pathways. Whole exome sequencing from patients has previously identified 16 unique pathogenic variants spanning *PPP2R5D*, with clusters of mutations identified in exons 5, 7, and 12. Within the current patient population, the two most prevalent variants that comprise ~50% of known cases, p.E198K and p.E200K respectively, are both caused by G>A transition mutations and reside in exon 5. To date, there are no disease-modifying treatments available for patients. Prime editing is a novel genome editing tool that offers a unique advantage over previous CRISPR/Cas9 technologies since it does not require the generation of double-strand breaks or the use of exogenous donor DNA templates. Additionally, previous work has demonstrated its ability to function in non-dividing cells, such as neurons. Therefore, we employed prime editing mediated gene correction as a novel therapeutic intervention for Jordan's syndrome. In our study, we designed enhanced prime editing guide RNAs (epegRNAs) that target and edit *Ppp2r5d*. Initial epegRNA screens using PE2max generated <1% editing. Incorporating an additional nicking gRNA (PE3max) generated a 28-fold increase in editing at the *Ppp2r5d* locus. We further optimized our epegRNAs by evaluating various reverse transcription templates and primer binding site lengths. Utilizing our optimized epegRNAs targeting *Ppp2r5d*, we demonstrate up to 20% editing efficiencies with minimal insertions or deletions (indels) detected in mouse Neuro2A cells. Moreover, a current drawback in utilizing prime editing systems for *in vivo* applications is the size of the prime editing components. With a minimum size of ~6.3 Kb, prime editors surpass the packaging capacity of gold-standard delivery vehicles such as adeno-associated virus (AAV). Therefore, to overcome the packaging capacity limitations of AAV and evaluate *in vivo* prime editing of *Ppp2r5d*, we developed an intein-split prime editing system that is within the packaging capacity of two AAV genomes. Utilizing novel murine models of Jordan syndrome that contain the human equivalent mutations, p.E198K and p.E200K respectively, we demonstrate in vivo correction of pathogenic *Ppp2r5d*

mutations in mouse brain. In summary, we establish prime editing as a corrective approach to target pathogenic disease-associated mutations within the central nervous system.

Wednesday Posters: Epigenetic Editing and RNA Editing

694. CasMINI-Mediated CRISPR Epigenome Editing for Cell Type-Specific Manipulation of Gene Expression

Mikah Green, Nikhil Hajirnis, Eric Choi, Ramesh Chandra, Shanmugasundaram Ganapathy, Mary Kay Lobo

Department of Neurobiology, University of Maryland School of Medicine, Baltimore, MD

CRISPR epigenome editing, using CRISPR-activation or CRISPR-interference (CRISPRa/i) allows for more physiologically relevant manipulation of gene expression. Previously, our lab has used Cas9 derived CRISPRa/i epigenome editing tools and light-inducible Opto-CRISPRa/i tools in Neuro2A cells to manipulate a transcriptional program that was altered in specific neuronal populations in the brain following exposure to an addictive substance. Recent advances in CRISPR have paved the way for using a Cas12f derived system (CasMINI) instead of Cas9. The advantages of this system are twofold - First, dead (d)CasMINI is less than half the size of dCas9 allowing for packaging in adeno-associated viruses (AAVs) instead of the previously used lentiviruses. Further, this smaller construct allows the possibility of including multiple guide constructs for manipulation of multiple genes at the same time. We are utilizing a CRISPRi system in which a KRAB domain is fused to dCasMINI, and a CRISPRa system in which VP64 is fused to dCasMINI. These constructs are paired with a dCasMINI-compatible guide RNA (gRNA) construct. With this system, we target hub genes previously found to be upregulated and downregulated in significant gene network modules in mouse brain neuron stubtypes following forced abstinence from fentanyl. We have validated for the first time in Neuro2a cells that the dCasMINI fusion genes can express at the mRNA and protein level. We have also shown that both the dCasMINI-KRAB & dCasMINI-VP64 constructs paired with gRNA can successfully downregulate and upregulate targeted gene expression levels, respectively. We are currently validating the light-inducible Opto-dCasMINI system for temporal-specific control of gene expression. For this platform, dCasMINI is fused to CIBN and KRAB or VP64 are fused to Cry2. Prior to light stiumation, the gRNA construct recruits dCasMINI-CIBN to the targeted gene locus and upon light stimulation, CIBN & Cry2 fuse, recruiting transcriptional regulator (VP64 or KRAB) to the promoter region of the target gene. The potential to use these tools to alter transcription of specific gene(s) in a cell type-specific manner in the brain will allow routine use of AAV CRISPR epigenome editing tools in vivo.

695. CRISPR-Mediated *Reln* Knockdown Disrupts Striatal Physiology and Cocaine Reward

Kasey Lynn Brida[1], Emily T. Jorgensen[1], Morgan E. Zipperly[1], Robert A. Pillips[1], Cathy E. Newman[1], Jennifer J. Tuscher[1], Kelsey Montgomery[2], Kristen R. Maynard[3], Keri Martinowich[2], Jeremy J. Day[1]

[1]Neurobiology, University of Alabama at Birmingham, Birmingham, AL,[2]Lieber Institute for Brain Development, Baltimore, MD,[3]Lieber Institute for Brian Development, Baltimore, MD

Reelin is a large, secreted glycoprotein with a well-characterized role in brain development and links to numerous neuropsychiatric disorders. While Reelin is abundant in the adult striatum, Reelin's functional role in this brain region remains poorly characterized. Using a recently generated cellular atlas of the rat nucleus accumbens (NAc) following cocaine experience, we identified *Reln* mRNA as a marker of cocaine-responsive *Drd1*+ medium spiny neurons (MSNs). Here, we sought to define Reelin's role in striatal functions, and to determine its contribution to cocaine behavioral response. We designed a CRISPR sgRNA targeting the *Reln*promoter to enable repression of *Reln* transcription with CRISPR interference (CRISPRi). We demonstrate minimal off-target effects in primary striatal neurons and efficacious *Reln* knockdown in vivo. To assess if *Reln* serves as a passive marker of cocaine-sensitive cells or facilitates cocaine response, we performed conditioned place preference following targeted *Reln* knockdown in the NAc. Compared to non-targeting gRNA controls, *Reln* knockdown animals had no preference for the cocaine-paired chamber. To understand Reelin's role in regulating activity in the NAc, we used whole-cell patch clamp following *Reln* knockdown. While we saw no changes in passive membrane properties or action potential properties, cells lacking *Reln* exhibited decreased intrinsic excitability and an inability to maintain sustained firing. Single-nucleus RNA-sequencing of the NAc following *Reln* knockdown reveals changes in the expression of genes important for maintaining calcium homeostasis and provide direction for future pharmacological assays. Together, these results reveal a key role for Reelin in striatal function and cocaine reward. Ongoing studies are modifying this CRISPRi strategy to be compatible with an adeno-associated virus delivery system for application in animal models and human-derived neuronal cultures.

696. The Integration of High-Resolution, Genome-Scale Epigenetic Mapping with Epigenetic Editing Technology Enables Precise Epigenetic Engineering of Cells

Rigel Joseph Kishton, Sam Gross, Pedro Mendez, Nien-Chen Wang, Nicholas P. Restifo, Justin Valley, Arash Jamshidi, Alex Aravanis

Moonwalk Biosciences, South San Francisco, CA

DNA methylation is prominent in determining cell type and is implicated in many disease processes. However, methylation states for most of the CpG sites in the human genome (~30 million sites) have never been measured for most cell-types. This has limited the field's ability to fully understand epigenetic biology and explore treatment modalities based on epigenome engineering. Here we describe for

the first time an epigenome engineering platform to enable complete, high-resolution single-cell mapping of the methylome in conjunction with targeted methylation modifications, a set of complementary technologies named EpiRead™ and EpiWrite™ respectively. The adoption of DNA sequence-targeted nucleases such as CRISPR/Cas, TALENS and ZFNs has mediated a new era of precise genome engineering, with novel therapeutic strategies for a variety of human diseases and disorders entering the clinic. Additional genome engineering strategies designed to confer higher degrees of editing specificity and limit the enzymatic cutting of DNA such as base editing, PRIME editing and epigenetic editing each exhibit great potential as therapeutic modalities. Epigenetic editing is a novel strategy to alter the phenotypic properties of cells or tissues through directed modifications of non-genetically encoded regulators of gene expression, such as histone acetylation and methylation or DNA methylation. Unlike traditional gene editing approaches which require cutting of DNA, epigenetic editing can be utilized to increase or decrease the expression of target genes, allowing for increased control over cell properties. Despite the considerable promise of epigenetic editing, current approaches often rely on targeting known genes through manipulation of gene promoters. The ability to both identify disease-specific epigenetic cell states and utilize targeted epigenetic editing techniques to modify cell epigenomes would have several advantages, including increased precision and specificity of epigenetic editing, the expansion of the target space for epigenetic editing to encompass regulatory regions of the genome such as enhancers and non-coding RNAs, and improvements to understanding the safety and off-target profile of epigenetically editing interventions. Further, enhanced understanding of the epigenetic biology of disease states may allow for rationally designed multiplex editing strategies to further enhance therapeutic benefit. Here we describe our development of dual, complementary technologies to enable high resolution epigenetic mapping in conjunction with targeted epigenetic editing. Our epigenetic mapping technology, referred to as EpiRead™, encompasses high resolution whole genome analysis of DNA methylation states, with the utilization of machine learning/artificial intelligence (ML/AI) to precisely identify and categorize cellular epigenetic states at single cell resolution. In parallel, we have developed EpiWrite™, a modular epigenetic editing technology that allows for the precision targeting of epigenetic edits to desired regions of the genome. We demonstrate the utilization of EpiRead™ and EpiWrite™ in the context of epigenetically editing primary human T cells, with epigenetic mapping-informed usage of EpiWrite™ to silence T cell genes in multiplex. We further show the increased utility of whole-genome epigenetic mapping of DNA methylation relative to commonly performed microarray based methylation platforms in characterizing the impact of epigenetic editing. Finally, we describe our utilization of EpiRead™ to create epigenetic maps of human T cell subsets, including the surprising finding that the in vitro differentiation of both CD8+ and CD4+ T cells does not recapitulate the epigenetic state of corresponding *in vivo*-derived subsets.

697. Tuned Upregulation of Diverse Gene Targets Using Programmable Epigenomic Controllers

Jeremiah Farelli, Chevaun Morrison-Smith, Marcus Gibson, Mayur Gurnani, Prachi Dhanania, Stephen Siecinski, Yaoyu Wang, Justin Chen, Adam Katz, Houda Belaghzal, Giuliana Coatti, Charles O'Donnell, Thomas McCauley

Omega Therapeutics, Cambridge, MA

Epigenetic mechanisms mediate DNA sequence and homeostatic gene expression, with dysregulation at this pre-transcriptional interface found at the root of myriad diseases. Therapeutic modulation of the epigenome presents a unique opportunity to leverage endogenous mechanisms for pre-transcriptional control of gene expression, avoiding biological and developmental hurdles inherent with existing modalities. Omega Therapeutics, a clinical-stage biotechnology company, has developed a modular platform for design of programmable mRNA therapeutics enabling precision epigenomic control of expression of single or multi-gene targets. Leveraging 3D chromatin architecture, we model cell-type- and context-specific epigenetic regulatory sites within conserved chromatin loop structures termed Insulated Genomic Domains (IGDs) to rationally design epigenomic controllers (ECs). ECs are mRNA-encoded proteins that target the epigenome with exquisite specificity for tunable and durable effect persisting after degradation of the mRNA product - decoupling pharmacokinetics (PK) and pharmacodynamics (PD) for an improved safety profile relative to existing modalities. ECs consist of one or more protein domains (effectors) selected from a validated library and tethered to custom, site-specific DNA-binding-domains to induce a desired regulatory effect. This fusion is optimally encoded in an mRNA therapeutic producing one or several effector-DBD fusion proteins, delivered tissue-specifically using custom lipid nanoparticles (LNPs). Complementing Omega platform ECs that have demonstrated durable gene downregulation for multiple months, here we demonstrate platform capabilities to up-regulate gene expression across a diverse set of gene types, spanning a broad spectrum of conditions. This includes (1) genes that have been inactivated in a cell type of interest, (2) activated but lowly-expressed genes whose augmented up-regulation would lead to therapeutic benefit, and (3) genes whose activation or further up-regulation is gated by regulatory processes awaiting stimuli or their cis regulatory elements (cRE). For cases (1) and (2), we have designed a library of effectors, rationally targeted these effectors to impact regulatory DNA sites within the IGDs of multiple genes, demonstrating expected epigenetic state change and resulting tunable increases in both transcription (fold-change) and duration of effect long after EC degradation. This includes the application of a human gene-targeting EC shown to maintain ≥2-fold upregulation through 55 days in K562 cells, while culture conditions could be maintained. In the context of case (3) we demonstrate the EC-driven epigenetic activation of distinct targets through modulation of cis-regulatory elements and by modulation of chromatin looping factors and IGD state change. Finally, we demonstrate the capability of an Omega EC to durably downregulate a gene of interest and then, using a different EC, to reverse the effect in the same cells, restoring expression of the gene.

Development of such a generalizable approach to epigenomic tuning and upregulation of a target gene enables broad application across a range of therapeutic targets and disease conditions.

698. Design and Characterization of Compact and Precise Cas Enzymes for Treating Diseases in Patients

Melanie Silvis[1], Xiao Yang[1], Ryan Swan[1], Tabitha Tcheau[1], Gabriella Alvarez[1], Julia Bachteal[1], Chris Still[1], Zaki Jawaid[1], Vincent Cutillas[1], Tengyu Ko[1], Blair Gainous[1], Brandon Liauw[1], Aayushma Gautam[1], Stanley Qi[1,2,3,4], Tim Daley[1], Dan Hart[1], Yanxia Liu[1]

[1]EpiC-BIO, South San Francisco, CA,[2]Department of Bioengineering, Stanford University, Stanford, CA,[3]ChEM-H, Stanford University, Stanford, CA,[4]Chan Zuckerberg Biohub, San Francisco, CA

Genome and epigenome editing each hold immense potential for making targeted, precise corrections to either our genes' sequences or how they are expressed, clearing new and accelerated paths toward therapies for previously untreatable diseases. However, there remain significant barriers to realizing this potential including the development of genome and epigenome editing tools that are both highly precise and have broad targeting scopes. By combining the precision and broad targeting potential of CRISPR-Cas enzymes with novel regulators of gene expression, Epic Bio's epigenetic editing platform is at the forefront of gene therapy to mitigate and reverse diseases that are beyond the reach of traditional genome editing. Our Cas engineering platform has led to the discovery of highly compact nuclease-deficient dCas enzymes, thus enabling efficient delivery by AAV due to their small sizes (one-third the size of SpyCas9), and avoiding double-strand breaks or permanent edits. All clinical applications of Cas-based genome engineering are constrained by the requirements for protospacer-adjacent motifs (PAM) in the target DNA. Importantly, our novel dCas enzymes display flexible PAM requirements and are easily programmed to target therapeutic genes, underlining their broad utility. Here we present a novel cellular assay for PAM characterization that comprehensively and quantitatively captures the PAM requirements of dCas enzymes in human cells. This assay enables accurate detection of greatly expanded PAM profiles for our lead dCas enzymes (dCasONYX, dCasRUBY, dCasTOPAZ), enabling the efficient targeting of disease-causing genes. This assay enables ongoing engineering and deep characterization of our novel dCas in relevant genomic contexts to facilitate their translation to therapeutics. Altogether, we present our work to optimize compact and precise Cas molecules at the core of our epigenetic editing platform and demonstrate their broad utility, representing a major advancement toward treating intractable diseases in patients.

699. Revolutionizing Safe Gene and Cell Therapy by Precise Epigenome Editing

Lei Stanley Qi

Bioengineering, Stanford University, Stanford, CA

The precise control of the epigenome is key to advancing gene and cell therapies for diseases beyond the scope of gene editing. While we have witnessed a rapid revolution in biotechnological advances towards gene therapy via gene editing, major challenges remain due to limited scope of disease indications and potential permanent editing side-effects. To address these critical challenges, we focus on the discovery of novel technologies that allow precise engineering of human epigenetics beyond gene editing. This effort is motivated first by the desire to bring the best tools to bear on human diseases of most need, and by the desire to do so in the safest and most efficacious manner. To address challenges in the field, we have developed highly compact nuclease-dead dCas systems (dCasMINI, 1/3 size of Cas9), as well as highly compact and potent epigenetic modulators (less than 200 bp). We have demonstrated their utility for the versatile control of gene transcription and epigenetic modifications and precisely targeting greatly expanded genomic contexts in living cells and in vivo. This platform is inherently safe, as it is designed to accomplish changes in gene expression without damaging or mutating genomic DNA. Recent studies have shown Cas9 nucleases or base editors can induce chromosomal translocation and genotoxicity. In comparison to gene editing-based approaches, we demonstrate epigenetic editing is safe without permanent off-targets or toxicities to cells. Additionally, the dCasMINI molecules we have developed have extremely low immunogenicity profiles, superior to larger and more immunogenic Cas9 or Cas12 proteins of bacterial origin. Although epigenetic editing has immense potential, we have addressed two critical challenges of applying epigenetic editing towards clinical applications: firstly we have generated a compact CRISPR-based epigenetic platform (reducing payload size by more than 2/3) capable of efficient delivery via either a single adeno-associated virus (AAV) or lipid nanoparticle (LNP) in vivo; secondly we have built a comprehensive library of epigenetic modulators, via both machine learning algorithms and large-scale screening, which allow us to activate and suppress multiple gene expression in a programmable way. We have developed an integrated platform based on these two major advances, termed Gene Expression Modulation System (GEMS), which realizes the full potential of epigenetic editing to precisely regulate gene expression. We demonstrate the clinical applications of our GEMS' persistent epigenetic editing platform to treat genetic disorders, including Facioscapulohumeral muscular dystrophy (FSHD), which are beyond the reach of conventional gene editing. Our FSHD program is poised to enter the clinic this year, potentially a first for epigenetic editing technologies. In a further demonstration of its utility, we can apply epigenetic editors to human T cells to enhance T cell functionality for better elimination of tumor malignancies. Notably, we have identified epigenetic modulators capable of durable and persistent target gene activation, opening the possibilities for novel therapeutic applications to address various haploinsufficiency diseases. In summary, precise epigenetic editing technologies hold great promises for developing next-generation gene therapies.

700. EPI-321: A Novel Epigenetic Gene Therapy for FSHD Targeting D4Z4 Epigenome

Alexandra Collin de L'Hortet, Abhinav Adhikari, Siddaraju Boregowda, Hao Zheng, Vishi Agarwal, Andrew Norton, Nalinda Wasala

Epic-Bio, South San Francisco, CA

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common types of adult muscular dystrophies with an annual incidence rate of about 1 in 10,000, affecting approximately 1 million people globally. With no cure available, current therapeutic strategies

only involve managing symptoms to improve overall quality of life. Misexpression of disease-causing protein, DUX4, in muscle leads to slow and progressive muscle degeneration through activation of apoptotic and other downstream pathways. DUX4 gene is encoded at the distal region 4q35 chromosome from D4Z4 macrosatellite array. In FSHD patients, the D4Z4 marosatellite array is hypomethylated, leading to stochastic and transient DUX4 expression, which makes the development of cure challenging. At Epic-Bio, we leverage our proprietary Gene Expression Modulation System (GEMS) platform to develop a treatment for FSHD that targets the D4Z4 epigenome and permanently suppress DUX4 expression. Our product, EPI-321 is a single vector AAV serotype rh74 encoding an ultracompact, catalytically inactive Cas protein (effector) fused to gene-suppressing modulators, under the expression of the muscle specific promoter, CK8e, and a guide RNA targeting D4Z4 locus. Our preclinical studies showed that EPI-321 administration leads to robust and dose-dependent suppression of DUX4 and DUX4-downstream gene expression in ten different FSHD patient-derived immortalized and primary myoblasts in vitro, irrespective of the number of D4Z4 repeats, and showed antiapoptotic activity as measured by Caspase 3/7 staining. Mechanistically, EPI-321 showed re-methylation of the D4Z4 target locus leading to suppression of DUX4 expression. Further, in vivo evaluation of EPI-321 in humanized FSHD mouse model showed a dose-dependent suppression of DUX4-pathway at the mRNA and protein level, and antiapoptotic activity in muscle tissues. Additionally, 3D engineered human muscle tissue (3D EMT) using FSHD patient-derived immortalized myoblasts transduced by EPI-321 resulted in efficient suppression of DUX4 and DUX4-pathway genes up to 46 days and demonstrated significant dose dependent improvement in muscle contractility, shown by increased twitch and tetanic force post-treatment. Taken together, our findings provide robust evidence for EPI-321 as a potential single-administration gene therapy for treating FSHD by permanently suppressing the pathogenic DUX4 gene through epigenetic silencing. We intend to submit an Investigational New Drug (IND) application this year and are looking forward to commencing first-in-human trials in 2024.

701. ADAR Mediated RNA Editing for Treatment of Collagen VI Related Muscular Dystrophy

Russell J. Butterfield, Manshu Tang

Pediatric Neurology, Univeristy of Utah, Salt Lake City, UT

Introduction: The collagen VI related muscular dystrophies (COL6-RD) are inherited disorders of muscle characterized by progressive weakness and a combination of distal joint laxity and proximal joint contractures. There are no treatments beyond supportive care. Mutations in any of the three genes encoding collagen VI can result in COL6-RD. Missense mutations resulting in substitution of the glycine residue in the conserved Gly-x-y repeat of the triple helical (TH) domain allow incorporation of abnormal chains into secreted tetramers resulting in the dominant negative effect. Allele-specific gene silencing has been proposed as a therapeutic mechanism for COL6-RD and several different approaches using antisense oligonucleotide (ASO) or small interference RNA (siRNA) are in early stages of development. The goal for this project is to demonstrate feasibility of an alternative treatment strategy with an in-situ RNA-editing using adenosine deaminase acting on RNA (ADAR) to correct mutations in COL6-RD at the transcript level. ADAR is a site-specific RNA editor converting adenosine (A) to inosine (I) in RNA transcripts. Since 'I' in the mRNA sequence is read by the translation machinery as guanine (G), A-to-I edits of G-to-A transitions result in a correction of the mutation at the transcript level. In contrast to ASO and siRNA-based approaches to treatment of COL6-RD which are limited to specific targeted mutations, ADAR editing may allow targeting of multiple adjacent sites using the same guide-RNA. This is especially attractive for COL6-RD where dominant negative mutations are clustered in the N-terminal end of the triple helical domain and a single guide-RNA could be developed targeting multiple adjacent mutations. **Methods:** We used cultured fibroblasts from skin biopsy samples from patients with COL6-RD due to glycine missense mutations in the TH domain of the *COL6A1* gene. Since the c.850G>A (p.G284A) mutation in exon 9 is the most common cause of COL6-RD, our initial studies were performed on fibroblast samples with this mutation. Candidate guide-RNA sequences were cloned into plasmids and transfected into cultured using lipofectamine. ADAR editing was measured using RNA-seq to compare the read counts of wild type and mutant transcripts. In our first experiments, we first used linear synthetic RNA oligonucleotides or RNA transcribed from plasmid vectors specific to *COL6A1*, exon 9. Next, we designed two circularized guide-RNA's by flanking the sequences targeting either pre-mRNA or spliced mRNA, both of which centering exon9, with sequences from Twister ribozyme and stem-forming sequences from pre-tRNA. The Twister ribozyme sequences undergo rapid autocatalytic cleavage, leaving termini that are brought together by the stem-forming sequences and ligated by the ubiquitous endogenous RNA ligase RtcB resulting in a more stable guide-RNA with longer half-life. **Results:** Using linear synthetic RNA oligonucleotides or RNA transcribed from plasmid vectors specific to *COL6A1*, exon 9, we were unable to identify a significant amount of editing. Circular guide-RNAs showed improved stability and longer half-life than the linear counterparts with a clear rise in A-to-I editing. RNA-seq revealed that the editing efficiencies reached about 20% for both pre-mRNA and mRNA guide-RNA's without detectable bystander editing. Our best candidate guide-RNA to date shifted the A:G ratio from 0.88 in control samples to 0.56 in treated samples. **Conclusions:** We successfully demonstrated that the c.850G>A mutation in *COL6A1* can be edited *in vitro* in patient-derived fibroblasts with a circularized guide-RNA. These experiments support further development of this therapeutic candidate by optimization of the guide-RNA sequence and secondary structure to shift the ration of wild-type and mutant transcripts to a level that can reliably reverse the retention of collagen VI protein in the fibroblast cells and correct the defect in the matrix deposition.

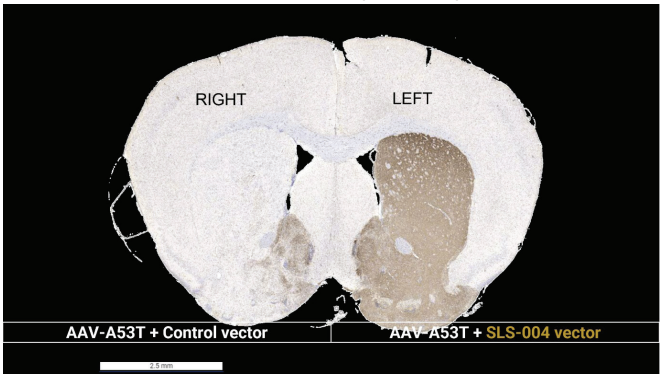
702. One Year *In Vivo* Safety After CRISPR Based Parkinson's Disease Treatment

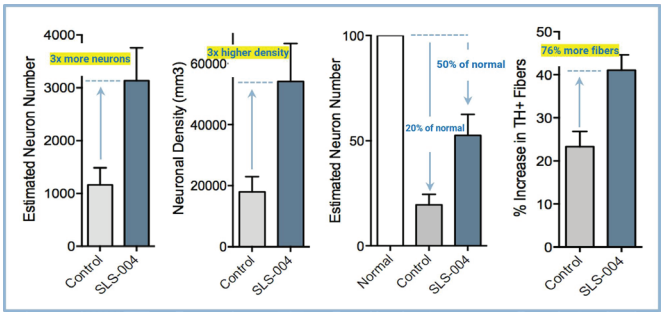
Krishna Subramanian[1], Raj Mehra[2]

[1]Seelos Therapeutics Inc., New York, VA,[2]Seelos Therapeutics Inc., New York, NY

Parkinson's disease (PD) is the second most common neurodegenerative disorder in the world after Alzheimer's disease. Neuronal α-synuclein disease defined by biology encompasses all α-synucleinopathies. These diseases include - incidental Lewy body disease, preclinical (without

clinical features), premotor, pure autonomic failure, idiopathic rapid eye movement-sleep behaviour disorder, prodromal (early signs or symptoms not yet fulfilling clinical diagnostic criteria), or as having a possible or probable clinical diagnosis of Parkinson's disease, Parkinson's disease dementia, or dementia with Lewy bodies. SLS-004 is a CRISPR based epigenetic modulator of α-synuclein gene expression. SLS-004 can be personalized individually for precision medicine application and can be tailored to be broadly used a therapeutic for downregulation of α-synuclein overexpression. SLS-004 utilizes a CRISPR based active epigenetic DNA methylation mechanism to restore homeostasis and reduce α-synuclein levels to normal physiological levels. SLS-004 can be applied across all neuronal α-synuclein diseases. In a humanized PD mouse model of A53T α-synuclein overexpression in vivo, SLS-004 was able to restore tyrosine hydroxylase (TH) levels in striatum in the treated hemisphere compared to control hemisphere. In the control hemisphere complete loss of TH-positive expression was observed, but in treated hemisphere TH-expression remained persistent, 76% more fibers. The stage of disease in control hemisphere is equivalent to the Stage 1A&1B of the newly coined neuronal α-synuclein disease integrated staging system (ND-ISS). TH-positive dopamine neurons in the substantia nigra pars compacta (SNpc) were recovered in the SLS-004 treated hemisphere compared to control hemisphere, 3X more dopamine neurons. Thus, dopaminergic dysfunction, but no evidence of clinical signs or symptoms (Stage 1b) was also recovered by treatment with SLS-004. SLS-004 was also administered directly into normal animals that were not induced with any targeted dopaminergic neuronal α-synuclein overexpression. In these animals the expression of TH levels in striatum in the treated hemisphere was retained. This was evident after one year of in vivo expression in mice. The animals were dosed with SLS-004 starting on Day 112 (Age) and were followed for one year up to at least 477 days. Thus, SLS-004 can be administered safely for a long duration of time in vivo using the novel CRISPR epigenetic modulation mechanism. The therapeutic lowering of α-synuclein with SLS-004 do not cause any adverse reactions in normal animals. This validates the broad application of SLS-004 across all neuronal α-synuclein diseases. No safety findings were observed both during in-life and at the extensive end-term necropsy in vivo. The safety and efficacy of SLS-004 are thus established in both normal and diseased animal models including in aged mice (>477 days). SLS-004 can be used to treat all neuronal α-synuclein diseases to restore the neurons back to normal physiological levels of α-synuclein through a safe and effective CRISPR based epigenetic regulation mechanism.





703. A Novel Rett Syndrome Patient-Derived Cerebral Organoid Model for Epigenetic Editing

Klaudia M. Braczyk[1,2], Julian Halmai[1,2], Casiana Gonzalez[1,2], Roy Ben-Shalom[2], Kyle D. Fink[1,2]

[1]Center for Interventional Genetics; Stem Cell Program; Gene Therapy Center; Institute for Regenerative Cures, Sacramento, CA,[2]MIND Institute; Department of Neurology, University of California, Davis, Health Systems, Sacramento, CA

Rett syndrome (RTT) is a rare neurodevelopmental disorder, primarily affecting girls, caused by loss-of-function mutations in *methyl CpG-binding protein 2* (*MECP2*). Mutations in *MECP2,* which is located on the X chromosome, lead to significant neurological deficits including speech and motor regression, cognitive impairment, and seizures. Currently, there is no cure for RTT and with only limited symptomatic treatments available, there's an urgent need for more therapeutic approaches targeting the underlying cause of this disease. Recent studies have shown that epigenetic reactivation of the endogenous, healthy copy of *MECP2* from the inactive X chromosome (Xi) can restore normal neurological functions in RTT-like neurons *in vitro* using two-dimensional (2D) ESC-derived neural cultures. However, the evaluation of this approach in a physiologically relevant brain microenvironment remains unexplored. Cerebral organoids formed from neuronal stem cells (NSC) derived from patient induced pluripotent stem cells (iPSC), grant close recapitulation of the human brain microenvironment due to cell three-dimensional organization, layer formation closely resembling brain architecture and increased neuronal maturation. Cerebral organoid model allows then for effective evaluation of epigenetic reactivation in a brain-like setting along with accurate assessment of viral-based delivery of therapeutics. **Methods**: RTT patient iPSCs were differentiated into NSCs and cerebral organoid cultures were established. Organoids were then investigated for RTT phenotype and neuronal maturation utilizing RT-qPCR and immunocytochemistry (ICC) to evaluate expression of neuronal molecular markers, and Microelectrode Arrays (MEA) for electrophysiological activity. Mature organoids were transduced with epigenetic editing tools to evaluate transduction efficiency and targeted reactivation. **Results:** RT-qPCR and ICC indicated that all established cerebral organoids indeed express various neuronal markers, and that RTT organoids successfully recapitulated RTT phenotype compared to healthy control organoids. MEA showed that all established organoids were capable of presenting electrophysiological activity with distinguishable firing pattern between RTT and control organoids. Viral transduction successfully delivered the CRISPR-dCas9 construct

to the mature, patient NSC-derived organoids with high efficiency. **Conclusions**: RTT patient iPSC NSC-derived cerebral organoids can serve as a brain-relevant model to investigate the epigenetic reactivation of the endogenous, healthy copy of *MECP2* gene. Future directions aim to rescue the RTT phenotype in RTT patient iPSC NSC-derived cerebral organoids, and to assess potential of the novel epigenetic editing therapy approach to treat Rett syndrome in patients.

704. Evaluation of CRISPR-CasRx Strategies for Targeted Gene Knockdown in Primary Hyperoxaluria Type 1: Unveiling Discrepancies between *In Vitro* & *In Vivo* Approaches

Laura Torella[1], Erwan Sallard[2], Jessica Del Campo Ayala[1], Mario Santafe-Méndez[1], Diego Herrero-Martínez[1], Guiomar Pérez-Otero[1], Cristina Gazquez[1], Cristina Olagüe[1], Africa Vales[1], Nerea Zabaleta[3], Gloria Gonzalez-Aseguinolaza[1]

[1]CIMA, University of Navarra, Pamplona, Spain,[2]University Witten/Herdecke, Witten-Annen, Germany,[3]Mass Eye and Ear/Harvard Medical School, Boston, MA

Primary hyperoxaluria type 1 (PH1) is a rare genetic disease characterized by oxalate overproduction in the liver and its toxic accumulation in the kidneys. CRISPR-Cas9-mediated *Hao1* gene disruption has been effective in reducing oxalate accumulation in a PH1 mouse model. However, permanent genome changes remain a safety concern. Here, we have investigated the use of CRISPR-RfxCasR13d (CasRx), a programmable RNA-guided, RNA-targeting technology that allows protein knockdown without modifying the DNA. First, we tested the efficacy of 11 guides that target different regions of the *Hao1* CDS and 3'UTR by analyzing the reduction in glycolate oxidase (GO) expression in transfected mammalian cells. GO expression was reduced to almost undetectable levels in all the conditions, interestingly, a reduction in expression of co-transfected reporter genes was also observed, reflecting the collateral cleavage of non-complementary bystander RNA. Despite a similar targeted knockdown efficiency, the gRNAs demonstrated different degrees of collateral activity and importantly, we did not detect evidence of this concerning activity in endogenous genes. Thus, we selected the most effective gRNAs with the lowest collateral cleavage effect to generate AAV8 vectors carrying single or multiple gRNAs in tandem to increase the knockdown efficiency and tested them *in vivo*. Mice were co-injected with an AAV expressing Luciferase to assess collateral activity *in vivo*. A reduction in Luciferase and CasRx expression confirmed collateral cleavage of exogenously expressed genes. While a reduction of *Hao1* mRNA levels was observed, it was insufficient to decrease GO protein expression. All-in-all, despite the promising *in vitro* results, we underscored the challenges and need for further optimization in applying CRISPR-CasRx for therapeutic purposes in the context of PH1.

705. Robust and Durable RNA Editing *In Vivo* with Axiomer™ Editing Oligonucleotides in Non-Human Primates

Nicole H. P. Cnubben, Katarzyna E. Kolodziej, Bruno Albuquerque, Saskia Crowe, Jim Swildens, Hans A. V. Kistemaker, Lenka van Sint Fiet, Peter de Visser, Sjef de Kimpe, Gerard Platenburg

ProQR Therapeutics B.V., Leiden, Netherlands

Axiomer, an RNA editing platform, uses chemically modified and complementary editing oligonucleotides (EONs) to recruit and guide endogenous Adenosine Deaminase Acting on RNA (ADAR) to perform A-to-I editing on double stranded RNA. To date, EONs have shown robust editing *in vitro* and *in vivo* (rodents), and we report here editing efficiency and tolerability of an EON targeting *ACTB* RNA in non-human primates (NHPs). In the primary experiment, cynomolgus monkeys were dosed with three intravenous infusions each at D1, D8, D15 with *ACTB* EON at a dose of 2mg/kg (N = 4) or control EON (N = 4). During this study animal welfare was closely monitored. Tolerability, including safety parameters, and *ACTB* editing efficiency in liver biopsies were assessed on D4, D18, D25, and D39. Editing efficiency of *ACTB* RNA, confirmed through RNAseq and dPCR, reached up to 70% in the *ACTB* EON group in comparison to 0.1% in the control group at D4 (1 dose) and showed an average of 61% and 62% editing efficiency observed at D4 and D18 (3 doses), respectively. Durable high double-digit editing was maintained within the *ACTB* EON group after the last dose. No significant off-target editing was observed in NHP liver upon treatment with EON targeting *ACTB* RNA. No abnormalities in animal welfare or signs of discomfort were noticed during treatment. Safety parameters did not show significant differences in the *ACTB* EON vs. EON control group, although some transient formulation-related changes compared to baseline were noted in circulating hepatic enzymes. Axiomer *ACTB* EON repeat dosing showed robust and durable editing efficiency at the RNA level in NHP *in vivo* with a similar safety profile known from other single-stranded oligonucleotides. These preliminary data confirm the potential of Axiomer EONs in preparation for clinical development and support discovery of new therapeutic applications.

706. Exon Editing of ABCA4 rNA in Human Retinal Explants and Non-Human Primate Retina for the Treatment of Stargardt Disease

Ting-Wen Cheng, Akiko Noma, John Bachmann, Tayla Cormier, Sangeetha Manokaran, Delaney Conor, Jim Faulkner, Justin Nelson, Brian Olshaw, Jason Thresher, Dennis Wu, Akiko Doi, Carrie Wager, Michael Ehlers, Robert Bell

Ascidian Therapeutics, Boston, MA

Exon editing is a promising strategy that utilizes RNA trans-splicing activity of endogenous spliceosomes to rewrite mutated RNA and thereby correct the underlying genetic causes of disease. Exon Editor RNA molecules can be designed to carry correct coding sequences of multiple exons, target a specific region of mutated pre-mRNA, and replace pathogenic mutations with the corrected sequence resulting in full-length, repaired mRNA molecules and wild-type proteins. Here we developed ACDN-01, an AAV-encoded RNA Exon Editor targeting

ABCA4, for the treatment of ABCA4-related retinopathies, including Stargardt disease. First, to model the Exon Editor expression and RNA replacement activity in the human retina, human retinal explants from 8 donors with no known ocular disease were treated with ACDN-01 *ex vivo,* to mimic bleb formation analogous to that seen following *in vivo* subretinal injection. The results demonstrated that ACDN-01 effectively transduced human retinal cells and efficiently replaced 22 exons in the 5' half of the 7 kb *ABCA4* coding sequence. Full-length ABCA4 protein expression levels in ACDN-01 treated human retinal explants were characterized by capillary electrophoresis. In addition, the full-length protein translated from ACDN-01 edited *ABCA4* mRNA was confirmed to retain functional ATPase activity. Next, durable and well-tolerated exon editing in non-human primate (NHP) retina were demonstrated following a single subretinal injection of ACDN-01 at therapeutically relevant doses. In an IND-enabling GLP toxicology study, ACDN-01 achieved stable vector transduction and Exon Editor RNA expression in NHP retina, mainly in the photoreceptor cells, at 3-months post-dosing and persisted up to 6-months post-dosing. Trans-splicing activity increased dose-dependently and effectively produced chimeric human/NHP ABCA4 mRNA and protein. ACDN-01 was well tolerated in the mid-dose group with minimal microscopic findings that did not affect eye function and therapeutically relevant levels of chimeric ABCA4 mRNA and protein were demonstrated. Retinal findings associated with the highest dose of ACDN-01 tested aligned with other previous reports that evaluated high doses of AAV in the eye. In summary, ACDN-01 can provide robust and durable *ABCA4* exon editing in *ex vivo* human retinal explants and NHP retina at well tolerated doses. These results support going forward with additional studies to fully understand the potential for ACDN-01 in the treatment of ABCA4-related retinopathies.

Wednesday Posters: Gene Targeting and Gene Correction New Technologies

707. A Novel Mutation-Agnostic Gene Therapy Using AAV-Delivered Heterologous Expression of Engineered Microbial Mechanosensitive Channel in Animal Model of Ocular Hypertension

Adnan Dibas, Lucero Garcia, Megan Aldape, Sanghoon Kim, Subrata Batabyal, Mohanty Samarendra
Nanoscope Technologies, Bedford, TX

Purpose Patient adherence to daily eye drop medications for control of ocular hypertension is a major challenge. The engineered mechanosensitive channels (EMC) derived from microbial systems can detect elevated pressure levels based on membrane tension. Introducing an AAV-carried EMC in the trabecular meshwork (TM) via intracameral injection offers an innovative approach for sustained reduction of intraocular pressure (IOP). In this study, we measure the aqueous humor (AH) outflow facility in an IOP-elevated TGFβ2 mouse model of glaucoma and assess the dose-dependent impact of EMC gene therapy in enhancing the outflow facility to lower IOP. **Methods** EMC was packaged in self-complementary (sc) AAV2 and produced using the triple transfection method. EMC expression in TM was driven by the MGP promoter upstream, while mCherry downstream served as a biomarker. Immortalized human TM cells were used for evaluating transduction by scAAV2-EMC. Since TGFβ (transforming growth factor beta) is known to increase intraocular pressure (IOP) by inducing changes in the TM, including extracellular matrix deposition and increased outflow resistance, intravitreal injection of Ad5-TGFβ-2 was carried out in adult C57BL/6J mice for IOP elevation. IOP was measured by TonoLab. Intracameral injection of scAAV2 was carried out to determine changes in the aqueous outflow (AH) facility and IOP due to EMC transduction of TM. The AH outflow measurement was conducted using manometry in EMC-treated and control groups. Visually evoked potential (VEP) measurement was conducted to evaluate the effect of EMC treatment on the health of retinal neurons. **Results** Our results show successful transduction of human TM cells by scAAV2-EMC, visualized by fluorescence microscopic imaging of mCherry reporter, and western blot. Intracameral delivery of scAAV2-EMC led to EMC expression in TM. Reduction of AH outflow through the conventional outflow pathway was observed in the IOP elevated TGFβ2 model. Intracameral scAAV2-EMC treatment led to a statistically significant increase in aqueous humor drainage in the TGFβ2 model along with a reduction in IOP. VEP measurement showed a 50% higher amplitude in the scAAV2-EMC treated group as compared to the control, which suggests protection of retinal neurons owing to the reduction of ocular hypertension. **Conclusions** Heterologous expression of EMC enables the reconstitution of innate pressure sensory and response capacity within tissues regulating pressure. When expressed in cells of the conventional drainage system of the eye, EMC gets activated by increased ocular pressure and modulates cellular changes that enhance the outflow facility and facilitate pressure relief through the conventional TM pathway. Ectopic expression of EMC represents a new therapeutic approach for the treatment of edema and hypertension in different organs.

708. B Cell Targeting by All-in-One Vectors for Cell Activation and Genome Engineering

Hsu-Yu Chen, Chun Huang, Atishay Mathur, Chan-Hua Chang, BingFei Yu, Paula M. Cannon
Molecular Microbiology and Immunology, University of Southern California, Los Angeles, CA

We have previously shown that genome editing at the Immunoglobulin Heavy chain locus can be used to reprogram human B cells to express custom antibodies, including broadly neutralizing antibodies that could provide an anti-HIV therapy. We typically edit B cells using the combination of CRISPR/Cas9 RNPs and AAV6-based homology donors, but we have now developed alternative 'all-in-one' integration-defective lentiviral vectors (All-IDLVs) that can also engineer IgH. These vectors package RNPs through an aptamer/ABP interaction, while the homology donor antibody cassette is encoded in the vector genome. Further adapting All-IDLVs for *in vivo* gene editing would provide significant advantages, especially as edited B cells can be amplified by immunization, suggesting that only a small number of edited cells may be necessary. However, this is made challenging by the lack of robust cell-type specific delivery systems, and the requirement for the cells to be cycling to support homology-directed genome editing. For example, VSV-G pseudotyped lentiviral vectors can only

transduce B cells *ex vivo* following incubation in activation media, which both upregulates the LDL receptor needed for vector entry, as well as driving the cell cycle changes that are necessary for homology-directed editing.To confer B cell targeting to All-IDLVs, we evaluated ligands directed against a series of B cell surface molecules. The ligands were displayed using either an engineered paramyxovirus system or in combination with a mutant VSV-G. As proof of principle, we inserted the broadly neutralizing nanobody, J3, into the IgG1 locus, where correct gene editing results in both secreted and membrane-anchored forms of a J3-Fc fusion antibody. The most effective combination was achieved by targeting a co-stimulatory molecule in the mutant VSV-G platform, which improved transduction of resting human B cells by more than 5-fold compared to wild-type VSV-G. Moreover, the vectors significantly activated the B cells, increasing expression of CD80 and CD86. As a result, All-IDLVs were able to engineer human tonsil B cells without pre-activation, detected by the surface expression of J3, and confirmed by specific PCR. Finally, these B cell targeted vectors were also able to transduce primary B cells from Rhesus macaques, supporting future investigations *in vivo* using this model. Taken together, we have developed a B cell targeted vector that can engineer B cells without requiring pre-activation, and which could therefore be compatible with *in vivo* engineering of B cells.

709. Expansion of *In Vivo* HDAd-Transduced HSCs by Constitutive Expression of tHMGA2 after Intravenous Vector Injection into Mobilized Mice without Additional Drug Selection

Hongjie Wang, Aphrodite Georgakopoulou, Sucheol Gil, Andre Lieber

University of Washington, Seattle, WA

We developed an *in vivo* hematopoietic stem cell (HSC) gene therapy approach that consists of HSC mobilization and intravenous injection of HSC-tropic HDAd vectors. To achieve therapeutically relevant numbers of corrected cells, the approach requires *in vivo* expansion of transduced HSCs. For *in vivo* HSC selection we are currently using a technique that involves an $mgmt^{P140K}$ mutant and treatment with low-dose chemotherapy drugs (O^6BG/BCNU). Clearly, a chemotherapy drug-free method for *in vivo* expansion would be desirable. We therefore generated a HDAd5/35++ vector system for stable integration of a transgene cassette consisting of the ubiquitously active EF1α promoter driving a 3′UTR truncated HMGA2 gene linked to GFP via a self-cleaving 2A peptide (HDAd-tHMGA2/GFP). In mice, HSCs were mobilized with G-CSF/AMD3100 and, at the peak of mobilization, intravenously injected with the vector (N=10). Twenty weeks after HDAd injection, GFP-positive PBMCs started increasing above the background of 1% in all the mice reaching an average of 50% (range 10-70%) by week 44. In mice that received a control HDAd-GFP vector, GFP marking in PBMCs was steady at <1%. During this time, in HDAd-tHMGA/GFP transduced mice, the number of white blood cells increased, mostly due to higher percentage of $GFP^+/CD19^+$ (B-lymphocytes) and $GFP^+/Gr1^+$ (granulocytes) cell fractions within PBMCs. The number of erythroid cells and erythroid parameters did not change. At week 44, the average GFP marking in bone marrow mononuclear cells (MNCs) was 4-fold lower than in PBMCs indicating that most of the tHMGA2-mediated expansion occurred after the cells exited the bone marrow. Within bone marrow cells, about 10% of LSK cells, a cell fraction that is enriched for HSCs, were GFP-positive. This is 10-fold higher than in mice that received the HDAd-GFP control vector, suggesting an expansion at the level of HSCs as well. In the spleen, on average 35% of LSK cells were GFP-positive in HDAd-tHMGA2/GFP mice. The vector copy number per cell was comparable in PBMCs, bone marrow, and spleen, implying that tHMGA2 did not increase vector integration, but only the number of transduced cells. Genomic DNA from bone marrow MNCs were subjected to genome-wide transgene integration site analysis. These studies showed random vector integration without signs of clonal dominance. Lineage-negative bone marrow cells from wk 44 HDAd-tHMGA2/GFP transduced mice were transplanted into lethally irradiated secondary recipients. Engraftments rates were nearly 100% and GFP marking in PBMCs was stable over 12 weeks at 40% on average. No hematological abnormalities were observed. Another *in vivo* HSC transduction study was performed in humanized mice, i.e. in mice that were transplanted with human HSCs and developed hematopoietic chimerism. These mice were mobilized and injected intravenously with HDAd-tHMGA2/GFP. GFP marking in peripheral human ($CD45^+$) leukocytes increased, starting at week 20, and reached on average 30% by week 24. Similar marking rates were found in the bone marrow in $CD34^+$ cells (HSCs), $CD33^+$ cells (myeloid), $CD19^+$ cells (B-lymphocytes) and $CD3^+$ cells (T-lymphocytes). The percentage of GFP^+ cells in these subfraction was 4-5 higher compared to mice that were *in vivo* transduced with the HDAd-GFP vector that did not express the tHMGA2 gene. In summary, we present an approach that mediated the autocatalytic, self-limiting expansion of *in vivo* transduced HSCs and myeloid/lymphoid progenitors without disturbance of hematopoiesis or neoplastic transformation. Considering concerns that increased HMGA2 expression is associated with certain cancers, safety and efficacy studies in non-human primate models are warranted.

710. Efficient and Precise Gene Editing by STAR (Seek-Tag-Amend-Release) Editing Enabled by Synergistic Actions of a Cas9 Nickase, a segNA (STAR Editing Guide Nucleic Acids) and a DNA Dependent Polymerase

Minghong Zhong[1], Qiong Zhao[2], Xiaoyun Lin[1], Ju-tao Guo[2]

[1]GeneLancet Biosciences, Inc., Doylestown, PA,[2]Baruch S. Blumberg Institute, Doylestown, PA

Clustered regularly interspaced short palindromic repeats (CRISPR)-associated protein (Cas) is a gene editing system directed by variants of small noncoding RNAs, each with a spacer sequence of approximately 20 nt long. CRISPR-Cas comprises one or two nucleic acid endonucleases, which cut one or both nucleic acid strands targeted by the spacer sequence. For precise gene editing, either a nucleic acid-templated editing synthesis, integration of exogenous DNA plasmids or viral vectors, or modifications of a nucleotide base by one or more protein domains are usually incorporated into actions of a naturally occurring system or a modified system with a chimeric guide RNA. Base-modifying protein effectors (base editors, BE) have bystander off-targets, which are guide-independent and rarely have

single-base resolutions, while templated gene editing (prime editing, PE) usually has decreased efficiency compared to wild-type enzymes. In particular, the guide RNA construct of a prime editor (pegRNA or epegRNA) is self-inhibitory because of its inner complementarity at the spacer region. In addition, plasmids and viral vectors are well known for integrating into the host genome. CRISPR editors utilizing base modifying protein effectors have a limited editing capacity, while gene editors based on a nucleic acid templated editing synthesis are greatly more versatile and capable of full-spectrum gene editing, including any single base change, short or long deletions, and insertions. Examples of such editors include prime editing, PASTE, and STAR editing. In contrast to a prime editor and PASTE, which use a reverse transcriptase that is error-prone and has low fidelity and low processivity, STAR editing (Fig 1) uses a single-strand DNA (ssDNA) template tethered to a guide RNA and either a cellular DNA-dependent DNA polymerase or a fusion protein comprising a Cas protein and a DNA-dependent DNA polymerase domain. DNA-dependent DNA polymerase is known to be precise and has high fidelity and processivity. STAR editing uses the nicked non-targeted DNA strand after single-strand nicking by a Cas protein as a DNA synthesis primer and an ssDNA template tethered to guide RNA, coined as a STAR editing guide nucleic acids (segNA), which is uniquely accessible by the chemically ligated guide RNA (LgRNA) technology. There is a potential self-inhibition in a segNA similar to a pegRNA or an epegRNA in prime editing; however, a double-stranded DNA/RNA is less stable than a double-stranded RNA/RNA, and thus the self-inhibition is decreased in comparison to a prime editor, and can be further reduced by structure optimization. To improve the editing efficiency and specificity of STAR editing, we have developed a screening platform for the optimization of segNAs (Fig. 2), consisting of a cleavage assay (by CRISPR-Cas9), an extension assay (by CRISPR-nCas9 and a DNA polymerase) and a confirmative cell assay. Various segNAs containing a committed stop codon were synthesized, and segNAs capable of efficient, precise anti-HBV gene inactivation have been identified. In addition, we observed cellular DNA polymerases are sufficient for DNA-templated precise gene editing. The screening platform is also useful for optimizing a chemically ligated epegRNA to improve prime editing.

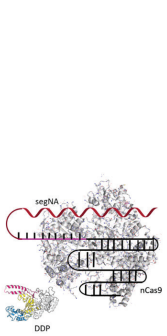


Figure 1. STAR editor

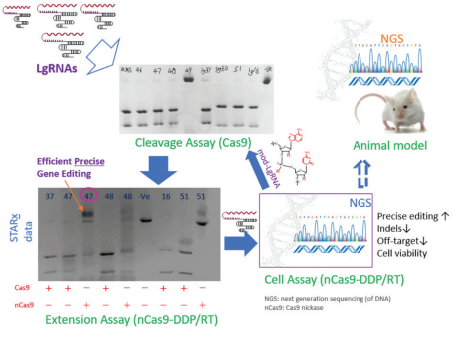


Figure 2. STARx/PEx screening platform for Efficient Precise Editing

Zhong, M. et al. Efficient and precise gene editing by STAR (Seek-Tag-Amend-Release) editing enabled by synergistic actions of a Cas9 nickase, a segNA (STAR editing guide nucleic acids) and a DNA dependent polymerase

711. Genome Editing Strategies for *In Vivo* Generation of Truncated EPO Receptors in HSCs to Expand Erythroid Progenitors Carrying Therapeutic Genetic Changes

Karthik V. Karuppusamy[1], Akshara S. Velmurugan[1], Chang Li[1], Hongjie Wang[1], Jack A. Queenan[2], Nouraiz Ahmed[3], David R. Liu[3], Robert Peters[4], Andre Lieber[1]

[1]University of Washington, Seattle, WA,[2]Harvard, Boston, MA,[3]Broad Institute, Boston, MA,[4]Ensoma, Boston, MA

Genome editing in hematopoietic stem cells (HSCs) has the potential to treat patients with β-thalassemia and sickle cell disease. We developed an *in vivo* HSC gene therapy approach that consists of HSC mobilization and intravenous injection of HSC-tropic HDAd vectors. To achieve therapeutically relevant gene correction levels, we are currently using an *in vivo* HSC expansion approach that is based on a mutant O^6-methylguanine-DNA methyltransferase ($mgmt^{P140K}$) gene that confers resistance to the low dose chemotherapy drugs O^6BG/BCNU. However, for non-oncological applications, especially for hemoglobinopathies, an *in vivo* selection approach that avoids chemotherapy or genotoxic agents would be preferrable. We have therefore been exploring a series of *in vivo* HSC selection/expansion methods that do not require chemotherapy drugs. One such approach is based on a truncation in the erythropoietin receptor (tEPOR), which causes benign congenital erythrocytosis—a condition marked by a non-pathogenic hyper-production of red blood cells. Others have shown that *ex vivo* tEPOR gene addition in HSCs or *ex vivo* editing to create a tEPOR can confer a selective advantage to the derived red blood cells, most likely due to mediating hypersensitivity to erythropoietin. Our goal is to create tEPORs in HSCs *in vivo* by base or prime editing together with the introduction of genetic changes that would cure hemoglobinopathies such as *in vivo* γ-globin gene addition, *in vivo* base editing to reactivate γ-globin, or *in vivo* prime editing to correct the sickle cell disease mutation. The approach would involve intravenous injections of mobilization agents and a therapeutic HDAd vector resulting in *in vivo* edited HSCs localized in the bone marrow. Upon differentiation into tEPOR-edited erythroid progenitors, an autocatalytic, (endogenous) EPO-mediated expansion would occur, resulting in corrected erythrocytes at levels that will be curative. For EPOR editing we used cytosine base editors that would introduce premature stop codons resulting in different C-terminal truncated EPORs. We have screened several single guide RNAs and found sgRNAs that result in the generation of a naturally occurring tEPOR variant, i.e. a stop codon at amino acid position 71 through converting TGG to TAA in HEK293T cells and found efficient editing at rates of ~30%. Next, we edited HUDEP-2 cells, an erythroid test cell line. and observed increased proliferation of cells with the desired modification up to 2.5-fold (within 5 days) with physiological concentration of erythropoietin. The second strategy involves prime editors that would create a tEPOR variant *in vivo* that was first reported by Harvey Lodish in 1990. Prime editing will replace the nucleotides for amino acids 467-Asp-Tyr-Ser-469 with nucleotides encoding Ala and Leu and creating a STOP codon, thereby removing the normal C-terminal 42 amino acids and adding alanine and leucine (for receptor stabilization). We used the latest prime editor enzyme PE6d and screened two nick-RNAs and eight pegRNAs. Preliminary screening in HEK293T cells showed desired prime editing with two of the pegRNA/nick-RNA

combinations. The generation of corresponding HDAd vectors is ongoing and results for *in vitro* and *in vivo* multiplex editing will be reported.

712. Highly Efficient Gene-Editing of *S. aureus* Cas9 in Patient Hematopoietic Stem and Progenitor Cells for Treating Sickle Cell Disease

ByoungYong Yoo[1], So Hyun Park[1], Vivien Sheehan[2], Gang Bao[1]

[1]Rice University, Houston, TX,[2]Emory University School of Medicine, Atlanta, GA

Introduction: Sickle cell disease (SCD) is a hemoglobinopathy caused by a single nucleotide mutation (A>T) in the sixth codon of the β-globin gene (*HBB*). Technologies based on CRISPR/Cas9 mediated gene-editing have been developed to provide a permanent cure for SCD by editing the *HBB* gene in hematopoietic stem and progenitor cells (HSPCs). Recently, Casgevy and Lyfgenia, *ex vivo* CRISPR/Cas9 gene-editing based therapies for SCD, have received FDA approval. However, the high cost of ~$2-3 million per treatment and the high-quality infrastructure needed for *ex vivo* editing limit access to these therapies, especially for patients in low-resource countries, such as Africa. Therefore, there is an unmet need for *in vivo* editing-based therapy that can significantly reduce the cost and eliminate the requirement of highly sophisticated infrastructure. One potential approach for *in vivo* gene-editing based therapy is to use adeno-associated virus (AAV) vector for delivery of the gene editing machinery to HSPCs in patients. However, the most widely used *S. pyogenes* Cas9 (SpCas9) cannot be packaged into a single AAV. Therefore in this study we systematically quantified the gene-editing outcomes of *S. aureus* Cas9 (SaCas9) which can be packaged into AAV to treat SCD, and compared the results with that of SpCas9. **Methods:** We edited two genes, *HBB* to correct the sickle mutation and *BCL11A* enhancer to induce fetal hemoglobin (HbF) with biological replicates in SCD HSPCs from different 2 donors. We delivered sgRNA with SpCas9 or sgRNA with SaCas9 as RNP, along with a corrective ssODN donor template to correct the sickle mutation in *HBB* and RNP of SpCas9 or SaCas9 to disrupt *BCL11A* enhancer by electroporation. *HBB* has a mutually permissive PAM sequence for both Cas9, and we screened 6 targetable sites for SaCas9 around the target site of SpCas9 and chose one with the highest editing efficiency. We analyzed the editing rate and small insertions and deletions (INDELs) profile by Next Generation Sequencing (NGS) and measured large deletions (> 61 bp) by ddPCR. We erythroid differentiated the edited SCD HSPCs for 14 days and measured % HbF by intracellular staining followed by flow cytometry. To evaluate the transduction efficiency of AAV in SCD HSPCs, we generated AAV6-GFP and transduced the SCD HSPCs, and measured % GFP^+ cells. **Results:** We found that SaCas9 is as highly efficient as SpCas9 in the sickle mutation correction (HDR rate by SpCas9: 42%, by SaCas9: 43%). With RNP only, the cutting efficiency of SaCas9 at *HBB* was comparable to that of SpCas9 (INDEL rate by SpCas9: 78%, by SaCas9: 80%). Large deletions, one of the unintended gene editing outcomes, were not significantly different (33% by SpCas9 RNP, 19% by SpCas9 RNP with the ssODN, 38% by SaCas9 RNP, and 18% by SaCas9 with the ssODN). SaCas9 was also as highly efficient as SpCas9 in *BCL11A* enhancer disruption (INDEL rate by SpCas9: 86%, by SaCas9: 88%). After erythroid differentiation, cells with *BCL11A* enhancer disruption by SaCas9 showed comparable HbF induction to SpCas9 (% HbF^+: 54% by SpCas9, 50% by SaCas9). The *in vitro* transduction efficiency of AAV6-GFP was 49% in SCD HSPCs. **Discussions:** In this work, we showed highly efficient gene-editing by SaCas9 and compared it with the highly optimized and widely used SpCas9 in sickle mutation correction and *BCL11A* enhancer disruption in SCD HSPCs. The results demonstrated the therapeutic potential of SaCas9 for treating SCD. We confirmed the high transduction efficiency of AAV6 in SCD HSPCs. Based on these results, we will generate AAV6-CRISPR with SaCas9 targeting *BCL11A* enhancer to investigate its potential for treating SCD by *in vivo* gene-editing.

713. Improving Therapeutic Homology Directed Repair Genome Editing Efficiency in Human Primary Cells through Optimal Guide RNA Selection

Sridhar Selvaraj, Nicole Marie Johnston, Ludwig Schmiderer, Nelson A. Amorin, Matthew H. Porteus

Pediatrics, Stanford University School of Medicine, Stanford, CA

Nuclease-based genome editing utilizing homology directed repair (HDR) pathway allows for precise integration of transgene sequences ranging from single nucleotide to multi kilobases in length. Genome editing platform employing Cas9/guide RNA (gRNA) ribonucleoprotein (RNP) and adeno-associated virus serotype 6 (AAV6) based HDR donor template delivery enables allelic HDR efficiency of 20-60% across different therapeutically relevant genomic loci in various human primary cell types, ex vivo. Further enhancement of HDR efficiency would greatly benefit both therapeutic and research applications of this technology. We have recently reported that transient treatment with a small molecule inhibitor of DNA-dependent protein kinase catalytic subunit (DNA-PKcs), AZD7648 during genome editing further improves the allelic HDR efficiency to about 50-90% through the inhibition of non-homologous end joining (NHEJ) DNA repair pathway. Since, NHEJ pathway plays a critical role in the maintenance of genomic integrity, there could be potential safety risks associated with this approach. Thus, it is essential to develop methods to enhance the HDR efficiency without modulating the endogenous DNA repair pathways. Recent studies have reported that gRNA selection could affect HDR efficiency due to the variability in DNA repair pathway choice between NHEJ, HDR and microhomology-mediated end joining (MMEJ). Here, we assessed whether alternate gRNA selection can affect HDR efficiency at previously characterized therapeutically relevant genomic loci in human primary cells. For this, we identified alternate gRNAs with PAM sites offset by 1 to 13 bases relative to the previously characterized gRNAs in the sense and antisense strands at *HBB, CCR5 and STING1* gene loci to allow for use of the same original HDR donor template. Following gene editing with RNPs of original/alternate gRNAs and AAV6 HDR donor in hematopoietic stem and progenitor cells (HSPC) and human pluripotent stem cells (PSC), we found the HDR efficiency to be variable between 10-90% across different gRNAs. The gRNAs with highest frequency of +1 INDELs (+1 gRNAs) post editing with RNP alone showed consistently lower HDR frequency of about 10-47% at all three loci upon editing with AAV6 template. The other gRNAs (var gRNAs) with variable INDEL patterns and lower +1 INDEL frequency showed higher HDR frequency of about 50-90%. Transient treatment with AZD7648 resulted in higher HDR

frequency across all gRNAs but the fold improvement was higher while using the +1 gRNAs indicating the preference for the NHEJ pathway (Fig. 1). Next, we assessed whether the kinetics of HDR and INDELs is affected by editing with +1 vs var gRNAs. In the samples edited with RNP only, we found that INDELs occur faster with the +1 gRNAs than the var gRNAs at all three loci starting at 4-8h. Upon editing with RNP and AAV6, we found that the HDR kinetics is faster with the var gRNAs than the +1 gRNAs from 4-8h which inversely correlated with the INDEL kinetics in the RNP only samples. Correspondingly, AZD7648 treatment normalized this difference in HDR kinetics. Thus, choosing gRNAs with lower +1 INDEL frequency could be ideal for achieving high HDR efficiencies by leveraging the alteration in choice of endogenous DNA repair pathways. In conclusion, optimal gRNA selection can aid in designing HDR-based high efficiency transgene integration strategies in human primary cells for therapeutic and research applications.

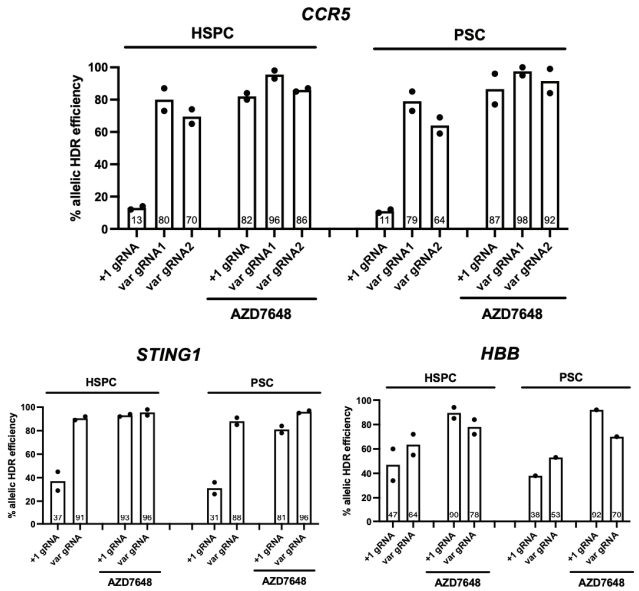


Fig 1: Improved HDR efficiency in HSPC and PSC with optimal gRNA selection. Allelic HDR efficiency in HSPC and PSC edited at the *CCR5, STING1 and HBB* loci with RNP/AAV6 gene editing using +1 or variable (var) INDEL gRNAs with and without AZD7648.

714. Making Change Possible: VLP-Mediated Editing in Primary Human Bronchial Epithelial Cells

Ariel Aspiras[1], Alisa Bell[2], Martin Mense[3], Calvin Cotton[1], Hillary Valley[1]

[1]Gene Editing, Cystic Fibrosis Foundation, Lexington, MA,[2]Cystic Fibrosis Foundation, Lexington, MA,[3]Cystic Fibrosis Foundation Therapeutics, Lexington, MA

Cystic fibrosis (CF) is a rare, life-shortening genetic disease caused by mutations in the *CFTR* gene. Gene editing strategies that can correct multiple CF-causing mutations are promising, as more than 2,000 genetic variants have been suggested to cause CF. Insertion of a partial cDNA (also known as a super exon (SE)) into the native *CFTR* genomic locus can restore the *CFTR* gene to a WT coding sequence for all mutations downstream of the insertion site. Targeted insertion of a super exon coding for exons (E) 2-27 into *CFTR* intron 1, could in theory benefit >99% of people with cystic fibrosis. Primary human bronchial epithelial cells (hBEs) are a frequently used model system for cystic fibrosis research, as they are derived from explanted lung tissue and differentiate into a pseudostratified epithelium over several weeks at air-liquid-interface (ALI) culture. However, delivery of gene editing reagents to well-differentiated hBE ALI cultures is difficult and hampers development of promising genetic correction strategies, such as the super exon. Virus like particles (VLP)s are composed of viral proteins to facilitate transduction but lack viral genomic material so are replication and integration incompetent. Here we show that in undifferentiated hBE cells, adenovirus with super exon donor DNA can be co-transduced with VLPs delivering complexed cas9 ribonucleoproteins (RNPs) without significant cytotoxicity and with 53% total editing efficiency. Additionally, we have demonstrated 17% editing of fully differentiated hBE cells in ALI cultures when RNPs were delivered via VLPs. These results suggest a way forward to genetically engineer primary hBE cells and to develop a durable cure for cystic fibrosis.

715. REMEDY Corrects Heterozygous Mutations Independent of Exogenous Donor Template with High Efficiency

Yasemin Sezgin[1], Noushin Saljoughian[1], Genesis Snyder[1], Colin Maguire[1], Ebru Erzurumluoglu Gokalp[2], Eleonora D'Ambrosio[1], Devi Jaganathan[1], Burcak Ozes[1], Zarife Sahenk[1], Sriram Vaidyanathan[1], Jerry R. Mendell[1], Nizar Y. Saad[3], Roshini S. Abraham[1], Juhi Bagaitkar[1], Susan D. Reynolds[1], Allison M. Bradbury[1], Meisam Naeimi Kararoudi[3]

[1]Nationwide Children's Hospital, Columbus, OH,[2]Eskisehir Osmangazi University, Eskisehir, Turkey,[3]Nationwide Children's Hospital and Ohio State University, Columbus, OH

One of the major limitations of the current gene correction approaches is the need of an exogenous donor template to introduce the desired changes in the targeted DNA. However, this donor template can have negative effects on cell viability and genome stability as it can be cytotoxic given the initiation of DNA sensing mechanisms and can cause unwanted integrations or recombination. Here we report the development of a novel genome editing strategy that allows REpair of Heterozygous Mutations Independent of Exogenous Donor Template with High EfficiencY efficient (REMEDY) in human and mouse cells. We hypothesized that CRISPR targeting of the mutant allele induces double strand break (DSB) and stimulates the movement of healthy homologous chromosome to serve itself as an endogenous DNA donor template. This initiates the correction of the CRISPR-targeted mutant allele via intra-homologous chromosome repair. To perform REMEDY, we designed mutant allele specific gRNAs (near-PAM or in-PAM) to introduce DSB. To test the idea, we designed multiple mutant specific gRNAs targeting the heterozygous phenylalanine 508 (F508del) mutations in CFTR gene to induce DSB. We electroporated the patient-derived human bronchial epithelial cells (HBEC) harboring one copy of the F508del variant with the Cas9/RNP. As the REMEDY correction is regulated by homology-directed repair (HDR), we treated the cells with HDR enhancers such as AZD7648, which further improved the efficiency of the correction. We observed a significant increase in the

frequency of wildtype allele in the cells treated with gRNA1 from 50% to 86%, and to 87% with gRNA2 measured with using Sanger sequencing and next generation sequencing (NGS). Data were analyzed by ICE (interference of CRISPR Edits) and CRISPResso2. We further demonstrated the utility of REMEDY in the context of other four different diseases with heterozygous mutations such as Hutchinson-Gilford progeria syndrome (correction of from 50% to 69% wild-type allele), inclusion body myopathy with early-onset Paget disease and frontotemporal dementia (IBMPFD) (correction of from 50% to 71% wild-type allele), inositol 1,4,5 triphosphate receptor 3 (ITPR3) (correction of from 50% to 62% wild-type allele), and tubulin folding cofactor D (TCBD) in human patient derived primary cells (correction of from 50% to 83% wild-type allele) and mouse model derived cells (correction of from 50% to 58% wild-type allele). REMEDY has immense potential to be used in basic science and pre-clinical studies to correct heterozygous mutations in vitro as well as to treat patients if corrections can be performed ex vivo for many cell therapy applications.

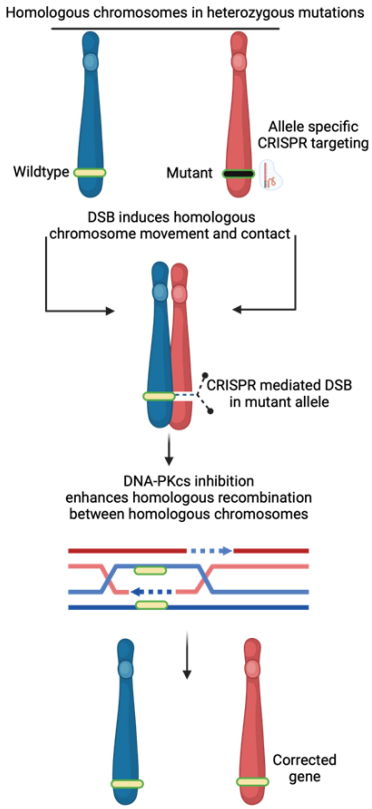


Figure 1. REMEDY results in highly efficient heterozygous mutation correction using HDR between homologous chromosomes and does not need an exogenous DNA template

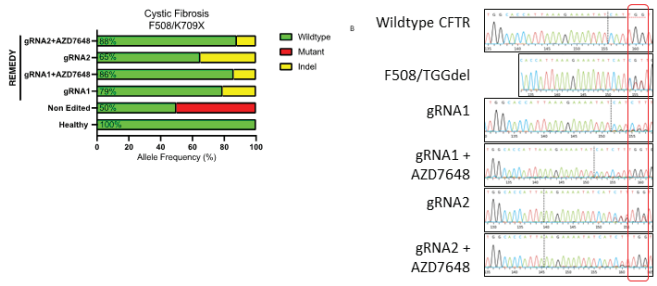


Figure 2. REMEDY corrects several heterozygous mutations in different cell types and diseases. REMEDY corrected heterozygous mutations in human airway epithelial cells with *CFTR* heterozygous mutation derived from a patient with CF.

716. A Catalogue of Novel Type II CRISPR Nucleases with Diverse PAM Preferences

Bang Wang[1], Yuanwei Cao[1], Miao Yu[2], Si Chai Liu[1], Lawrence Yuk Ting Ip[1], Ken Chau[1], Hoi Yee Keung[1], Zongli Zheng[2]

[1]GenEditBio Limited, Hong Kong, China,[2]Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet, Hong Kong, China

Clustered regularly interspaced short palindromic repeats and associated proteins (CRISPR-Cas) system holds great promise to revolutionize genome editing and therapy in basic and translational settings. Various CRISPR-Cas systems have been explored and engineered, such as CRISPR-Cas9 and CRISPR-Cas12a that belong to the Class 2 of CRISPR-Cas system. However, therapeutics applications of these tools can be limited due to the restriction of the protospacer adjacent motif (PAM)-related targetability and potential off-target effects. To address these challenges, we established and streamlined an efficient, fast, high-throughput platform for screening natural novel CRISPR Cas nucleases. With this new platform, we identified and characterized dozens of novel type II Cas nucleases with diverse PAM restrictions and protein sizes. We selected three representatives with A-rich, C-rich, and AC PAM recognitions for detailed optimization and engineering. The A-rich PAM nuclease (GEBxII108) showed generally comparable editing potency compared to the well-documented *Streptococcus pyogenes* (SpCas9) at over ten endogenous loci, and with higher fidelities at tested sites than SpCas9 as evaluated by GUIDE-seq. We engineered the sequences of Cas and single guide RNA (sgRNA), which remarkably enhanced the potency in primary cell using RNA format, enabling fully functioning. For the C-rich PAM nuclease (GEBxII920), we mutated key amino acids at the endonuclease domain to be used as a nickase. The results showed that it was functional when fused with an adenine base editor, with an expanded base editing window (A1 to A12), which might be useful for base editing at the *cis*-regulatory region (e.g. promoter) or splicing site. There are numerous scenarios where disease-causing mutations are not in an ideal distance to NGG - the PAM sequence of the most widely used SpCas9, making it necessary to use nucleases with alternative PAM recognitions. We identified a nuclease (GEBxII901) that recognizes a 5'-NRHACT-3' PAM, where the 'ACT' bases are highly restrictive. We utilized this restriction property for an allele-specific targeting application. The result showed a significant discriminative power on

editing mutation-containing allele than the WT allele. A base editor based on this GEBxII901-engineered nickase showed higher potencies compared to SpCas9-base editor at over ten tested target loci. Based on our novel type II Cas nuclease mining platform, we have identified a collection of type II Cas nucleases with diverse PAM restrictions that could be useful alternatives to SpCas9 in cell and genome editing therapy development.

717. Advanced Gene Editing with an Enhanced Site-Specific Nuclease for Knock-Out and Knock-In Applications

Oscar Alvarez, Lucas Homa, Khalid Hajj, Zhibo Ma, Cindy Negron, David Ebeid, Matthew Nitzahn, Mauricio Barajas-Mora, Arturo Barcenas, Alicia Davis, Mu Li, Nicholas Cam, Ming-Yue Lee, Blair B. Madison
Poseida Therapeutics, San Diego, CA

Our Cas-CLOVER™ Gene Editing System is a high-fidelity system that relies on the Clo051 nuclease domain and dCas9 RNA-guided DNA binding. Dual guide RNA targeting greatly restricts double-strand breaks (DSBs), conferring high specificity to the Cas-CLOVER nuclease, which is approximately 10-fold greater than Cas9, and thus significantly reduces the risk of unwanted off-target mutations. Across multiple candidate sites, the Cas-CLOVER nuclease delivers high on-target activity (84% average) and low off-target editing (<0.5%). Through efforts exploring the potential of the Cas-CLOVER nuclease, we pursued biased phylogeny-informed and structure-based mutagenesis of the Clo051 nuclease domain. One mutation (S44P) conferred a 2 to 3-fold enhancement of on-target activity, with confirmatory studies showing higher editing activity among multiple genomic targets. Notably, very low off-target editing (<0.2%) was maintained with the S44P variant, even with dose escalation, reflecting full preservation of Cas-CLOVER fidelity. The enhanced activity and fidelity were likewise observed in vivo following LNP-mediated delivery of Cas-CLOVER mRNA. We then investigated the ability of this 2nd generation Cas-CLOVER nuclease (CCv2) to yield site-specific knock-ins. Targeting an intron in the mouse *Alb* gene, a bi-directional promoterless and a uni-directional promoter-containing strategy were each evaluated for the integration of akaluciferase and phenylalanine hydroxylase (PAH) genes of interest (GOI). Each DNA donor cassette was delivered using an AAV vector. At a dose of 5E12 vector genomes per kg (vg/kg) and 1.5 mg/kg CCv2 LNP, akaluciferase bioluminescence imaging (BLI) yielded a >300-fold increase in signal relative to AAV-only controls. Liver immuno-staining indicated successful targeting of >67% of nucleated cells in the liver. For the PAH GOI delivery and integration, mRNA levels of hPAH reached 100 to 150% of normal mouse PAH levels. In the context of *Pah* knockout PKU animals, normal serum phenylalanine levels were achieved within 7 days of dosing. These data highlight the utility of the CCv2 nuclease for efficient knock-ins. In conclusion, the high-fidelity Cas-CLOVER nuclease system represents a key advancement in gene editing technology, boasting exceptional efficiency and specificity compared to conventional nucleases. The unique dimeric structure of the Cas-CLOVER high-fidelity nuclease provides the core foundation for its specificity, which greatly minimizes the occurrence of double-strand breaks at off-target sites. The S44P variant has proven to be a key enhancer of Cas-CLOVER activity, through modulation of Clo051 nuclease structure, yielding a 2 to 3-fold improvement in on-target editing. Moreover, this 2nd generation Cas-CLOVER nuclease exhibited remarkable capabilities for site-specific knock-ins, as evidenced by successful integration of a relevant GOI in mouse liver cells and phenotypical rescue in a PKU mouse model. The high fidelity coupled with the enhanced CCv2 activity could lead to greater safety and efficacy benefits for gene editing therapeutics compared to traditional Cas9.

718. Domesticating Retron Reverse Transcriptases for Precise Genome Editing

Seth Shipman
Bioengineering and Therapeutic Sciences, UCSF & Gladstone Institutes, San Francisco, CA

The use of chemically synthesized DNA sequences has accelerated biomedical research. We now recode genomes, barcode molecular events, and modify cell proteomes using synthesized DNA. However, limitations in synthetic DNA delivery prevent us from realizing the full power of these approaches. Unlike engineered RNA or protein, which we may produce inside cells, synthetic DNA must be physically delivered to the cells that we aim to modify, using methods that are inefficient, lack specificity, and offer no temporal control. As a solution, my lab is harnessing components of a bacterial immune systems called a retron to produce designed DNA inside cells by reverse transcription. I will present work showing how we have domesticated and modified retrons for use as a platform biotechnology via high-throughput, quantitative assays. I will discuss implications of this technology for genomic medicine and also highlight new approaches for fundamental biology that the retron enables, including precise and trackable genome editing screens and molecular recording of transcriptional events.

719. CRISPR-Enabled Autonomous Transposable Element (CREATE) for RNA-Mediated Gene Editing and Delivery

Yuxiao Wang[1], Ruei-Zeng Lin[1], Bruce McCreedy[2], Meghan Harris[1], Bianca Lavayan[1], Daniel Getts[1]
[1]Myeloid Therapeutics, Cambridge, MA,[2]ONK, Durham, NC

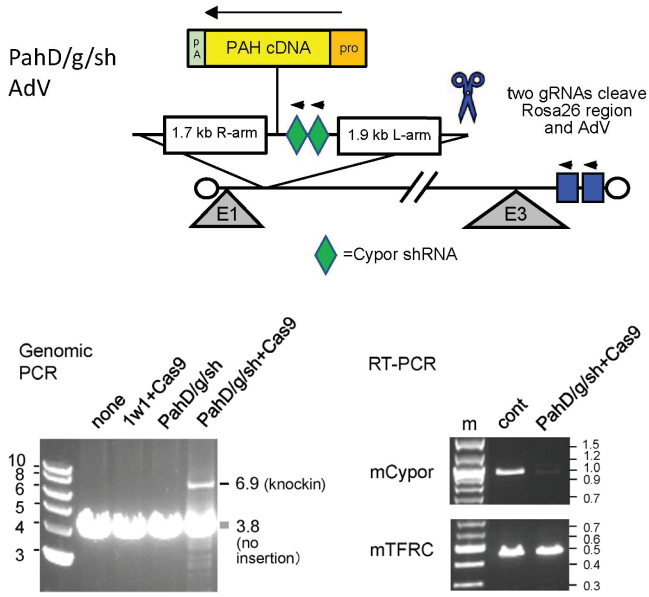
To realize the potential of genome editing, tools that can achieve targeted delivery of multi-kilobase gene payloads without inducing double-stand breaks (DSB) are necessary. The combination of Prime Editing with a DNA transposase overcomes the size limitation but requires co-delivery of multiple components including a DNA donor plasmids. Here, we introduce the CRISPR-Enabled Autonomous Transposable Element (CREATE), an RNA-based genome editing system that merges CRISPR/Cas9 with the capabilities of the human L1 retrotransposon to insert gene-sized payloads without DNA donors or double strand breaks. This is achieved by endowing the L1 mRNA with features that enable Cas9-guided reverse transcription and specific integration of an RNA-encoded payload gene without size limits. To demonstrate targeted gene delivery in mammalian cells, we insert a 1.1 kb gene comprising a promoter and green fluorescent protein (GFP) into several loci in human genome. Mechanistic studies reveal that the CREATE system is highly specific with no observed off-target events. The fully RNA-based delivery of CREATE can achieve efficient payload integration in Huh7 liver cells. Together, these findings

establish CREATE system as a programmable gene delivery platform solely based on RNA components enabling large-scale in vivo genome engineering with broad therapeutic potential.

720. The Development of Knock-In Adenovirus Vectors Aiming for Safe *In Vivo* Genome-Editing Therapy of Genetic Diseases Using Multiplex CRISPR Guide RNAs and Short-Hairpin RNA

Tomoko Nakanishi, Megumi Yamaji, Nakamura Mariko, Izumu Saito, Sigeto Sato, Nobutaka Hattori
Juntendo University, Tokyo, Japan

Introduction: Phenylketonuria (PKU) is one of the most common metabolic diseases, which results in the accumulation of phenylalanine (Phe) caused by a deficiency of the hepatic enzyme phenylalanine hydroxylase (PAH). Currently, there is no cure for PKU. The prevailing treatment is predominantly through dietary restriction of Phe to the minimum required level for normal growth, supplemented with specifically designed medical foods. CRISPR/Cas9 genome editing system, the most popular and powerful tool for gene-editing technology, is expected for clinical application to therapies for genetic diseases such as PKU. We have developed adenovirus vectors (AdVs) expressing eight multiplex guide RNAs (gRNAs) and found that these AdVs can efficiently disrupt multiple genes of human papillomavirus and hepatitis B virus *in vitro* and *in vivo* by simultaneous expression with Cas9 nickase using double-nicking strategy. The strategy reduces unwanted off-target effects, and, in fact, off-target mutations decreased below the detection limit in our study. Here, we showed a novel approach aiming for efficient and safe knock-in therapy. **Results**: An animal model for PKU, the Pah^{enu2} mouse, has a missense mutation (F263S) in exon 7, which inactivates PAH. To treat these mice, we first constructed an AdV containing a 4-kb donor DNA, including the normal exon 7 sequence, together with eight multiplex gRNA expression units. The former six gRNAs cleave genomic DNA near the mutation site and the latter two cleave the AdV genome to excise the donor DNA from the backbone. When the AdV was infected to culture cells together with the AdV expressing native Cas9 or Cas9 nickase, the knock-in efficiency increased by excision of the donor DNA but was still 3% at maximum. By administration of the AdVs to Pah^{enu2} newborn mice, a 30% reduction of the serum Phe levels was observed after 5 weeks. However, knock-in efficiency was low in the liver cells, at less than 1%, and the treatment was not sufficient. Therefore, we tried to develop a novel, more efficient knock-in system where AdV delivers the entire expression unit into the Rosa26 safe-harbor region. This AdV carries the donor DNA consisting of the expression unit of normal Pah cDNA, homology arms of 1.7 and 1.9 kb, and two gRNAs targeting at Rosa26 and two shRNAs against Cypor mRNA (graphic1) (the cells successfully knocked-in lack Cypor mRNA and can be selected *in vivo* by high dose acetaminophen). The knock-in efficiency of this AdV was about 7% *in vitro*, and the level of Cypor mRNA decreased by more than 90% (graphic2). **Conclusion**: We developed AdVs simultaneously expressing multiplex gRNAs and shRNAs and carrying a large donor DNA, which can be selected in the knocked-in liver cells *in vivo*. Utilizing the method, we established an efficient system of "cDNA knock-in", in which the entire expression units of normal cDNA are inserted efficiently into the Rosa26 region. Our results suggest that the cDNA knock-in strategy using AdVs described here could be applied to the treatment of various inborn metabolic disorders by exchanging the cDNA in the expression unit.



721. Intron Editing of HSPC Enables Lineage-Specific Expression of Therapeutics

Eduardo Seclen[1], Aminah Lawal[1], Hana Cho[1], Alexandre Juillerat[1], Sylvain Pulicani[2], Philippe Duchateau[2], Julien Valton[2]
[1]Innovation, Cellectis, INC, New York City, NY,[2]Innovation, Cellectis, SA, Paris, France

Gene therapy on hematopoietic and progenitor stem cells (HSPC) has the potential to provide a lifelong supply of genetically encoded therapeutics. Gene editing strategies for supra-endogenous expression often rely on constitutive promoters resulting in transgene overexpression irrespective of cellular differentiation, which could be detrimental for HSPC function. We have developed a TALEN mediated promoter-less intron editing technology that relies on the endogenous cellular RNA splicing machinery to induce lineage-specific transgene expression exclusively after HSPC differentiation. By inserting a promoterless eGFP cassette into different intronic regions of the genome, we demonstrated a lineage-specific expression pattern for several hematopoietic lineages *in vitro* and *in vivo*. In addition, we modeled the potential translatability of this technology by inducing myeloid-specific expression of alpha-L-iduronidase (IDUA), the deficient enzyme in Mucopolysaccharidosis type I. Our *in vitro* data confirmed that editing the introns of B-cell, T-cell or myeloid/microglial lineage-specific genes did not lead to significant eGFP expression in HSPC prior to cell differentiation. For our myeloid gene candidate, *in vitro* differentiation of edited HSPC confirmed the expected myeloid-specific expression pattern of the transgene, while maintaining the endogenous expression of the targeted gene. For our B- and T-cell gene candidates, successful lineage-specific eGFP expression was confirmed after editing activated peripheral blood and

mononuclear cells (PBMCs). eGFP-edited HSPC were injected into a conditioned immunodeficient mouse model that recapitulates bone marrow transplantation including *in vivo* differentiation into myeloid, B cell, T cell and microglia lineages. Sixteen weeks after HSPC injection, animals were euthanized and eGFP expression was characterized in blood, brain and bone marrow. A thorough characterization of human cells engraftment, level of editing and transgene expression in multiple tissues will be presented. Lastly, we edited our myeloid gene candidate in HSPC to support IDUA expression after differentiation. *In vitro*, differentiated IDUA-edited HSPC displayed a 10-fold supra-endogenous IDUA expression in comparison with the endogenous IDUA expression of non-diseased cells, highlighting the high translational potential of this technology for therapeutic approaches based on cross-correction. *In vivo*, IDUA-edited HSPC effectively populated blood, spleen, bone marrow and brain of conditioned immunodeficient mice, and retained the capacity to secrete IDUA *ex vivo*. We believe this intron editing approach is an important addition to the HSPC gene editing toolbox that might unlock new strategies for the treatment of metabolic and neurological diseases.

722. Highly Specific Zinc Finger Proteins with Synthetic Target Sites Enable Self-Regulated Expression of Dosage-Sensitive Transgenes

Gillian Houlihan, Matthew Mendel, Qi Yu, Kimberly Marlen, Jisoo Lee, Yolanda Santiago, Mohammad Samie, Patrick Dunn, Amy Pooler, Bryan Zeitler

Sangamo Therapeutics, Inc., Richmond, CA

Limiting overexpression of AAV delivered cargo is an important safety consideration particularly for indications with dosage sensitive genes which require transgene expression in a precise therapeutic range to balance efficacy and potential toxicity. To address this therapeutic challenge, we developed a self-regulating expression technology using zinc transcriptional repressor (ZFRs) proteins that serve as vector elements to precisely control transgene expression. The combined ZFR and target site payload is <1kb, does not require exogenous components and should be compatible with any promoter and transgene construct in which fine control of expression is required. Highly specific orthogonal ZFs were engineered to bind unique target DNA sequences not found in preclinically or clinically relevant mammalian genomes. When tethered to a KRAB repressor domain, these (ZFRs) exhibited exquisite specificity with no detectable differentially expressed genes identified in mouse or human neurons transduced with AAVs encoding the engineered ZFRs. Top candidate ZFRs were assessed for tolerability in vivo. AAV mediated ZFR delivery to wild type mice revealed these proteins were well tolerated with no neuronal loss or neuroinflammation observed in brain or spinal cord. Next, we developed a gene circuit regulated by a ZFR feed-forward loop using ZF target sites engineered into a promoter to tune the level of transgene expression. A panel of self-regulated expression cassettes controlling the expression of GFP were evaluated by flow cytometry and microscopy in transfected cells and AAV transduced neurons. A reduction in the population of cells with the highest levels of GFP expression was observed compared to unregulated expression cassettes. Transgene levels in AAV transduced neurons were measured by RT-qPCR which demonstrated the magnitude of ZF mediated self-regulation directly correlated with the number of ZFR target binding sites in the expression cassette. Furthermore, modulating ZF affinity for a given target site directly influenced transcriptional output. ZFR cooperativity should reduce expression proportionally to the level of delivered transgene cells thus enabling uniform transgene expression on a per cell basis. Further development and in vivo validation will be performed to evaluate this ZF platform as a solution for safe and effective gene therapies with dose-sensitive transgenes.

Wednesday Posters: Oligonucleotide Therapeutics

723. Enhancing Antisense Oligonucleotide Efficacy with Small Molecule Oligonucleotide Activity Enhancers

Saeed Anwar[1], Scott David-Bittner[2], Annie Tang[1], Rika Maruyama[1], Hong M. Moulton[2], Toshifumi Yokota[1]

[1]Medical Genetics, University of Alberta, Edmonton, AB, Canada,[2]Biomedical Sciences, Oregon State University, Corvallis, OR

Antisense oligonucleotides (ASOs) are synthetic oligomers that are adept at binding to RNA and modulating gene expression through various mechanisms, including mechanisms include RNase H-mediated cleavage, direct steric blockage, splicing modulation, non-coding RNA inhibition, and gene activation. These oligomers show promise for treating a range of complex genetic and rare diseases. However, their suboptimal activity in targeted cells and organs, particularly *in vivo*, represents a significant challenge. Following systemic administration, ASOs predominantly accumulate in the liver and kidneys, with markedly reduced accumulation in other tissues. Moreover, ASOs often become sequestered in nonproductive intracellular sinks, e.g., endosomal compartments, impeding effective gene expression modulation. This study investigates the hypothesis that small molecule oligonucleotide activity enhancers (OAEs) can significantly amplify ASO efficacy and safety. We have developed a mini-library of OAEs by repurposing molecules already approved by the FDA or extensively researched for other indications. These molecules are hypothesized to aid ASO escape from endosomal entrapment and enhance ASO activity in a non-toxic manner upon co-administration. We demonstrate the impact of OAEs on ASOs' therapeutic potential in three distinct *in vitro* disease models. Our findings include a significant (~3-fold) increase in exon-skipping efficiency of phosphorodiamidate morpholino oligomers (PMOs) targeting exon 27 of the *DYSF* gene in dysferlin-deficient limb-girdle muscular dystrophy recessive type 2 (LGMDR2) patient-derived myoblasts, restoring an out-of-frame transcript to in-frame and indicating potential functional improvements as evidenced by an enhanced myotube fusion index. Additionally, we observed that OAEs markedly increased the efficacy of *DUX4* transcript knockdown (71-97%) by locked nucleic acid (LNA) and 2′-O-methoxyethylribose (MOE) gapmers in facioscapulohumeral muscular dystrophy (FSHD) patient-derived myotubes. This was accompanied by a significant reduction in the expression of *DUX4* downstream genes, including *ZSCAN4*, *TRIM43*, and *MBD3L2*. Furthermore, we demonstrate OAEs' capacity to improve allele-selective LNA and MOE gapmer ASOs targeting the $ACVR1^{R206H}$ allele (15-25%) in fibrodysplasia ossificans

progressiva (FOP) patient-derived fibroblasts. Across these cell-based disease models and ASO chemistries, OAE co-administration resulted in highly significant enhancements in ASO activity. OAEs thus emerge as a practical tool for intensifying the activity of diverse ASO chemistries, potentially broadening the therapeutic applicability of ASO-based strategies. Ongoing research focuses on the *in vivo* efficacy and safety of these OAEs in relevant mouse models, with the aim of translating these findings into clinically viable treatments.

724. Optimization and Application of the Endosomal Escape Vehicle (EEV™) Platform for Enhanced Delivery of Oligonucleotides to Skeletal and Cardiac Muscle

Xiang Li[1], Patrick G. Dougherty[1], Mah Kheirabadi[1], Nelsa L. Estrella[1], Ajay Kumar[1], Amy N. Hicks[1], Ashweta Sahni[1], Mark Wysk[1], Kimberli J. Kamer[1], Wenlong Lian[1], Nanjun Liu[1], Ning Li[1], Matthew Streeter[1], Kyle Totaro[1], Ming Zhou[1], Christopher M. Brennan[1], Anushree Pathak[1], Alison Burman[1], Sara L. Blake[1], Mohanraj Dhanabal[1], Andy Stadheim[1], Mahasweta Girgenrath[1], Ziqing Leo Qian[2]

[1]Entrada Therapeutics Inc, Boston, MA,[2]Entrada Therapeutics, Boston, MA

Intracellular delivery of oligonucleotide therapeutics is challenging because of poor cell entry and limited escape from the endosome in the target cell. These limitations often necessitate high therapeutic doses and can be associated with less-than-optimal therapeutic activity. One strategy to overcome these limitations is conjugation of oligonucleotides to cell-penetrating peptides (CPPs). Compared with linear CPPs, cyclic CPPs have been shown to efficiently penetrate the cell membrane and are more resistant to proteolytic degradation. We have designed a family of proprietary cyclic CPPs that form the core of our Endosomal Escape Vehicle (EEV™) technology, which is capable of delivering covalently conjugated cargo to several tissue types. First generation EEV cyclic CPPs underwent several specific chemical modifications to optimize their ability to deliver antisense oligonucleotides. Notably, potentially cytotoxic residues were substituted, and nuclear localization signal was added to improve the delivery of oligonucleotides. These studies demonstrate that the medicinal chemistry underlying the structure of cyclic CPPs are integral in their ability to efficiently deliver therapeutic cargo. The therapeutic potential of EEV-oligonucleotide conjugates has been confirmed in preclinical models of Duchenne muscular dystrophy (DMD) with enhanced exon skipping and broad dystrophin protein expression in skeletal and cardiac muscles of mice and nonhuman primates. These results demonstrate the ability of the EEV platform to efficiently deliver exon skipping oligonucleotides to skeletal and cardiac muscle in preclinical models of DMD and support the potential for further study in patients with DMD. The first-in-human, phase 1 clinical trial of ENTR-601-44 in healthy male subjects is ongoing in the United Kingdom, with study results anticipated in the second half of 2024.

725. S10 Peptide Conjugated to Phosphorodiamidate Morpholino Oligomer Mediates Exon Switching in Mouse Airway Epithelial Cells

Luis Sorroza Martinez[1], Maud Auger[1], Nadine Brahiti[2], Carole-Ann Huppé[3], Laurence Faucher-Giguère[4], Imen Arbi[4], Maxime Hervault[4], Xue Cheng[4], Bruno Gaillet[5], Frédéric Couture[6], David Guay[4], Soultan Al-Halifa[4]

[1]Chemical Engineering, Université Laval, Quebec, QC, Canada,[2]Fledan Therapeutics, Quebec, QC, Canada,[3]TreansBIOTech, Quebec, QC, Canada,[4]Feldan Therapeutics, Quebec, QC, Canada,[5]Chemical Engineering, Université Laval, Québec, QC, Canada,[6]TransBIOTech, Quebec, QC, Canada

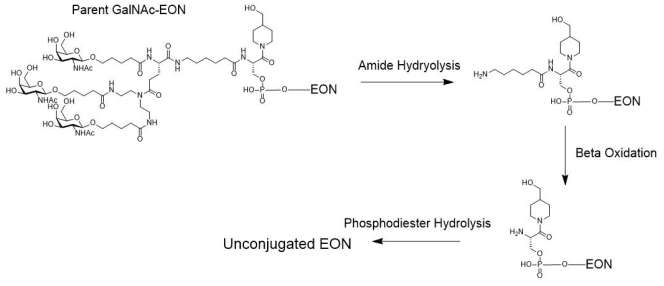
Among the various inhaled biomolecules investigated to treat lung diseases, antisense oligonucleotides (ASOs) are attractive due to their ability to mediate RNA degradation, alternative splicing, or suppress translation of targeted RNA sequences without affecting genomic DNA. Several ASOs have shown promising results in treating respiratory diseases such as asthma and cystic fibrosis (CF). Among the different ASO derivatives, phosphorodiamidate morpholino oligomers (PMOs) have great potential as therapeutic biomolecules. PMOs are ASOs in which the phosphodiester is replaced by a phosphorodiamidate backbone and the ribose by a morpholino subunit. PMOs have been successfully approved by the FDA to restore dystrophin protein expression in muscles of Duchenne muscular dystrophy (DMD) patients after systemic administration. Nevertheless, except in the case of altered tissues such as deteriorated muscle membranes in DMD, *in vitro* and *in vivo* studies show that PMOs are internalized into cells but remain mostly trapped in endosomes, preventing them from reaching intracellular RNA molecules. S10 is a peptide-based delivery agent that enables the intracellular delivery of biomolecules such as GFP, CRISPR-associated nucleases, base editor ribonucleoproteins (RNPs), and a fluorescent peptide into lung cells after intranasal or intratracheal administrations to mice, ferrets, and rhesus monkeys. Herein, we demonstrate that covalently attaching S10 to a labeled peptide or a functional splice-switching PMO improved their intracellular delivery to airway epithelia in mice after a single intranasal instillation. Data revealed a homogenous delivery from the trachea to the distal region of the lungs, specifically into the cells lining the airway. Quantitative measurements further highlighted that S10-PMO conjugation via a cleavable bond strategy was the most beneficial compared to conjugation via a non-releasable bond. We believe that S10-based conjugates bring new opportunities to enable the intracellular delivery of peptides and ASOs with therapeutic properties to the lungs.

726. Complex Metabolism and Prolonged PK/PD of a GalNAc-Conjugated Editing Oligonucleotide (EON) in Mice

Everett J. Perkins[1], Ryan Hill[1], Ellese Carmona[2], Shrutokirti De[1], Simone Bigler[3], Nicole Cnubben[3], Wouter Beumer[3], Cherie Kemmel[3], Christian van't Hekke[3], Lenka van Sint Fiet[3]

[1]Eli Lilly and Company, Indianapolis, IN,[2]Eli Lilly, Cambridge, MA,[3]ProQR Therapeutics, Leiden, Netherlands

Background: Editing oligonucleotides (EONs) are single stranded oligonucleotides designed to utilize ADAR (Adenosine Deaminase Acting on RNA) to induce single nucleotide (A to I) changes in mRNA. GalNAc-EON11 is a triantennary N-acetylgalactosamine-conjugated EON being explored for the treatment of a liver metabolic disorder. EON11 has demonstrated effective editing of the target mRNA *in vitro* and *in vivo* in mice. **Methods:** To assess the relationships between EON concentrations and pharmacological effect, a PK/PD study was conducted with GalNAc-EON11 in a mouse disease model. The drug was administered subcutaneously at a single dose of 30 mg/kg. Plasma, liver, spleen, and kidney were collected from 4 mice at time points ranging from 0.5 hr to 28 days post-dose. Parent drug and its metabolites were assessed in plasma and tissues by high resolution LC-MS and hybridization-HPLC. Additional study arms assessed liver concentrations and pharmacodynamic effects after repeat dose paradigms. Pharmacodynamics were determined by measuring the target enzyme activity in liver. **Results:** The EON11 oligonucleotide was rapidly cleared from plasma by extensive liver and kidney uptake. The EON was stable *in vivo*, with a long tissue half-life and no *in vivo* metabolites of the nucleic acid moiety observed. However, 10 metabolites of the GalNAc linker still attached to EON11 were observed. Very low levels of fully unconjugated EON11 were present in all tissues. The metabolic pathway of the GalNAc portion was generally consistent with previous reports of triatennary GalNAc molecules, with rapid initial loss of the sugars after tissue uptake and sequential cleavage of the amide bonds (Figure 1). But the predominant and long-lived metabolites in tissues were full-length EON11 with an intact portion of the alkyl heterocycle-based linker, in addition to sequential oxidation products of the 6-carbon alkyl chain. These metabolites are all presumed to retain editing activity, based on preliminary *in vitro* studies with GalNAc and non-GalNAc EONs. Further analysis was conducted by hybridization-HPLC to increase sensitivity and simplify the evaluation of PK/PD relative to total EON, assuming all GalNAc metabolites will be detected equally. Plasma concentrations of total oligo were low relative to liver but were reflective of the liver elimination profile. Spleen concentrations of total EON increased over the course of the study, suggesting redistribution from other tissues. The resulting PK profile of total EON indicates a terminal half-life that exceeds 28 days in mice, which coincided with prolonged durability of effect and accumulation with repeated dosing. Enzyme activity in liver increased over the 4-week period and was further increased with repeated dosing, indicating potential utility of this GalNac-conjugated EON approach in the treatment of metabolic genetic disorders.



727. *In Vitro* Assessment of GalNAc-Conjugated siRNA Cellular Uptake and Target Knockdown in Primary Human Hepatocyte Culture Models

Karissa E. Cottier[1], Jeannemarie Gaffney[2], Devika Bhalerao[1], Candice Lewis[1], John Griffith[1], Scott Heyward[1]

[1]BioIVT, Halethorpe, MD,[2]BioIVT, Medford, MA

New therapeutic modalities, including oligonucleotide therapies, comprise a growing share of drugs in development due to their improved target affinity and selectivity, and a longer duration of action as compared to small molecules. For liver diseases, additional site selectivity can be achieved using N-Acetylgalactosamine (GalNAc)-conjugated to therapeutic RNA to direct liver delivery by its strong affinity for the hepatocyte-specific asiaglycoprotein receptor (ASGPR). In these studies we characterized the time, ASGPR expression, and hepatocyte donor-dependent cellular uptake and knockdown efficiency of a GalNAc conjugated siRNA in standard plated hepatocytes as well as in the HEPATOPAC ® model, a micropatterned co-culture for long-term primary human hepatocyte culture. This model retains hepatic functions for up to 28 days including high levels of albumin, urea, and stable metabolic enzyme activity. To observe treatment time-dependent differences in GalNAc-siRNA efficiency, standard plated hepatocytes were treated with 50nM of Cy-3 GalNAc-siRNA targeting GAPDH for 24 hours on day 0, 1, and 2 of plating. In standard plated cultures, the greatest siRNA uptake and percent knockdown was seen when cells were treated on the day of plating (day 0). A significant difference in siRNA uptake was observed between the hepatocyte lots. Previous HEPATOPAC studies assessing ASGPR expression, siRNA uptake as measured by Cy3 signal, and target knockdown (KD) showed a strong linear correlation between these variables. To confirm ASGPR dependent uptake in HEPATOPAC, ASGPR was knocked down prior to GalNAc siRNA treatment. ASGPR siRNA transfection knocked down ASGPR mRNA by >80%. ASGPR KD reduced GalNAc-siRNA uptake by approximately 2-fold and target KD by approximately 3-fold. When HEPATOPAC cultures were exposed to GalNAc siRNA for increasing durations (0.25, 1, 4, 24, 48, or 72 hours), GalNAc-siRNA uptake and target KD peaked after 48 hours of exposure. No toxicity was observed in the hepatocytes as measured by urea output. A significant difference in both maximal uptake and KD was observed between hepatocyte lots. In this study, siRNA uptake and knockdown kinetics were similar within the hepatocyte lots tested, however the magnitude of effect varied significantly between donors. This suggests that the use of multiple lots may be important when assessing GalNAc-siRNA *in vitro*. Together, these data demonstrate the suitability of both standard cultures for short term assays, as well as HEPATOPAC cultures for longer term assays including extended incubations or repeat dosing, knockdown of multiple targets, and assessment of toxicity.

728. Oligonucleotide-Based AML Immunotherapy through Targeted STAT3/DNMT1 Inhibition Combined with TLR9/IRF8 Activation

Dongfang Wang[1], Karol Jacek[2], Damian Kaniowski[3], Yu-Lin Su[4], Haiqing Li[1], Tomasz Adamus[3], Susanta Hui[5], Bozena Kaminska[2], Vittorio DeFranciscis[6], Carla Esposito[6], Annalisa DiRuscio[7], Bin Zhang[8], Guido Marcucci[8], Ya-Huei Kuo[8], Marcin Kortylewski[1]

[1]Immuno-Oncology, Beckman Research Institute at City of Hope Comprehensive Cancer Center, Duarte, CA,[2]Nencki Institute of Experimental Biology, Warsaw, Poland,[3]Beckman Research Institute at City of Hope, Duarte, CA,[4]Beckman Research Institute City of Hope, Duarte, CA,[5]City of Hope Comprehensive Cancer Center, Duarte, CA,[6]Institute for Experimental Endocrinology and Oncology "Gaetano Salvatore" (IEOS), Naples, Italy,[7]Harvard Medical School Initiative for RNA Medicine, Harvard Medical School, Boston, MA,[8]Beckman Research Institute at City of Hope Comprehensive Cancer Center, Duarte, CA

Acute myeloid leukemia (AML) cells resist differentiation stimuli despite high expression of innate immune receptors, such as Toll-like Receptor 9 (TLR9). We previously demonstrated that targeting STAT3 using TLR9-targeted decoy (CpG-STAT3d) oligodeoxynucleotide increases immunogenicity of human and mouse AML cells. Here, we elucidated molecular mechanisms of AML cell reprogramming driven by STAT3-inhibition/TLR9-activation. The transcriptional profiling of AML cells isolated from mice after intravenous administration of CpG-STAT3dODN or leukemia-targeted *Stat3* silencing with TLR9 co-stimulation, upregulated similar sets of myeloid cell differentiation (*Irf8, Cebpa, Itgam*) and antigen-presentation (*Ciita, Il12a, B2m*) related genes while downregulating leukemia-promoting *Runx1*. Single cell transcriptomics revealed that CpG-STAT3d induced multilineage differentiation of leukemic cells into monocytes/macrophages, erythroblastic and B cell subsets. As shown by an inducible *Irf8* silencing in AML cells in mice, IRF8 upregulation was critical for monocyte-macrophage differentiation of leukemic cells. TLR9-driven AML cell reprogramming was likely enabled by downregulation of STAT3-controlled methylation regulators, such as DNMT1 and DNMT3a/b. In fact, the combination of DNMT inhibition using a combination of DNMT1 aptamer inhibitor (AptaDiR) or hypomethylating agent (azacytidine) with CpG immunostimulation mimicked CpG-STAT3d effects, resulting in AML cell differentiation, T cell activation and systemic leukemia regression. These findings highlight immunotherapeutic potential of bi-functional oligonucleotides to unleash TLR9-driven differentiation of leukemic cells by concurrent STAT3 and/or DNMT inhibition.

729. Antisense Oligonucleotide Targeting FUS-DDIT3 Oncogene for RNase H Degradation

Erin F. Hall[1], Simon Wentworth[2], Jillian Gallagher[1], Toloo Taghian[1], Yvette Diaz Lopez[1], Abigail W. McElroy[1], Hector Ribeiro Benatti[1], Sarah Nath[1], Neeta Somaiah[3], Gina D'Amato[4], Jonathan K. Watts[5], Miguel Sena-Esteves[6], Heather L. Gray-Edwards[1]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Department of Neurology, University of Massachusetts Medical School, Worcester, MA,[3]Department of Sarcoma Medical Oncology, University of Texas MD Anderson Cancer Center, Houston, TX,[4]Sylvester Comprehensive Cancer Center, University of Miami Health System, Miami, FL,[5]RNA Therapeutics Institute, UMass Chan Medical School, Worcester, MA, Canada,[6]Department of Neurology, UMass Chan Medical School, Worcester, MA

Introduction: Myxoid Liposarcoma (MLSc) is soft-tissue sarcoma caused by a translocation between the FUS and DDIT3 genes on chromosomes 12 and 16 respectively (96% of cases) or more rarely between the EWSR1 and DDIT3 genes (4% of cases). The aberrant fusion protein FUS-DDIT3 is thought to act as a transcription factor inhibiting adipocyte differentiation. Tumor growth is slow in MLSc and prognosis is typically good for patients with low grade MLSc, however the disease often becomes chemotherapy resistant upon metastasis. Primary tumors typically occur in the limbs. There are 11 documented fusion transcript variants of myxoid liposarcoma, with most transcripts consisting of the N-terminus of FUS and the entire coding sequence of DDIT3. Type 1 and 2 are the most common fusion transcripts, with breakpoints at FUS exon 7 and 5 respectively. Our lab has been working to develop antisense oligonucleotides (ASO) for type 1 and type 2 MLSc chimeric FUS-DDIT3 mRNA that will target the transcript for RNase H degradation, resulting in terminal differentiation of MLSc tumor cells. **Methods:** To screen ASOs for knockdown efficiency, we acquired the tumor-derived MLSc type 1 DL221 cell line. To model the type 2 MLSc cell line, we generated a stable HEK-293T cell line expressing the type 2 FUS-DDIT3 fusion by infecting cells with a lentivirus expressing the type 2 fusion transcript. ASOs were designed by tiling across the type 1 and type 2 breakpoints between FUS and DDIT3 sequences in the chimeric mRNA to engage specifically the chimeric mRNA without affecting the normal transcripts. We screened several different ASO chemistries in our cell lines, including 2'O-Methoxyethyl (2'-MOE) and Locked Nucleic Acid (LNA) using lipid transfection. **Results:** We have identified a top 2'-MOE and candidate in our type 2 cell line. The 2'-MOE ASO resulted in 37% average knockdown compared to a non-targeting control. We have experiments ongoing to identify a top LNA ASO in our type 2 cell line. We also have experiments ongoing in NSG mice to select the lead ASO candidate for type 1 MLSc using the DL221 cell line. Looking towards the clinic, we are planning future human clinical trials for both type 1 and type 2 patients.

Wednesday Posters: Nonviral Therapeutic Gene Delivery and Synthetic/Molecular Conjugates

731. AmplifyBio Non-Viral Gene Editing (NVGE™) Platform

Andrew Conroy, Benjamin J. Meckiff, Craig Fett, Diana Nguyen, Evelyn Vilchez, Hemi Shah, John Greer, Justin Win, Kevin Shao, Marcela Briones, Zheng Pan, Eric J. Kunkel, Theresa R. Stueve

AmplifyBio, LLC, South San Francisco, CA

Adoptive T cell therapies have achieved unprecedented response rates for hematological malignancies, and multiple trials are recruiting for solid tumors. While many therapies are manufactured using viral vectors, the benefits of targeted non-viral approaches are clear: improved insertional specificity; a better understood safety profile; reduced cost of manufacturing; and greater flexibility in simultaneous knock-in and knock-out strategies. AmplifyBio has developed an industry-leading, CRISPR-based Non-viral Gene Editing Platform[1], NVGE™, to support the cell therapy industry from discovery through commercialization. *Targeted engineering for improved genomic safety:* Viral editing produces cells with variable transgene copy numbers and unknown insertion sites, and reductions in integrating vector copy number are recommended by the FDA to reduce risk of insertional mutagenesis. For TCR-T therapies, AmplifyBio's targeted knock-in of TCR payloads of known copy number (2 copies/diploid cell) results in transgenic TCR expression at physiologic levels, without reliance on foreign promoters. Further, simultaneous knock-out of endogenous TCR undercuts competition for CD3 molecules requisite for surface TCR expression and minimizes wildtype and transgenic TCR α and β chain mispairing, enhancing TCR-T function and safety. *Multiplexed edits for increased therapy function:* The modular NVGE™ Platform can be leveraged to multiplex edits including addition of co-receptors and selection markers to the payload. Further, we show the flexibility of the NVGE™ Platform allows for simultaneous knock-out of endogenous TCR and one of six immunosuppressive targets in TCR-T cells. Moreover, the platform can accommodate payloads up to 5.3 kB and potentially larger with modifications to the backbone plasmid used for homology-directed repair. *Rapid cycling time for product selection:* The versatility and speed afforded by single-step NVGE™ produces TCR-T cells with features that can be iterated, vetted, and prioritized in a battery of functional and phenotypic assays we offer clients to inform product selection decisions. *Superior genomics safety profile and testing services:* NVGE™ allows for precision genome engineering with minimal off-target events, as evidenced by FDA-responsive genomic safety assays on offer with AmplifyBio. *cGMP cell therapy manufacturing and lot release testing:* The NVGE™ Platform has been optimized for clinical-scale cGMP manufacturing, and this process has generated materials tested in humans. In addition, AmplifyBio offers comprehensive lot release testing services responsive to FDA guidance to support regulatory filings and clinical trials. *Conclusion:* AmplifyBio offers the advanced cell therapy community a portfolio of solutions from TCR discovery to product release rooted in its CRISPR-based, NVGE™ precision engineering platform. Relative to non-targeted editing, NVGE™ produces TCR-T cells with more predictable phenotypes and superior safety profiles, critical for clinical success and lower-cost manufacturing at scale. 1. Foy *et al*. Non-viral precision T cell receptor replacement for personalized cell therapy. *Nature* (2023); PMID: 3635659.

732. *In Situ* Modification of Macrophages for Targeting PDL-1 in Triple Negative Breast Cancer

Armaan Siddiqui[1], Hemavathi Dhandapani[1], Aijaz Rather[1], Arvind Ingle[2], Deepak Sharma[3], Prakriti Tayalia[1]

[1]Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India,[2]Tata Memorial Centre Advanced Centre for Treatment, Research and Education in Cancer, Mumbai, India,[3]Bhabha Atomic Research Centre, Mumbai, India

Background: Chimeric Antigen Receptor (CAR) T-cells have demonstrated remarkable success in treating hematological malignancies. However, hindrance in the ability to infiltrate the solid tumor and exhaustion by tumor microenvironment has impeded their success in treating solid tumor. On the other hand, circulating macrophages and monocytes form a substantial part of the tumor milieu and efficiently infiltrate solid tumor masses. This unique capability of circulating monocytes and macrophages has gained significant interest in extending CAR therapy to macrophages for the treatment of solid tumor. **Aim:** The clinical scaling of chimeric antigen receptor therapy poses challenges related to the maintenance of high quality, logistical complexities, and substantial financial investments. To address these issues, we propose the utilization of a non-viral approach for in situ modification of peritoneal macrophages to express CAR for targeting Triple negative breast cancer (TNBC). This approach will facilitate development of a cost-effective approach to target solid tumor using immunotherapy and will also help avoid the risk of tumorigenic potential of virus-modified cells. **Method:** Chitosan, a biocompatible and biodegradable cationic polymer was modified with mannose to make it specific to monocytes and macrophages. Mannosylated chitosan, a cationic moiety was utilized as a carrier of plasmid DNA (Polyplex system) encoding CAR against PDL-1 which is highly expressed in TNBC. Macrophages were modified using the polyplex system and evaluated for their anti-tumor activity against murine and human triple negative breast cancer tumor models both in vitro and in vivo. STING agonist was used along with the polyplex based CAR macrophage therapy to enhance the efficacy of the study. **Results:** Mannosylated chitosan based polyplex was prepared at different weight ratios of mannosylated chitosan (Mannosylated chitosan: pDNA expressing CAR) and the size was well below 200 nm and had zeta potential of +30mV at higher weight ratios. These polyplexes were analysed for their ability to transfect both murine macrophage and human macrophage. Phagocytic ability of these modified macrophages was evaluated upon co-culture of transfected macrophages with murine and human breast cancer cell lines and the modified macrophages displayed PDL-1 specific phagocytosis. Further, the polyplex system was tested in immunocompetent murine triple negative breast cancer model and the polyplex system showed a potent anti-tumor activity when used along with STING agonist. The anti-tumor activity was further supported by the elevation in the levels of pro-inflammatory cytokines in the tumor microenviroment. **Conclusion:** The study

presents significant advancements in gene and cell therapy, showcasing an effective *in situ* immunotherapeutic approach for cancer treatment. These findings contribute substantially to the field and propose a promising candidate for advanced therapeutic strategies.

733. Internalization of a Peptide-Based Nanoplatform for Cas9 Ribonucleoprotein Delivery and Gene Editing

Alyson Schwartz[1], Joey LaValla[1], Audrey Wessinger[1], Jessica Larsen[1,2], Angela Alexander-Bryant[1]

[1]Department of Bioengineering, Clemson University, Clemson, SC,[2]Department of Chemical and Molecular Engineering, Clemson University, Clemson, SC

Introduction: The CRISPR/Cas9 system is a rapidly evolving gene editing tool for treating genetic diseases. Ribonucleoprotein (RNP) complexes eliminate the need for transcription or translation once internalized within the cell, reduce off-target effects, and have a higher delivery efficiency compared to plasmid and mRNA delivery. However, due to their large size, negative charge, and susceptibility to degradation within the endosome, RNP complexes require a delivery vehicle for successful internalization. Peptides have emerged as a promising delivery system due to their low immunogenic response, high versatility, and self-assembling properties. The fusogenic peptide, DIV3W, is able to electrostatically bind with the negatively charged RNP due to a large cationic tail. Our lab has previously demonstrated that DIV3W is biocompatible, able to facilitate endosomal escape, and protect RNA cargo. The objective of this project is to evaluate the ability of the DIV3W peptides to deliver bioactive Cas9 RNP complexes and induce knockout of green fluorescent protein (GFP) in HEK293 cells. Methods: The RNP:DIV3W system is formed by spontaneous complexation of Cas9 and gRNA at equimolar ratios, and then adding DIV3W at increasing molar ratios. The complexation of the RNPs and peptides was observed using an agarose gel electrophoresis assay. To analyze cytocompatibility, RNP:DIV3W complexes were formed and incubated with HEK293 human embryonic kidney cells for 48 hours, after which, an MTS assay was performed. The internalization of the complexes was visualized by forming RNP complexes using Cy5-labeled non-targeting siRNA (siNT) and Cas9 at equimolar ratios, and then adding DIV3W at varying molar ratios. The complexes were added to HEK293 cells and incubated for 4 and 24 hours, before analyzing using fluorescence imaging and flow cytometry. Results and Discussion: We observed that the RNP/DIV3W complexes successfully self-assembled at a minimum molar ratio of 1:1:20 (gRNA:Cas9:DIV3W) as shown in the agarose gel shift (Figure 1A). The RNP/DIV3W complexes were cytocompatible with HEK293 cells, demonstrating over 90% viability when analyzed via MTS (Figure 1B). Fluorescent imaging revealed that the RNP/DIV3W complexes were efficiently internalized into HEK293 cells at 4 and 24 hours. Flow cytometry quantified this data as shown in Figure 2. Conclusions: The results demonstrate that the DIV3W peptide is able to successfully self-assemble with RNP and the complex is cytocompatible with HEK293 cells. Furthermore, the DIV3W peptide facilitates internalization of RNP into HEK293 cells. The initial studies have shown the promise of the DIV3W peptide delivery system for gene editing. Future studies for the project include quantification of GFP knockout, identifying indel mutations, and evaluating various routes of internalization.

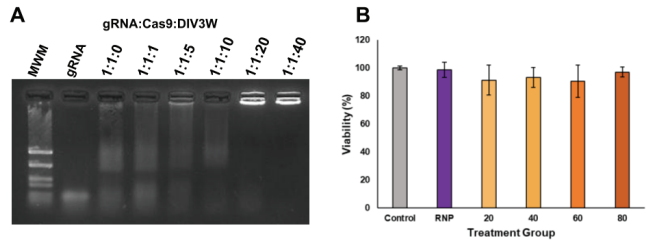


Figure 1: RNP:DIV3W Complex Characterization. Equimolar RNPs were complexed with increasing concentrations of DIV3W and electrophoresed in an agarose gel (A). HEK293 cells were treated with equimolar RNPs complexed with increasing concentrations of DIV3W and incubated for 48 hours before assessing biocompatibility using MTS (B). N=3. Data are mean ± SEM.

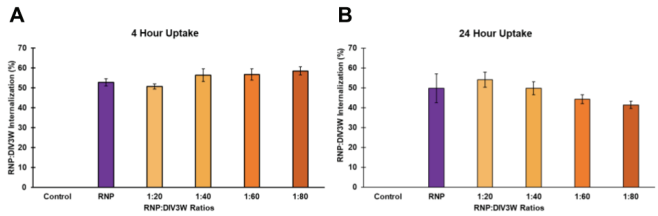


Figure 2: Uptake Flow Cytometry. HEK293 cells were treated with equimolar Cas9 and Cy5 siRNA RNPs complexed with increasing concentrations of DIV3W. (A) 4 hour flow cytometry. Data are mean SEM± (N=2). (B) 24 hour flow cytometry. Data are mean SEM± (N=3).

734. Comparing the Performance of Different Double-Stranded DNA Donor Templates in CRISPR-Cas9 Genome Editing Applications

Vaughn Thada[1], Tyler Kozisek[2], Karthik Murugan[1], Jim Williams[2], Rolf Turk[1], Garrett Rettig[1]

[1]Integrated DNA Technologies, Coralville, IA,[2]Aldevron, Fargo, ND

Genome editing using the CRISPR-Cas9 system can be harnessed to disrupt gene function, correct a pathogenic mutation, or insert an exogenous sequence into the genome. To facilitate insertion, a donor template with homology arms that flank the guide RNA (gRNA) target site must be introduced into cells along with the genome editing machinery. While synthetic oligonucleotides affecting SNP changes for small insertions can be quite efficient in mediating 'perfect' repair, knock-in of long (> 500 base pairs) double-stranded DNA (dsDNA) donor templates is generally inefficient. However, through innovations in donor DNA design, donor DNA modifications, and inhibition of proteins in competing double-strand break (DSB) repair pathways, we have observed additive and incremental improvement of dsDNA donor knock-in efficiencies via homology-directed repair (HDR). To this end, knock-in rates can be increased up to ten-fold by treating cells with small molecules and/or peptides that enhance HDR. Within the Danaher Life Sciences operating companies, IDT Alt-R™ HDR donor blocks are end-modified linear dsDNA templates for research use only (RUO), and Aldevron's Nanoplasmid™ is an RUO through cGMP-grade circular dsDNA template. In CRISPR knock-in experiments in K562, HEK293, and U2OS cells, Alt-R™ HDR donor blocks and the Nanoplasmid vector achieve similar knock-in rates in the absence of HDR enhancers, but in the presence of HDR enhancers, higher knock-in percentages are achieved with Alt-R™ HDR donor blocks, demonstrating up to 35% knock-in efficiency (with a 2 kb insert) in HEK293 cells with Cas9 mRNA and donor

delivery achieved via electroporation in the presence of multiple HDR enhancers. However, in induced pluripotent stem cells (iPSCs), Cas9 mRNA and Nanoplasmid vector delivery by electroporation in the presence of a single HDR enhancer results in 35% knock-in efficiency of a 2 kb insert, indicating donor template performance may be cell-type specific. As such, due to the scalability and cGMP manufacturing capabilities of Aldevron's Nanoplasmid vectors, we are interested in developing methods to improve Nanoplasmid vector knock-in efficiencies in the presence of multiple HDR enhancers in all cell types. To do this, we designed a "Nanoplasmid vector derivative" for use in CRISPR knock-in experiments. In K562, HEK293, and U2OS cells, use of the Nanoplasmid vector derivative results in knock-in rates similar to those observed with the original Nanoplasmid vector and Alt-R™ HDR donor blocks in the absence of HDR enhancers, but in the presence of HDR enhancers, knock-in percentages achieved with the Nanoplasmid vector derivative are comparable to those achieved with Alt-R™ HDR donor blocks and are more than double those achieved with the Nanoplasmid vector. Thus, future cGMP manufacturing of the Nanoplasmid vector derivative may allow for the large-scale production of a donor template that exhibits increased performance in CRISPR knock-in applications in a multitude of cell types, including the production of non-viral CAR T-cells. In this poster, we assess how different gRNA target sites, donor template insert sizes, and donor template homology arm lengths affect knock-in efficiencies of the aforementioned dsDNA templates in both immortalized and therapeutically-relevant cell types.

735. Highly Efficient Genome Editing of Human Primary Immune Cells Using Scalable Microfluidic Droplet-Enabled Cell Mechanoporator

Byeongju Joo[1], You-Jeong Kim[2,3], Dayoung Yun[1,4], Sungbin Im[1], Cheulhee Jung[4], Aram Chung[1,2,3]

[1]MxT Biotech, Seoul, Korea, Republic of,[2]School of Biomedical Engineering, Korea University, Seoul, Korea, Republic of,[3]Interdisciplinary Program in Precision Public Health, Korea University, Seoul, Korea, Republic of,[4]Department of Biotechnology, Korea University, Seoul, Korea, Republic of

Gene and cell therapy have emerged as pivotal modalities in clinical practice, marking transformative advancements in the disease treatment paradigm. Representative examples, including CAR-T, CAR-NK, and CRISPR treatment, underscore the shift towards genetically modified therapeutic approaches. The efficacy of these interventions relies on the precise delivery of external genetic molecules such as mRNA, plasmid DNA, and/or CRISPR into target cells. Current methods for ex vivo gene modification predominantly employ established techniques such as viral vectors and electroporation. However, these widely adopted methods encounter limitations related to high cost, low scalability, high cytotoxicity, and/or loss of cell functionality. This study introduces a novel genome delivery platform characterized by high efficiency and high scalability, facilitated by our microfluidic droplet-enabled cell mechanoporator. Cells and target genetic materials are co-encapsulated within droplets in the microfluidic platform. As these droplets pass through a narrow constriction, mechanical cell stretching leads to the transient discontinuity of the cell membrane and nuclear envelope. The recirculation flows generated within the droplets facilitate the internalization of external cargos through the generated membrane discontinuities, enabling highly efficient delivery of macromolecules, which is limited to viral transduction. Also, the permeabilization of cell membrane and nuclear envelope is recovered within droplets, which minimizes cell death. Leveraging our platform, we successfully achieved the internalization of GFP-expressed mRNA (996-nt) and plasmid DNA (9.1 kbp), demonstrating a highly effective cell transfection. Furthermore, through the delivery of CRISPR-Cas9 ribonucleoproteins (RNPs) for non-homologous end joining (NHEJ), double NHEJ, and homology-directed repair (HDR), we demonstrated a significantly improved genome editing efficiency in comparison to conventional methods such as electroporation and lipofection. Also, employing a parallelized microfluidic platform enabled a rapid and high cell processing capability which is challenging to achieve with electroporation and viral vectors. Compared to a single-channel platform, the parallelized channel showed equivalent gene delivery efficiency and cell viability while proving a high throughput process. This underscores the applicability of our system in the production of cell therapies, which typically require processing on the order of 10^8 cells. In summary, this study highlights the platform's capability for achieving high delivery efficiency, scalability, low analyte consumption, and cost-effective operation in the context of CRISPR-based genome editing. These findings affirm its significant potential for applications in genome editing and cell engineering-based therapies.

736. Manufacturing Off-the-Shelf Allogeneic NK Cell-Based Immunotherapy via Hydroporator®

Hyelee Kim[1,2], Mujin Lee[3], Bohwa Han[3], Jinho Kim[4], Duck Cho[4], Junsang Doh[3], Aram Chung[1,2]

[1]Korea University, Seoul, Korea, Republic of,[2]MxT Biotech, Seoul, Korea, Republic of,[3]Seoul National University, Seoul, Korea, Republic of,[4]Sungkyunkwan University, Seoul, Korea, Republic of

CAR-T cell therapy, characterized by its efficient therapeutic effects, has received six approvals from the Food and Drug Administration (FDA) for the treatment of blood cancer. However, as autologous T cells from patients frequently exhibit functional deficiencies due to lymphopenia, there is a substantial demand for allogeneic off-the-shelf CAR immunotherapies. Therefore, the focus has shifted to natural killer (NK) cells exhibiting high cytotoxicity against tumor cells, independent of antigen specific TCRs. As a result, allogeneic off-the-shelf CAR-NK cells have the potential to yield an equivalent or more effective therapeutic outcome than autologous CAR-T cells. To manufacture CAR-NK cells, successful transfer of CAR-encoding genes is imperative. However, conventional viral transduction is impeded by genotoxic off-targeting. Furthermore, non-viral approaches such as electroporation and lipofection are limited by low cell stability after the process and suboptimal transfection efficiency. In response to these limitations, we present a novel microfluidic intracellular delivery platform named Hydroporator® to address the drawbacks associated with current methods and enhance the manufacture of highly efficient NK cell-based immunotherapy. Hydroporator® demonstrated high transfection efficiency in delivering GFP mRNA of primary NK cells without compromising their viability and phenotype. Therefore, we employed this approach to manufacture anti-CD19 CAR-NK cells by transfecting CAR mRNA. These CAR-NK cells showed highly enhanced

cytotoxicity against CD19-positive lymphoma cells. To further validate the capability of Hydroporator® to produce next-generation NK cell-based immunotherapy, CRISPR-Cas9 ribonucleoprotein (RNP) targeting an inhibitory receptor was introduced into primary NK cells. The successful knockout of inhibitory receptors in processed NK cells resulted in enhanced killing capacity against solid cancerous cells. These results suggest that Hydroporator® can deliver various types of cargo without affecting the stability of hard-to-transfect primary NK cells. In conclusion, our study introduces a novel microfluidic intracellular delivery platform that may set a new standard for manufacturing highly stable and efficient NK cell-based immunotherapy.

737. The JL Transposon System: A Novel, Efficient, and Safe Tool for Gene and Cell Therapy with Lower Cytotoxicity and Enhanced Transposition Efficiency

Pingjing Zhang[1], Kejia Kan[2], Chengyi Song[3], Tao Liu[1], Bo Gao[3], Yan Sun[1], Ning Wu[2], Qijun Qian[1], Wei Wang[2]

[1]Shanghai Cell Therapy Group, Shanghai, China,[2]Shanghai Waker Bioscience Co., Ltd., Shanghai, China,[3]College of Animal Science and Technology, Yangzhou University, Yanghzou, China

Effective gene therapy requires robust delivery of the desired genes into relevant target cells, long-term gene expression, and minimal risks of secondary effects. The challenges of the DNA transposon-based gene delivery with respect to integration efficiency and insertional mutagenesis facilitate the development of a novel hyperactive JL transposon system through a large-scale genetic screening within the genome of the Three-Spined Stickleback (Gasterosteus aculeatus). The JL system includes the Tiniplasmid, a minimized antibiotic-free transposon plasmid with backbone DNA of less than 500bp for efficient cell nucleus entry. It also has the JL Transposase, an mRNA-based engineered transposase that produces over 10-fold transposition integration levels with significantly lower mRNA dosage and reduced safety concerns. To better understand the JL transposon system, we conducted a comprehensive assessment of its biological properties of CAR-T cell manufacturing. Our findings demonstrate that the JL transposon system has more efficient transposition than classical systems such as PiggyBac (PB) and Sleeping Beauty (SB). It achieves comparable efficiency with significantly lower plasmid doses and less cytotoxicity to T cells. As a result, it substantially increases CAR-T proliferation. To investigate the potential genotoxicity, we compared the terminal inverted repeat (TIR) non-specific promoter activity between the JL and conventional transposon systems using luciferase assay. The results showed that the 28bp TIR of the JL system has lower non-specific promoter activity. Moreover, we performed a genome-wide integration site analysis (ISA) using ligation target amplification PCR (LTA-PCR), next-generation sequencing, and in-house developed bioinformatics tools to analyze the integration sites of JL transposon-based system in transfected primary T cells. More than 10,000 unique integration sites (UISs) were retrieved from 5 samples on day 10 post-transfection. The results showed that UISs were randomly distributed throughout the genome, and no clustering on a particular genome was observed. On average, 69.14% of the UISs occurred within genes (79.67% in genes ± 10 kb). Interestingly, the relative count ratio of all sites related to cancer-associated genes was below 0.5%, and no genes related to known adverse events of cell and gene therapy were found within the integration sites. Furthermore, no clustered hotspot was found from the above results. These findings suggest the JL transposon system could be an effective and safe delivery method for human gene and cell therapy, and a promising tool for future research and clinical applications.

738. Precision T Cell Engineering through a Non-Viral Microfluidic Gene Delivery Platform, Hydroporator®

Seohyun Kim[1], Eunji Kwon[1], Dayoung Yun[1], Aram Chung[1,2]

[1]MxT biotech, Seoul, Korea, Republic of,[2]Bioengineering, Korea University, Seoul, Korea, Republic of

For decades, the cornerstone of cancer treatment has been surgery, chemotherapy, and radiation therapy. Nevertheless, there is a rapid paradigm shift in the therapeutic landscape, moving away from conventional methods to the realms of gene and cell therapies. One notable breakthrough is CAR (chimeric antigen receptor)-T cell therapy, demonstrating extremely high efficacy, especially for patients with hematological malignancy. Despite its success, they face challenges in safety, scalability, and cost due to the use of viruses as a gene delivery carrier, limiting the widespread accessibility of the therapy. In this study, we present our novel non-viral microfluidic gene delivery platform, named Hydroporator®. Through this platform, we achieved the efficient production of CD19 targeted CAR-T cells with high scalability. These CAR-T cells have been substantiated to exhibit potent cytotoxicity against CD19-positive lymphoma cells, while preserving innate cell functionality. To further demonstrate the capability of Hydroporator® in facilitating the production of CAR-T cells with enhanced cytotoxicity, we employed CRISPR-Cas9 ribonucleoprotein (RNP) to target an inhibitory receptor PD-1. Successful knockout of PD-1 in processed T cells was achieved. Additionally, to validate the potential use of Hydroporator® for allogenic T cell production, we demonstrate efficient TRAC knockout. In summary, our findings suggest that our novel gene delivery platform holds promise as a new tool for developing diverse cell therapies.

739. Potent and Metabolically Fit CAR T Cell Manufacture Enabled with Novel Solupore® Non-Viral Transfection

Jamie Frankish

Avectas, Maynooth, Ireland

Background and aim: Retention of cell health and function following the delivery of cargo is of pivotal importance in the advancement of next generation gene-modified cell therapies. Current gene delivery technologies for cell therapy applications rely on mechanical or electrical mechanisms which can negatively impact cell quality and result in wide-spread unregulated gene expression. Solupore is an automated, reproducible closed physicochemical transfection system for the delivery of a wide variety of cargo (RNA, DNA, protein) to primary immune cells as well as CD34+ cells for use in the development of clinical cell therapies. The aim of this work was to assess Solupore and another standard technology, electroporation, in the manufacturing of gene modified T cells by analysing the effect cargo delivery has on

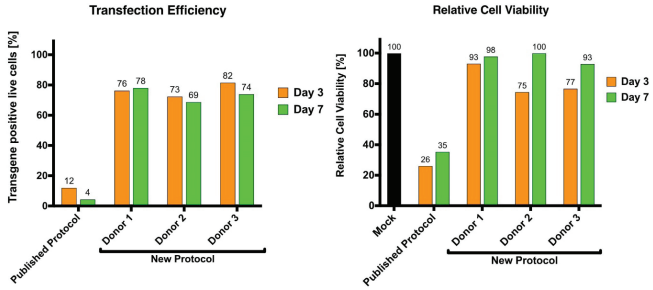
the health and functionality of those cells post-transfection. **Methods, Results, & Conclusion:** To determine the impact transfection has on the cell quality, function, and ultimately the therapeutic potential, Solupore and electroporation were assessed in several in vitro and in vivo assays, such as metabolic fitness, surface marker analysis for T cell phenotype, cytokine secretion, apoptosis response, the immunological synapse with target cells, and tumor burden reduction. Using CRISPR-Cas9 RNP based knockout of TRAC, β2M, and CD7 as proof of concept, we demonstrate that Solupore efficiently delivers cargo for multiplexed editing of primary T cells. Transduction of the clinically approved anti-CD19 CAR was used to benchmark functionality of transfected and edited cell populations. The measured metabolic profile, OXPHOS, is higher in both basal and maximal respiration for Solupore transfected populations. Solupore retains 16% more TSCM cells, IFN-γ is increased 1.45x fold more per cell and apoptosis is reduced from 25% to 12% when compared to electroporation. Significant improvement in cellular avidity is observed, demonstrating over 70% of cells binding to target cancer cells for Solupore transfected cells, compared to just 37% for electroporation. This leads to enhanced cytotoxicity of target cancer cells in vitro and performs as a more potent and significantly more effective therapy in vivo, with more than a 30x fold enhanced tumour growth inhibition (pval = <0.0001). Solupore transfected cells engrafted 5.4x fold more compared to electroporation in NSG mice and retained their gene KO profile at terminal endpoint. Taking the in vitro data together with the in vivo data highlights that the Solupore technology retains a more metabolic fit, functional, and potent cell compared to an electroporation standard.

740. Persistent Genetic Modification of K562 and Human Hematopoietic Stem and Progenitor Cells Using S/MAR DNA Nanovectors

Patrick Derigs[1], Malte Thomas[1], Alicia Roig-Merino[2], Julia Denise Peterson[1], Luisa Burger[1], Toros Tasgin[1], Anna Hartley[1], Michael Schmitt[3], Carsten Müller-Tidow[3], Richard P. Harbottle[1]

[1]DNA Vector Lab, German Cancer Research Center, Heidelberg, Germany,[2]MaxCyte, Gaithersburg, MD,[3]Department of Internal Medicine V, University Hospital Heidelberg, Heidelberg, Germany

Transplantation of hematopoietic stem and progenitor cells (HSPCs) electroporated with cytotoxic drug resistance inducing genes would ensure effective tumor suppression by applying respective anticancer therapy while maintaining sufficient hematopoiesis in cancer patients. Effective strategies for the persistent genetic modification of HSPCs are needed for this treatment approach. Recombinant integrating viruses are a potent and widely used tool. However, their random integration into the genome introduces a risk of insertional mutagenesis. Their innate immunogenicity presents another impediment to clinical application. To achieve the goal of an efficient and safe generation of stably transfected cells of a variety of cell types, we have developed a non-viral DNA vector platform based on Scaffold/Matrix-Associated Regions (S/MAR). The episomal maintenance of S/MAR DNA nanovectors (nS/MAR) eradicates the risk of insertional mutagenesis, providing a major advantage compared to already clinically applied approaches using CRISPR/Cas9 systems or viral vectors. Additionally, with its unlimited capacity, even large therapeutic transgenes or other sophisticated genetic elements can be delivered with nS/MAR. Furthermore, genetic engineering based on n/SMAR with its inherent mitotic stability should minimize costs for the manufacturing of genetically modified HSPCs. In this study, we have evaluated the efficiency and persistence of reporter transgene expression in the K562 cell line and HSPCs using nS/MAR. Additionally, we have cloned nS/MAR containing a mutant human dihydrofolate reductase transgene (*hDHFR*) to facilitate resistance against methotrexate (MTX) and tested them in K562 cells. For electroporation the MaxCyte ExPERT® platform was used. Transfection efficiency and cell viability were analyzed by flow cytometry. Our study demonstrates the feasibility of using nS/MAR for persistent genetic modification of K562 and HSPCs. Using nS/MAR containing reporter transgenes, initial transfection efficiencies of primary human HSPCs of three different donors were high with up to 82% positive cells. Cell viability was sufficient ranging from 75 to 93 % three days after electroporation (**Figure 1**). Transfected HSPCs retained reporter transgene expression for at least 21 days in culture. In K562 nS/MAR containing double-mutant *hDHFR* facilitated resistance against MTX successfully. Further studies are ongoing to modify primary HSPCs with these nS/MARs and to characterize engraftment potential and MTX resistance. These findings provide a promising first step towards the persistent genetic modification of HSPCs with nS/MAR and their usage in hematopoietic cell transplantation for the treatment of cancer patients or monogenic hematological disorders.



741. One Stop Solution for Circular RNA Synthesis and Lipid Nanoparticle Delivery

Xiaofei Long, Youshang Zhao, Zhen Sun, Kangming Chen, Qingwei Meng, Fengmei Pi

GenScript Biotech Corporation, Nanjing, China

The approval of messenger RNA (mRNA) based COVID-19 vaccines was a scientific turning point, establishing mRNA as a new drug modality with great therapeutic potential. However, linear mRNAs have short half-life and require base modification to reduce their immunogenicity, imposing a limitation to the broad application. Circular RNAs (CircRNAs) are single-stranded, covalently closed RNA molecules with non-modified base that presents a better alternative for prolonged expression of the proteins. Here we developed a new in vitro self-splicing intron system to efficiently produce circRNAs by screening and rationally designing sequences that aid in splicing. Not only that, we also screened for highly effective expression elements, the circRNAs we synthesized are quite stable and highly expressed, with low immunogenicity and fewer exogenous sequence residues, enabling higher safety and extended protein translation in cell-based assays in vitro. In addition, the circRNA encoding a CCR9 antigen,

a transmembrane protein, was encapsulated in lipid nanoparticles (LNP) and intramuscularly injected into mice without adjuvant in a single immunization. The in vivo result showed that strong antibody productions were induced and detected in serums by flow cytometry using a CHO-CCR9 stable cell line. In summary, Genscript developed a one-stop solution for circular RNA including sequence design, RNA synthesis & purification, and LNP encapsulation.

742. Nanoparticle Immunoadjuvant Complexes Augment Antigen-Specific Germinal Center Responses to Enhance Humoral Immunity

Nicholas J. Tursi[1,2], Toshitha Kannan[1], Jennifer Londregan[2], Katlyn Lederer[2], Michaela Helble[1], Ebony Gary[1], Nicole Bedanova[1], Ivan Maillard[2], David Allman[2], David Allman[2], Amelia Escolano[1], Andrew Kossenkov[1], Daniel W. Kulp[1], David B. Weiner[1]

[1]Vaccine and Immunotherapy Center, The Wistar Institute, Philadelphia, PA,[2]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

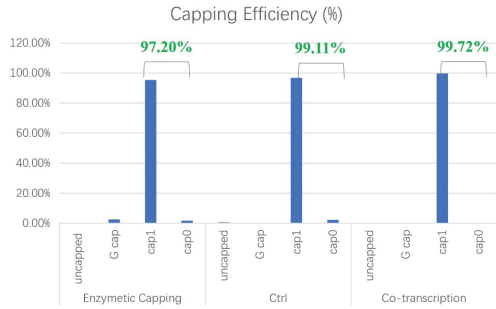
An alluring strategy for improving protective humoral immunity against infectious diseases would be to directly target the germinal center (GC). Robust GC responses are associated with high titer and high affinity antibody responses. Despite advances in formulation strategies and vaccine design, there remains a critical need for next generation approaches to elicit potent humoral immunity. Multimeric nanoparticles offer enhanced immunogenicity compared with monomeric antigen partially due to potent B cell activation and increased antigen density on the nanoparticle. Here, we investigated whether GC cytokine IL-21 could be displayed on nanoparticles to further enhance immune responses. We designed a chimeric nanoparticle using the GT8-60mer, a germline targeting HIV immunogen, as a model to scaffold IL-21. GT8 and IL-21 scaffolded nanoparticles, termed GT8 IL-21 nanoparticle immunoadjuvant complexes (GT8 IL-21 NIC), increased antigen-specific GC B cells, functional Tfh cell responses, and serum antibody titers relative to the antigen-only GT8-60mer. Additionally, GT8 IL-21 NICs reorganized antigen-specific GC B cell responses to the light zone (LZ) of the GC, the canonical site of antigen-driven selection. Single-cell RNA sequencing of antigen-specific GC B cells from GT8 IL-21 NIC immunized mice demonstrated upregulation of selection-associated gene signatures and a shift in the clonal repertoire. Thus, GC targeting NICs may provide value to drive improved humoral immunity.

743. Early-Stage Considerations for mRNA Drug Development: Addressing Production Technologies, Stability, and Safety

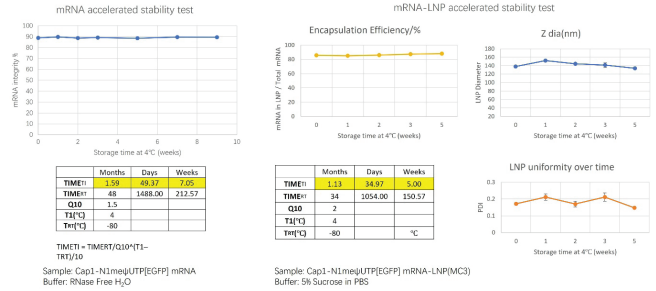
Zhiming Yang[1], Ye Bu[1], Qifan Liu[1], Xinfa Shi[1], Irene Song[1], Luyan He[1], Xin Swanson[1], Paul Li[2]

[1]PackGene Biotech INC, Houston, TX,[2]PackGene Biotech, Houston, TX

mRNA serves as a versatile platform for vaccine, gene, and cellular therapy, offering advantages in quick production, easy delivery, scalability, and native post-translational modification. However, before scaling up production, considerations of technology, cost, quality, stability, and safety are crucial. Our study explores key aspects in the early stages of mRNA drug development, focusing on later production processes. In choosing capping technology, the ownership of co-transcription technology by Trilink prompts consideration of licensing fees for CleanCap. A comparison with enzymatic capping reveals comparable efficiency, making enzymatic capping a viable, potentially fee-avoiding alternative. Addressing stability, purified mRNA from in vitro transcription (IVT) exhibits a shelf life of 5 years, while mRNA-LNP, susceptible to freeze-thaw cycles, remains stable at -80°C for over 2 years, maintaining size, uniformity, and encapsulation efficiency. Stable mRNA expression persists even after 2 years. Concerning dsRNA-induced immunogenicity, HPLC purification is suggested to reduce the dsRNA : mRNA ratio. Our optimized IVT system consistently achieves <0.1% dsRNA : mRNA ratio, with ongoing exploration into further reduction through additional HPLC purification. These insights provide crucial considerations for mRNA drug development, guiding informed decisions for successful and safe production processes.



Samples	name	Response	Observed mass (Da)	Percentage(%)	Capping efficiency (%)
Enzymetic Capping	uncapped	3450	7260.2773	0.04%	
	G cap	207567	15232.9291	2.40%	
	cap1	8241611	15289.0807	**95.39%**	**97.20%**
	cap0	156524	7619.5136	1.81%	
Ctrl	uncapped	7822.5411	7822.8023	0.63%	
	G cap	8167.7465	8168.0769	0.26%	
	cap1	24647.471	24648.1792	**96.72%**	**99.11%**
	cap0	8181.7731	8182.0386	2.39%	
Co-transcription	uncapped	7226.1402	7226.3641	0.29%	
	G cap	0	0	0%	
	cap1	22618.3286	22619.0484	**99.72%**	**99.72%**
	cap0	0	0	0%	



Wednesday Posters: Other Nonviral Delivery

744. Engineered Virus-Like Particle to Deliver CRISPR-Cas9 Ribonucleoprotein *In Vivo* with Saturated Efficacy and Minimal Off-Target Effects

Zihua Jiang[1], Hua Lu[2], Martin Chan[2], Bang Wang[2], Zongli Zheng[1]

[1]Ming Wai Lau Centre for Reparative Medicine, Karolinska Institutet, Hong Kong, China,[2]GenEditBio Limited, Hong Kong, China

The revolutionary CRISPR-Cas9 system offers a glimmer of hope for treating a vast array of genetic diseases that currently have no effective treatments. Delivery of the CRISPR-Cas9 system should be transient and prompt to minimize off-target effects, especially when delivered in vivo systemically. To achieve this, an engineered virus-like particle (eVLP) system that delivers Cas9 ribonucleoprotein (RNP) is under development. This system can enable rapid targeting of the genome and degradation of the Cas9 protein, thereby minimizing the off-target effects of CRISPR-Cas editing systems. Here we engineered a highly efficient VLP containing the wildtype SpCas9 and sgRNA. We conducted an evaluation of the off-target effect of the SpCas9-sgRNA RNP eVLP in various targeting sites. We compared its performance at a saturated dosage with that of the liposome-based CRISPR RNA, both *in vitro* and *in vivo*. Two methods were utilized to assess genome-wide off-target effects, namely the GUIDE-Seq and a novel method for editing event detection (EDITED-Seq). Our team developed the EDITED-Seq method to enable concurrent identification and verification of potential off-target sites, which is compatible with regular gene editing protocols and does not require additional cell manipulations. To produce the eVLP, four plasmids (VSV-G, MMLV gag-pol, MMLV gag-SpCas9 and sgRNA plasmids) were co-transfected into LentiX-293T cells. At 48h post-transfection, the supernatant was harvested, purified, and concentrated 100-fold for cell transduction or 1000-fold for animal administration. The optimized eVLP after purification and concentration can easily achieve over 90% editing efficacy at several targeting sites (HEK3, TTR, PD-1, *Pcsk9, Dnmt1*) in different species cell lines (HEK293T, HepG2, Jurkat, Vero, Hepa1-6, Neuro-2a). The eVLP targeting mouse *Ttr* gene was utilized to do liver gene editing on the C57BL/6 mice by tail vein injection, achieving a 60% indels in the bulk liver with a 95% reduction of serum *Ttr* protein at day 7 post-administration, which performed compatible with that of the method based on liposome delivered CRISPR RNA. we also assessed SpCas9 editing efficiencies in other tissues and observed 2.9% indels in the spleen, 1.5% indels in the lung, and minimal indels in the muscle, tail and heart. In the off-targets profiling results, the eVLP method had no detectable off-targets in cell lines, while the liposome-based RNA method showed several off-target sites according to GUIDE-Seq and EDITED-Seq results. Furthermore, EDITED-Seq results of *in vivo* edited liver samples showed that the eVLP method had significantly less off-targeting editing compared to the liposome-based RNA method. The data suggests that using eVLP to deliver CRISPR-Cas9 ribonucleoprotein can significantly reduce off-target effects compared to liposome-based RNA delivery both *in vitro* and *in vivo*.

745. High-Throughput Screening Powers the Discovery of Tissue-Specific Lipid Nanoparticles *In Vivo*

Qing Xia[1], Zhixiong Zhou[1], Bang Wang[1], Zongli Zheng[2]

[1]GenEditBio Limited, Hong Kong, China,[2]Karolinska Institutet, Shatin, Hong Kong

Efficient and safe delivery of CRISPR/Cas9 gene editing agents to the target tissue/cells is in high demand. Lipid nanoparticles (LNPs) have become a popular nonviral delivery tool in the nucleic acid-based therapies due to their low immunogenicity and modularity. Developing LNPs to enable tissue-specific delivery is desperately needed and extremely challenging. To rationally design highly effective LNP and pinpoint its optimal formulation has been proven difficult. Cell-based evaluations do not necessarily predict in vivo performance. Therefore, to effectively evaluate a large amount of LNP candidates in vivo is highly desirable. We herein present a high-throughput screening platform that simultaneously evaluates delivery efficiencies of up to 96 barcoded LNPs for in vivo gene editing. The delivery efficiencies of different LNPs were tracked and quantified by their corresponding barcodes. The barcode sequence *per se* has no effect on delivery and can only be detected when both *Cas9* mRNA and sgRNA are co-delivered and functional. We show that the barcoding system works effectively in both small and large animals, as well as in cells. Particularly, delivery efficiencies in small animals do not correlate well with large animals, highlighting the importance of direct evaluation in non-human primates for potential clinical applications. Using the barcoding technology, we were able to identify highly performatively LNPs for hepatic delivery in NHPs, with industry-leading dose-dependent efficacy. Moreover, we were able to expand our LNP discovery for extrahepatic delivery.

746. Optimization of Lipid Nanoparticles for Gene Editing of the Liver via Intraduodenal Delivery

Christine Wei[1], Yining Zhu[1], Shuting S. Cai[2], Jingyao Ma[1], Leonardo Cheng[1], Kam W. Leong[2], Hai-Quan Mao[1]

[1]Johns Hopkins University, Baltimore, MD,[2]Columbia University, New York, NY

Introduction: Lipid nanoparticles (LNPs) have emerged as a promising non-viral gene delivery vehicle for diverse therapeutic strategies, including *in vivo* gene editing. Despite notable progress in gene delivery research, the predominant focus remains on optimizing traditional administration modes, such as intravenous and intramuscular injections. The potential of oral delivery to enhance the quality of life for patients is constrained by challenges associated with physiological barriers. In this study, we explore the feasibility of gene editing in the liver using pDNA LNPs through intraduodenal injection as an indicative route for oral administration. Our optimized LNP-based all-in-one CRISPR-Cas9 pDNA gene-editing tool demonstrated targeted liver delivery, efficient gene editing, therapeutic efficacy, and safety in a hypercholesterolemia-related mouse model following intraduodenal injection, underscoring the potential for an oral gene editing approach in the treatment of familial hypercholesterolemia and other genetic diseases. **Methods:** By employing a multi-step screening approach that

addresses various physiological barriers and a machine learning-guided framework, we systematically assessed a diverse library of ca. 1,000 pDNA LNP formulations and optimized the type of helper lipid and component ratios for liver-targeted gene delivery via intraduodenal injection. Using the optimized LNP encapsulating a pDNA construct encoding both *Strep. pyogenes* Cas9 and gRNAs targeting loxP sites, we demonstrated successful non-viral *in vivo* gene editing of the liver post intraduodenal injection in an Ai9 reporter mouse model. Utilizing an all-in-one SpCas9 pDNA construct containing gRNAs targeting *PCSK9* and *ANGPTL3*, we evaluated the therapeutic efficacy of the optimized pDNA LNP formulation in a hypercholesterolemia-related mouse model. **Results:** The top-performing LNPs from the library screening achieved highly preferential liver transfection one week post intraduodenal injection, with over 87% of the bioluminescent flux localized in the liver (**Fig. 1a**). At a dose of 1 mg/500 µL, CII-β3, a DOPE-based LNP with over 95% liver accumulation one week after intraduodenal administration, achieved the highest transfection efficiency in the liver among all formulations and doses, and demonstrated efficient gene editing capability in the liver in the Ai9 model (**Fig. 1b-c**). In the hypercholesterolemia-related mouse model, mice with elevated serum cholesterol levels (> 300 ng/µL) experienced a significant reduction in serum cholesterol following treatment with CII-β3 pDNA LNPs, with no significant change in serum ALT (a liver toxicity marker) compared to the untreated group (**Fig. 1d**). Notably, there was an average of 27% decrease in serum cholesterol ($p < 0.01$) one week post intraduodenal LNP administration in comparison to the untreated group (**Fig. 1e**). **Conclusion:** Our optimized pDNA LNP formulation, CII-β3, demonstrated efficient gene editing capability in the liver and therapeutic efficacy in lowering serum cholesterol levels post intraduodenal administration. This all-in-one LNP-based pDNA-mediated gene editing strategy via intraduodenal administration not only showcases its potential as an oral therapeutic solution for familial hypercholesterolemia, but also unveils new possibilities for DNA-based medicine.

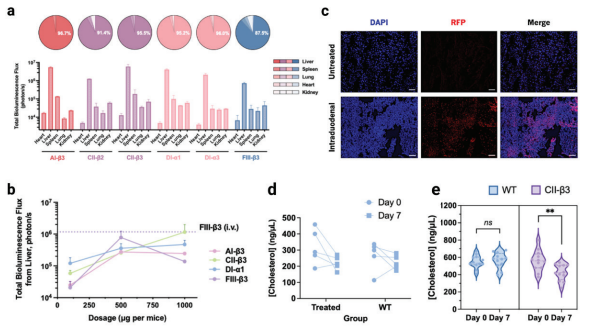


Fig. 1 | **a**, Expression percentage by organ and *ex vivo* bioluminescence flux of top six individual LNP formulations one week after intraduodenal injection (n = 4). **b**, Dosage response curve of top performing LNPs. **c**, Fluorescence confocal microscopy images of liver sections collected one week following CII-β3 pDNA LNP administration. Scale bar = 200 µm. **d-e**, Paired serum cholesterol concentrations before and one week after intraduodenal administration of CII-β3 pDNA LNP (n = 12).

747. Development of Self-Assembled Functional RNA Nanoparticle for CRISPR-Cas9 System Delivery

Yeochan Yun, Chang-yun Lee, Hyeon-Yeol Cho
Kookmin University, Seoul, Korea, Republic of

Recently, the CRISPR-Cas9 system has been extensively researched in genetic research, biotechnology, and medicine. It allows for precise gene editing and has potential applications in the development of novel therapies, the study of gene function, and the treatment of genetic diseases. Various approaches, including DNA, RNA, and ribonucleoproteins, have been developed to improve intracellular delivery of the CRISPR-Cas9 system. In this study, we proposed self-assembled functional RNA nanoparticles for mRNA delivery of the CRISPR-Cas9 system. Magnetic nanoparticles (MNPs) have been widely used in the biomedical field, with applications in bioimaging, biomolecule extraction, cancer treatment, and targeted drug delivery systems. The introduction of T7 polymerase conjugation to the MNP surface has significantly enhanced mRNA delivery via rolling circle transcription (RCT). The 10 nm zinc-doped MNPs that were synthesized exhibit strong magnetic properties and a significant surface area. A cationic polymer coating was applied to provide amine groups on the MNP surface, which allowed for conjugation with the carboxyl group of T7 polymerase. These functionalized MNPs played a crucial role in the synthesis of mRNA by replacing T7 polymerase in RCT and forming an RNA layer. Significantly, by displaying T7 polymerase on the surface of magnetic nanoparticles, we enhanced the encapsulation efficiency of mRNA creating an RNA shell that envelops the magnetic core. Successful mRNA delivery and expression were demonstrated through the observation of green fluorescent protein expression inside the cells using confocal laser scanning microscopy. In addition, self-assembled functional RNA nanoparticles enables the co-delivery of Cas9 mRNA and target gene sgRNA into cells, resulting in an efficient knockout of the target gene. This mRNA delivery system has potential for advanced gene editing and therapeutic applications.

Acknowledgement: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT) (RS-2023-00211360), and the "National Institute of Health" research project (2023ER180100).

748. A Fiber Based Adsorbent to Address Purification Requirements in the Delivery of mRNA Therapies via Extracellular Vesicles

Ian Scanlon, Christie Childers, Daniella Steel, Marc Hummersone
Research and Development, Astrea Bioseparations, Cambridge, United Kingdom

Introduction/ Background: Extracellular vesicles (EVs) and exosomes are currently being investigated as useful tools for the delivery of various C> therapeutics across several areas. Particularly, in oncology and areas where their native tropism, stability, and lack of inherent immunogenicity can deliver therapeutic payloads with increased efficacy. mRNA sequences such as CRISPR cas 9 can be delivered using EVs as an alternative to the systemic delivery using Lipid nanoparticles (LNPs). This avoids the potential issues with LNP instability, toxicity, and the limits around their low drug load efficiency. Purification of

the raw materials used to produce both the payload and the vector in these new systems can be challenging since both modalities are larger than can be dealt with easily by most chromatographic adsorbents. We present a study showing that the AstreAdept fiber adsorbent can be used to produce pure linearised plasmid for mRNA production and can be used to isolate EVs from their biological sources to support early process development in quickly accomplishing the critical quality attributes required for clinical use. **Experimental:** A fiber mat functionalised with ion exchange or hydrophobic ligands was encapsulated in a disposable housing which was sanitized, and used to purify raw material components for the non-viral vector gene therapy delivery. **Results and Discussion:** Hydrophobic material was used to purify linearised plasmid DNA produced from E Coli lysate. Anion exchange material was used to purify EVs expressed in a biological system. Processes were evaluated by capacity, recovery, and removal of product and process related impurities.

749. Novel Immune Tropic LNP-Based mRNA Delivery in Non-Human Primates

Juliet N. Crabtree, Ganapathy S. Sankaran, Karolina Kosakowska, Grace Chen, Shinu John, Muthusamy Jayaraman

ReNAgade Therapeutics, Cambridge, MA

mRNA therapies have emerged as the new frontier of genetic medicines and have shown early clinical promise in treating pathologies with established genetic targets in oncology and autoimmunity. Lipid nanoparticles (LNPs) are a clinically validated and effective delivery platform for mRNA *in vivo*. The promise of LNP-mediated RNA delivery has been demonstrated for vaccines and liver-directed therapies. Despite recent clinical success, RNA medicines are limited by insufficient delivery to extra-hepatic tissues, and cell-specific delivery remains a large unmet need in the field. Here we describe a high-throughput LNP discovery engine capable of unlocking LNP-mediated RNA delivery to extra-hepatic tissues. We utilized >300 unique lipids which further resulted in the development of >200 LNPs across multiple structural and chemically distinct classes of ionizable lipids, helper lipids, structural lipids, and hydrophilic lipids, utilizing various LNP formulation ratios. We employed a systematic barcoding screening approach to evaluate the engineered LNP libraries, alongside performing functional mRNA delivery assessments in mice and non-human primates (NHPs). Using this approach, we identified a suite of novel immune tropic LNPs. One of the lead LNPs identified by our barcoding efforts, when evaluated individually in an NHP model, demonstrated ~60% delivery in total splenic T cells and ~50% in peripheral blood T cells. Our data opens opportunities for exploration of *in vivo* chimeric antigen receptor (CAR) T cell therapies in both oncology and autoimmune disease settings.

750. Ligand-Conjugated Poly (Beta-Amino Ester) Nanoparticles for Targeted mRNA Delivery to Primary T Cells

Manav Jain, Savannah Est-Witte, Stephany Tzeng, Sarah Neshat, Sydney Shannon, Francis Yu, Leo Cheng, Jonathan Schneck, Jordan Green

Johns Hopkins University, Baltimore, MD

Introduction: Despite the promise of adoptive T cell therapies, the translational potential of these treatments is hindered by a long and expensive ex vivo manufacturing process whereby patient T cells are isolated, genetically engineered using a viral vector, expanded for several days, and then reinfused into the patient. In contrast, non-viral gene delivery approaches can be used to transfect endogenous T cells and generate therapeutic cells in situ, thereby addressing these manufacturing concerns. Poly(beta-amino ester)s (PBAEs) are biodegradable cationic polymers that can be complexed with nucleic acids like mRNA to form polymeric nanoparticles for efficient non-viral gene delivery *in vitro* and *in vivo*. Although PBAE nanoparticles have demonstrated robust transfection across different cancer cell lines and certain myeloid cells, this class of materials has had limited efficacy in transfecting primary T cells. Here, we outline the generation of polymeric nanoparticles comprised of a novel, hybrid lipophilic PBAE formulation and conjugated to several T cell targeting ligands. These targeted PBAE nanoparticles demonstrate higher T cell transfection than standard PBAEs, highlighting the potential of this non-viral gene delivery platform for T cell engineering.**Materials and Methods:** Nanoparticles were formed by self-assembly by mixing the PBAE polymer with GFP-encoding mRNA and a functional PEG linker. Different T cell targeting ligands were then conjugated to the functional PEG, thereby generating ligand-targeted nanoparticles. The targeting ligands investigated in this study were aCD3 and aCD28, which are the canonical "Signal 1" and "Signal 2" required for T cell activation. Moreover, CD3-TCR engagement has previously been implicated in T cell endocytosis, which informed our choice of aCD3 as the primary T cell targeting moiety used with our particles. Nanoparticle size and polydispersity was characterized use dynamic light scattering (DLS) and transmission electron microscopy (TEM). An overview of this nanoparticle fabrication process can be seen in Figure 1A.Primary murine T cells were co-incubated with polymeric nanoparticles that were conjugated to either aCD3 and aCD28, aCD3 alone, or no ligand. After 24 hours, transfection (as measured by GFP expression) and cell viability were measured using flow cytometry.**Results and Conclusions:** As shown in Figure 1B, nanoparticle size, as measured by DLS and TEM is consistent across the three different conditions with all particles being ~200 nm in size. Despite the uniformity in size between conditions, particles with multiple targeting ligands (aCD3 and aCD28) achieved significantly higher T cell transfection than particles with only one or no targeting ligands, as seen in Figure 1C. This trend was seen to be consistent in a dose dependent manner. Moreover, there were no significant differences in T cell viability across these conditions (Figure 1D), suggesting that PBAE NPs with multiple targeting ligands can achieve robust mRNA delivery with minimal toxicity. While previous studies have investigated the use of targeted PBAE NPs for T cell engineering, this is the first demonstration that particle functionalization with multiple ligands, namely the two key signals needed for T cell activation, significantly enhances transfection. The ability of these targeted polymeric nanoparticles to transfect endogenous T cells are currently being evaluated in vivo.

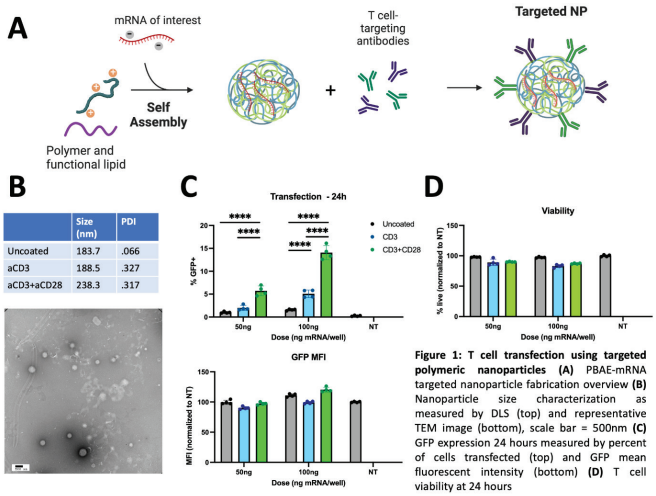


Figure 1: T cell transfection using targeted polymeric nanoparticles (A) PBAE-mRNA targeted nanoparticle fabrication overview **(B)** Nanoparticle size characterization as measured by DLS (top) and representative TEM image (bottom), scale bar = 500nm **(C)** GFP expression 24 hours measured by percent of cells transfected (top) and GFP mean fluorescent intensity (bottom) **(D)** T cell viability at 24 hours

751. Ionizable Lipid Selection and Targeted LNP Formulation Development for *In Vivo* CAR-T Therapy

Mir Mukkaram Ali

Non-Viral Gene Therapy and Genome Engineering, Mir Mukkaram Ali, Cambridge, MA

CAR-T (chimeric antigen receptor - T cell) therapy has shown significant clinical benefit to patients with hematologic malignancies such as leukemia and lymphoma. However, the treatment is currently performed ex vivo with a complex manufacturing process that results in barriers to patient access. To obviate the complexities of ex vivo manufacturing, we are developing therapeutics that enable *in vivo* specific cell reprogramming to provide re-dosable, off-the-shelf gene therapies to patients without the need for pre-conditioning treatments. We have developed a novel ionizable lipid series for *in vivo* reprogramming of T-cells via targeted delivery of mRNA using lipid nanoparticles (LNPs). αCD3 and αCD8 targeted LNPs were developed via post-insertion of an antibody (Fab/VHH) PEG-lipid conjugate into pre-made "parent" LNPs based on novel ionizable lipids. We found that targeted LNP performance was dependent on lipid chemistry including head group and tail group structure. Specifically, the hydrophobic chain length, branching, structural isomerism, and/or degree of saturation within the lipid head group and tail group impacted LNP potency. We selected ionizable lipid based on a combination of LNP physiochemical properties (size, polydispersity, RNA encapsulation and freeze-thaw stability) and the efficiency of reporter protein (GFP or mCherry) expression in primary human T cells transfected ex vivo and optimized LNP performance via ionizable lipid structure. We evaluated a subset of lead lipids for *in vitro* potency using mRNA encoding αCD20 Chimeric Antigen Receptor (CAR) protein assessing potency based on the level of membrane bound αCD20 CAR protein expression in transfected T cells and by T cell cytotoxicity towards CD20+ Raji B-cells in Raji/CAR-T cell co-culture experiments. Additionally, we demonstrated the specificity of the αCD8 targeted LNPs to the CD8+ T cell population in human whole blood and *in vivo* in a human T cell engrafted NSG mouse model. The targeted LNP compositions exhibited promising *in vivo* T cell reprogramming and tolerability, supporting further development towards the clinic.

752. Bioanalytical Development and *In Vitro* Characterization of mRNA LNP Formulation for *In Vivo* CAR-T Therapy

Allison Caron[1], Aaron Griset[1], Mir Ali[1], Viktor Lemgart[1], Austin Boesch[1], Ulrik Nielson[1], Daryl Drummond[1], Christian Mueller[2], William Kuhlman[1], Christopher Borges[2]

[1]Sanofi, Cambridge, MA,[2]Sanofi, Waltham, MA

CAR-T (chimeric antigen receptor - T cell) therapy has shown significant clinical benefit to patients with hematologic malignancies such as leukemia and lymphoma. However, the treatment is currently performed ex vivo and requires a complex manufacturing process that results in barriers to patient access. To obviate the complexities of ex vivo manufacturing, we are developing therapeutics that enable *in vivo* specific cell reprogramming, providing redosable, off-the-shelf gene therapies to patients while eliminating the need for pre-conditioning treatments. We developed several analytical methods to characterize and optimize the final lipid nanoparticle (LNP) formulation. To determine the effect of various lipid compositions and the association, specificity, and functionality of different moieties for CD3, CD4, and CD8 targeting, we incubated fluorescently labeled (DiI) LNPs containing reporter (GFP and mCherry) mRNAs with primary human T cells *in vitro*. Transfection of LNPs composed with ionizable lipids DLn-KC3-DMA maintained T-cell viability at various concentrations and displayed >60% GFP+ CD8+ cells and <5% GFP+ CD4+ cells when conjugated with an αCD8 (e.g., TRX2) targeting moiety. We observed similar trends in specificity for CD4+ T cells with αCD4 (e.g., Ibalizumab Fab) moieties. Additionally, we verified transfection, translation, and subsequent surface expression of a functional aCD20 CAR protein by incubating T cells with LNPs encapsulating functional mRNA cargo and using an anti-FLAG antibody to detect a FLAG-tag sequence. Finally, we assessed the cytotoxic potential of CAR-LNPs by co-culturing CAR-LNP transfected T cells with a Raji cell line expressing the antigen of interest and found CAR-LNPs that transfected 20% of CD8+ T-cells with a detectable level of αCD20 CAR resulted in 3-fold higher Raji killing than the same LNP formulation with a non-functional mCherry mRNA. In summary, our suite of in vitro characterization assays enables us to assess the impact of many modifications to a LNP formulation thus supporting the further development of these candidates towards the clinic.

753. Evaluation of Lipopolymers as Therapeutic Sirna Delivery Agents in Non-Malignant and Malignant Primary Cells

Aysha S. Ansari[1], Cezary Kucharski[1], Mahsa Mohseni[1], Daniel N. M. Sundaram[1], Joseph M. Brandwein[1], Xiaoyan Jiang[2], Hasan Uludag[1]

[1]University of Alberta, Edmonton, AB, Canada,[2]University of British Columbia and BC Cancer Research Institute, Vancouver, BC, Canada

Targeting leukemia-driving oncogenes via short interfering RNA (siRNA) offers an alternative approach to leukemia treatment that can overcome the limitations of conventional chemotherapy as well as specific small molecule inhibitors. Low molecular weight polyethyleneimine (PEI) grafted with lipid substituents (lipopolymers) have emerged as promising carriers for siRNA delivery to leukemia

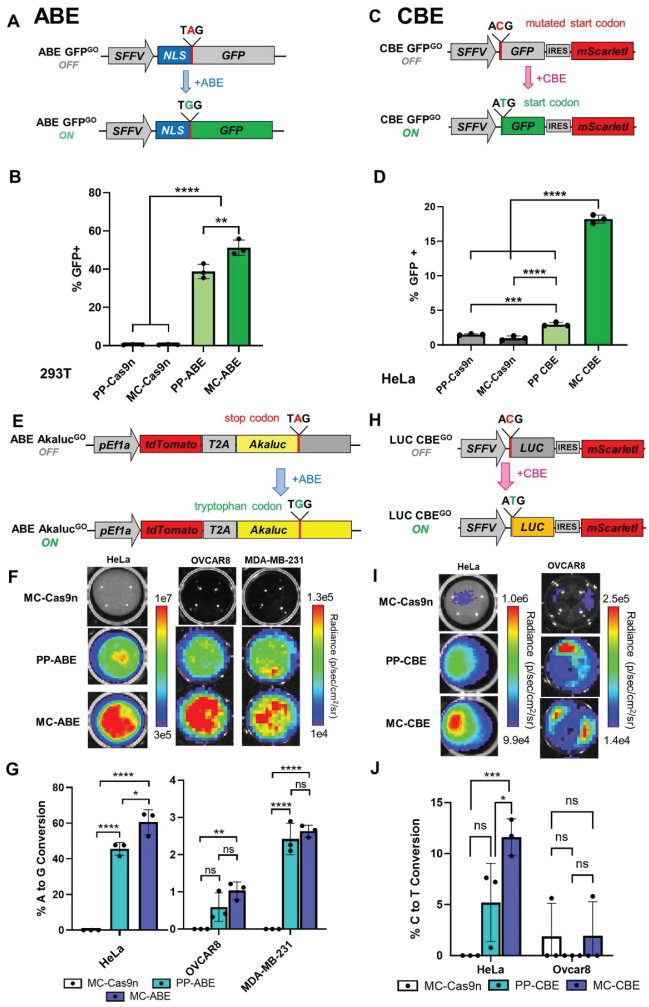
cells (Valencia-Serna J. et. al. J. Control. Release. 2019, Landry B. et. al. J. Control. Release. 2016). Lipopolymers harboring select aliphatic lipids were recently shown to downregulate target oncogene leading to significant anti-leukemic activity in both *in vitro* and *in vivo* cell line-based leukemia models (Ansari A. et. al. Blood. 2022). The goal of this study was to evaluate these lipopolymers in (i) peripheral blood mononuclear cells (PBMCs) obtained from healthy donors to determine the potential impact on non-malignant cells, and (ii) primary leukemia patient cells to assess therapeutic efficacy. The direct effect of lipopolymer/siRNA complex treatment on normal PBMCs was examined by quantifying the target gene (FLT3) transcript levels by RT-qPCR. At day 1 post-treatment, no changes in target mRNA levels were observed in all six PBMC samples with the two candidate lipopolymers (LeuFect B and LeuFect C) tested. Among 11 PBMC samples evaluated at day 2, only one sample showed a significant reduction in oncogene transcript levels on treatment with LeuFect B. The impact of lipopolymer/siRNA complex treatment on progenitor cells in the PBMC population was assessed by measuring changes in their colony forming ability. Of the seven PBMC samples tested, significant reduction in colony numbers was observed in one sample with LeuFect B and two samples with LeuFect C. On an average, the lipopolymer/siRNA complexes were well tolerated by normal healthy cells as changes observed in oncogene expression and colony forming ability were not consistent across the whole pool**.** Primary acute lymphoblastic leukemia (ALL) patient cells treated with complexes targeting STAT5A showed marked reduction in STAT5A transcript levels in four out of seven samples with both the lipopolymers tested. The subsequent impact of STAT5A downregulation on the clonogenicity of leukemia stem cells was observed in the significant decrease in colony numbers seen in three samples (out of eight) with LeuFects. Additionally, combined downregulation of STAT5A and BCR-ABL genes in BCR-ABL positive ALL patient samples led to a notable decrease in cell proliferation and viability. Moreover, 2 out of 3 acute myeloid leukemia (AML) patient samples harboring the FLT3-ITD mutation showed significant retardation in their colony forming ability on treatment with FLT3-targeting siRNA complexes. Collectively, the ALL and AML primary samples demonstrated promising preliminary results, suggesting a therapeutic effect on the stem cell population which is critical for mitigating relapse or prolonging remission. These findings provide further proof-of-concept for clinical translation of lipopolymer mediated siRNA therapy of leukemia, showing relative safety and selectivity of these complexes formed by lipopolymer/siRNA combinations.

754. Non-Viral Pathways to Precision: Utilizing DNA Minicircles to Deliver Adenine and Cytosine Base Editors in Cancer Cells

Melissa M. Evans[1,2], Shirley Liu[1,2], Joshua S. Krautner[1,2], Caroline Seguin[2], Rajan Leung[1], John Ronald[1,2,3]

[1]Medical Biophysics, Western University, London, ON, Canada,[2]Robarts Research Institute, London, ON, Canada,[3]Lawson Health Research Institute, London, ON, Canada

Introduction: Curing genetic diseases hinges on our ability to modify DNA precisely. CRISPR-Cas9 has revolutionized this field, enabling exact RNA-guided DNA targeting but also reveals challenges, like unintended genetic changes and safety concerns. A critical aspect is safely delivering editing tools into cells. We explore non-viral DNA minicircles (MCs) for this purpose. Smaller and lacking prokaryotic backbone elements, MCs are safer alternatives to traditional viral vectors. Our study examines MCs for delivering adenine and cytosine base editors (ABEs and CBEs) to cancer cells, employing the innovative 'Gene On' (GO) activatable reporter system and development of a new bioluminescent GO reporter with the sensitive luciferase Akaluciferase (Akaluc), to precisely monitor edited cells in vitro. **Methods:** We engineered 293T, HeLa, OVCAR8 and MDA-MB-231 cells for ABE and CBE studies to express GFP^{GO} (Fig 1 A, C), $Akaluc^{GO}$, or LUC^{GO} (Fig 1 E,H). These cells were transfected with MCs or plasmids (PPs) containing ABE/CBE/Cas9n. 48 hours post-transfection, GFP^{GO} expression was assessed via flow cytometry (Fig 1 B, D), and LUC^{GO}/$Akaluc^{GO}$ activation was measured using bioluminescence imaging (Fig 1 F, I). DNA was extracted for PCR and Sanger sequencing to analyze editing efficiency (Fig 1 G, J). **Results:** MC-ABE or MC-CBE transfected cells showed significantly higher GFP^{GO} expression compared to PP-ABE/CBE or PP and MC Cas9n control-transfected ones at 48 hours (Fig 1B, D). $Akaluc^{GO}$ or LUC^{GO} cells receiving MC-ABE or MC-CBE displayed significantly stronger signal than MC-Cas9n controls ($p < 0.0001$, Fig 1F, $p<0.001$, 1I). All MC-ABE cells had significantly higher bioluminescence than PP-ABE ($p < 0.05$). The delivery efficiency and percent editing varied across cell lines (Fig 1G, J). In some lines, it was difficult to detect editing with Sanger sequencing, but we were still able to detect an increase in bioluminescence over controls. **Conclusions:** Our findings demonstrate the effective use of DNA MCs in delivering ABEs and CBEs to cancer cells, tracked by the activation of the reporter gene GO systems. As the first study to use MCs for base editor delivery, we show their advantage over traditional plasmids but look to advance beyond this single comparison in the future, as well as employ inducible promoters to better control base editor expression. The flexibility of this system also allows for the assessment of newer base editor versions and tracking their activation as well as comparing them to MCs.



756. Multiplex CRISPR/Cas9 Knockout Utilizing Sequential Delivery via Microfluidics Transfection System

Melis Keceli, Sewoon Han, Alla Zamarayeva, Todd Sulchek, Chih-Wen Ni

CellFE, Alameda, CA

Introduction: Multiplex genome editing is an emerging technology that can broaden the application of gene therapies to perform multiple gene edits within therapeutic cells. However, targeting multiple loci using CRISPR/Cas9 ribonucleoprotein (RNP) complexes can result in chromosomal translocations. The aim of this study is to address the potential of cell genotoxicity by establishing a cGMP manufacturing workflow for sequential gene editing. Our platform uses a microfluidics-based cell process that enables rapid cell compressions to deliver gene-modifying payloads intracellularly in native cell medium. Using this technology, we have demonstrated a process to sequentially deliver payloads at different time intervals to prevent genotoxicity. The benefit of the workflow is to achieve multiple genetic edits while maintaining cell viability. **Methods:** Human primary T-cells were transfected at multiple timepoints to evaluate transfection and damage to cells. Human primary T cells, isolated from fresh leukopak and subsequently frozen, were thawed and activated for the indicated time points. We tested two major applications: transient gene expression using mRNA (eGFP and mCherry for different reporters) and CRISPR/Cas9 knockout using RNP complex targeting TRAC and B2M loci. The transfection efficiency and cell viability were measured after sequential editing. Different time intervals (1hr and 24hr) were also tested for use in specific applications. To establish a baseline, a single transfection of one payload or co-delivery of both payloads was performed as a control. **Results:** Our platform effectively conducted two mRNA transfections sequentially in one hour interval without causing extra damage to cells comparing to single transfection. Using 48hr activated T-cells, cell viability post transfection ranged from 40% to 60% both for single and sequential transfections. Transfection efficiency was also comparable between single and double transfections in a range of 60% to 80%. Similar results were observed for CRISPR knockouts. Importantly, cells from both single and double transfections recovered fully 5 days post transfection to equivalent viability and cell numbers, suggesting this workflow is feasible for multiple gene editing in T-cell manufacturing. We further developed a workflow for 24hr transfection interval between gene knockouts to address the risk of chromosomal translocations. **Conclusion:** Our microfluidic platform demonstrated a capacity to perform multiplex transfections in a sequential manner for multiple payload delivery. The efficiency of such sequential transfections is comparable to single transfection while maintaining similar cell viability. Our platform and workflow provide a solution for T-cell manufacturing to address the challenges of complex gene editing.

757. Machine Learning-Design of mRNA Delivery Vehicles for *In Vivo* T Cell Therapies

Shashi Murthy

Nanite, Inc., Boston, MA

A major challenge in the design of in vivo T cell therapies is the delivery of nucleic acid payloads, such as chimeric antigen receptor-encoding mRNAs, to T cells with high specificity. While lipid nanoparticles (LNPs) have shown potential in addressing this challenge, there exists a need for alternatives capable of minimal concurrent delivery to the liver and other organs, and lower toxicity. Polymers offer appealing attributes that directly address the limitations of current LNP as well as viral vector gene delivery vehicles. While there have been sporadic attempts to exploit polymeric delivery vehicles, the field has thus far been severely hindered by the vast chemical design space that must be navigated, ranging from atomic level design of repeating units, identification of optimal molecular weights, and structural considerations such as branching and crosslinking. Additional challenges include control of polymer-cargo interactions for effective packaging and ultimately, demonstration of effective delivery in vivo. Advances in machine learning, materials informatics, and accurate, high throughput chemical synthesis techniques now make it possible to not only cover this vast design space but also generate new predictions of previously unknown delivery vehicle candidates. We report how this approach can be applied to delivery of mRNA payloads to T cells. Candidate delivery vehicles are identified through an iterative process that consists of generating a small number of predictions from an established model, testing the predicted set for delivery to T cells, feeding that data back into the model, and generating a new set

of predictions. Effective candidates can be identified with only a few such cycles. An additional attribute of the predicted vehicles is the ability to engineer different types of polymer backbone breakdown characteristics. Such characteristics play an important role the design of therapies that need to be redosable without concerns of immunogenicity or bioaccumulation.

758. Designing Programmable Nanotechnologies for *In Vivo* CAR T Cell Generation

Diego Sergio Banuet Gonzalez[1], Ruby A. Sims[2], Anya Yakimenko[3], Ruth A. Foley[4], Suwen Li[5], Jigar Patel[3], Christopher S. Seet[5], Satiro N. DeOliveira[2], Steven J. Jonas[2]

[1]California Nanosystems Institute, University of California, Los Angeles, Los Angeles, CA,[2]Department of Pediatrics, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA,[3]University of California, Los Angeles, Los Angeles, CA,[4]Bioengineering, University of California, Los Angeles, Los Angeles, CA,[5]Department of Medicine, Division of Hematology-Oncology, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA

Cell-based immunotherapies are transforming the treatment of relapsed and/or refractory pediatric cancers, with next generation approaches exploring non-viral methods for engineering T cells with chimeric antigen receptors (CARs) *in vivo*. While the delivery of CAR expression constructs *via* lipid nanoparticles (LNPs) has emerged as a promising technology to mediate CAR expression in human T cells, a key barrier to their clinical, *in vivo* translation lies in their non-specific delivery. Here we report the design of a modular, antibody conjugated LNP platform for direct targeting, activation, and engineering of immune cell populations. Optimization of our non-targeting platform was achieved through microfluidic synthesis of several LNP formulations integrating different ionizable lipids for mRNA delivery. Our highest performing LNP configurations contained the ionizable lipid SM-102, with nanoparticle diameters <200 nm measured by dynamic light scattering and green fluorescence protein (GFP)-encoding mRNA cargo encapsulation efficiencies >80% by Ribogreen fluorescence-based assay. The surface of the LNPs were then decorated with αCD3 monoclonal antibodies utilizing a bioorthogonal conjugation approach. Live cell imaging and flow cytometry analysis of activated T cell populations revealed GFP expression in as little as 4 h post LNP treatment. At least 80% of treated cells went on to express GFP for up to 72 h during active T cell proliferation. Addition of αCD3 monoclonal antibodies showed comparable transfection to that of optimized, non-targeting LNP platforms; while, in addition, our preliminary studies indicate the potential of our αCD3-modified LNPs to both stimulate and transfect naïve T cells. These nanotechnologies serve as a modular, foundational platform for T cell engineering, enabling target specificity to empower next generation cellular immunotherapeutic approaches with logic-gating and other advanced capabilities.

759. Teaching Old Lipids New Tricks: Engineering LNP Composition for Extrahepatic Delivery of DNA for Lung Cancer Imaging and Treatment

Badriprasad Ananthanarayanan, Blaine McCarthy, Sushil Lathwal, Suthara Ramachandran, Anderson Lee, Christine Pedersen, Xiaobin Wu, Robby Chandra, Nadege Morisot, David Suhy

Earli, Inc, Redwood City, CA

Early detection of lung cancer significantly improves survival rates. Yet only 27% of all lung cancers are diagnosed at Stages 1-2. This is partly due to the lack of highly accurate, non-invasive diagnostic imaging methods that can differentiate malignant cancers from benign lesions. To overcome this limitation, Earli's platform uses cancer-activated DNA vectors to force tumors to produce synthetic biomarkers. Unlike most gene therapy applications, transfection of a tiny fraction of tumor cells often can produce sufficient biomarker expression for diagnostic imaging, provided there is minimal expression in normal cells. This necessitates the development of nanoparticles that can deliver DNA to lung tumors. While lipid nanoparticles (LNPs) have achieved clinical success for the delivery of RNA to hepatocytes, delivering DNA to lung tumors entails solving two fundamental challenges: preventing rapid clearance of intravenously injected LNPs by phagocytes in the liver and spleen, and delivering DNA into the nucleus of tumor cells for gene expression. Comprehensive bioengineering of the LNP surface and core lipid components were used to address these challenges. To model DNA delivery to the nucleus, we developed an *in vitro* assay based on serum-starved, slowly dividing cell lines, including H1299 cells derived from lung tumors, and screened LNP formulations encapsulating dye-labeled nanoplasmid DNA expressing GFP. We then applied high-throughput, Design-of-Experiments methodologies to screen a library of sterol and helper lipid variants to optimize expression for each ionizable lipid tested. We also evaluated a library of 30 lipid-polymer variants to define LNP surfaces that could minimize serum opsonization and concomitant cell uptake while retaining high cell transfection rates. Through these engineering approaches, we identified LNPs that increased GFP expression by 40-fold while exhibiting lower uptake into cells compared with a canonical composition based on the FDA-approved drug Patisiran. In murine models, preliminary pharmacokinetic studies established that our lead LNP formulations exhibited strikingly longer circulation, achieving up to 15-fold higher DNA copies in serum at 48 hr post injection compared to canonical liver-directed LNPs. In a subcutaneous H1299 xenograft model, this resulted in up to 4-fold greater tumor exposure. LNPs encapsulating DNA encoding firefly luciferase driven by a constitutive CAG promoter were evaluated by *ex vivo* bioluminescence imaging (BLI) of the tumor and other major organs 24-48 hr post administration. The lead LNP formulations produced high levels of expression and achieved 17-fold higher BLI in the tumor, and 50-fold greater tumor-to-liver BLI ratio, than Patisiran-like formulations of the same ionizable lipid. In an orthotopic lung xenograft model, these formulations produced BLI 220-fold higher than PBS-dosed mice, whereas non-tumor-bearing mice produced no BLI above background, verifying the ability of these LNPs to selectively express DNA in lung tumors. Thus, comprehensive engineering of LNP composition enables extrahepatic delivery of DNA to lung tumors. When combined with cancer-activated expression of

PET reporter genes and further validation in preclinical mouse models and toxicology studies, this represents a promising diagnostic imaging platform for early detection of lung cancer.

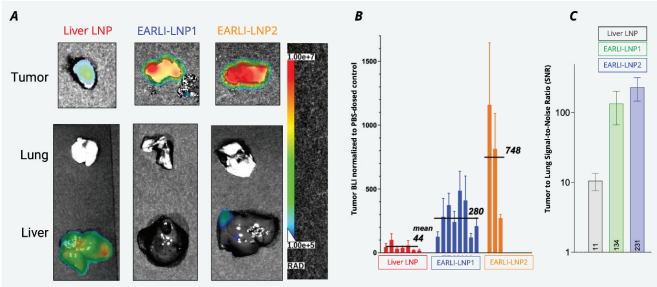


Figure 1. Extrahepatic delivery and expression of DNA by engineered LNPs. (A) Representative images of tumors, lungs, and livers from mice dosed intravenously with indicated LNPs encoding firefly luciferase and imaged 48 hr after administration. (B) Tumor BLI for indicated LNPs normalized to background in PBS-dosed mice. Each bar represents a distinct study. (C) Signal-to-Noise Ratio of average tumor to lung BLI for indicated LNPs. For (B) and (C), n=5 mice per group, error bars represent S.E.M.

760. Rapid Screening of Exosomal RNA Cargo for Therapeutic Research Applications

Chris DJamoos

Promega Corporation - Madison, WI, Brighton, MI

Aim: Exosome RNA therapy is emerging as a pivotal tool in cell and gene therapy, offering several advantages over traditional cell-based methods. Exosomes, as naturally occurring nanocarriers, provide a non-immunogenic, stable, and efficient means of delivering therapeutic nucleic acids. In addition, there are significant advantages in their manufacture, storage and distribution. Exosomes, when engineered through host cell transfection, can effectively deliver therapeutic nucleic acids such as mRNA, miRNA, siRNA, and circRNA. A crucial element in harnessing the full therapeutic potential of exosomes is the development of rapid, efficient methods for their isolation and the characterization of their RNA payload. The aim of this study was to validate a rapid and scalable exosomal miRNA isolation platform for characterizing nucleic acid payloads. **Methods:** Fully automated exosomal miRNA isolated from 500 µl of plasma (n=60) was performed using a magnetic bead-based affinity capture system (EXO-NET®, Inoviq Ltd) and a high-throughput miRNA extraction t chemistry (Maxwell® HT miRNA Plasma and Serum, Promega Corporation) on an automated platform (KingFisher APEX, ThermoFisher). Western blot analysis was used to confirm the presence of canonical protein biomarkers. The EV-RNA content was assessed by RNA seq using NovaSeq X Plus system (Illumina) and then targeted miRNAs were validated by RT-PCR. **Results:** Exosomal miRNA isolation from 60 plasma samples was performed in less than 2 hours. Western blot analysis confirmed the presence of exosomal protein biomarkers (CD9 and flotillin-1). Small RNA seq analysis identified more than 400 miRNAs in plasma derived exosomes. Further targeted miRNA analysis by RT-PCR showed abundancy and consistent expression of miR191 (Ct: 22.43±1.87), miR-21 (Ct: 21.98±1.96) and miR-186 (Ct: 24.59±2.03) in all plasma samples. **Conclusion:** We have established and validated a rapid, scalable and automated platform for exosomal RNA isolation for downstream analysis and characterization. The platform holds great promise for facilitating the development and translation of exosome-based companion diagnostics and therapeutics into routine clinical practice.

761. Peptide Based Nanoparticle-Platform for Extrahepatic Delivery of Functional mRNAs

Gilles Di Vita[1,2], Audrey Grunenberger[1,2], Elodie Czuba[1], Léa Cabrera[1], Veronique Josserand[3], Melanie Guidetti[3], Neil Desai[2]

[1]DIVINCELL, Nimes, France,[2]AADIGEN, Pacific Palisades, CA,[3]IAB, Grenoble, France

Therapeutic mRNAs constitute a new generation treatments to restore functional version of mutated or missing proteins in patients. We have developed a unique RNA Therapeutic platform that leverages novel peptide technology able to systemically target and deliver the RNA therapeutic into the pathologic cell to fight or prevent diseases. ADGN technology is based on short amphipathic peptides that form stable nanoparticles with mRNAs. The molecular composition and sequences of ADGN nanoparticles have been rationally designed for selective organ targeting of functional mRNAs, to overcome hepatic accumulation and to induce robust mRNA expression in the targeted tissue. ADGN/mRNA nanoparticles are stable both in solution and in dry powder formulations, with an average size of 100 nm and a high encapsulation efficiency (>90%). ADGN nanoparticles have been successfully used for the rescue of lost function of tumor suppressor functions resulting from mutations or alterations as a potential therapeutic approach in cancers. Tumor suppressors p53 and BRCA-1 play essential roles in tumorigenesis. Indeed, p53 or BRCA-1 mutations resulting loss of function are common across variety of tumors and influence tumor cell proliferation and metastasis in more than 50% of cancers. ADGN-531 combines proprietary P53 derived mRNA with a tumor selective peptide nanocarrier to rescue of p53 tumor suppressor function in p53 mutated cancers. ADGN-531 markedly inhibits the growth of a large panel of cancer cells harboring p53-missense or nonsense mutations or p53-deletion, by rescuing p53 transcriptional activity and subsequent expression of its target proteins. Systemic IV-administration of ADGN-531 resulted in dose responsive tumor growth inhibition in SaOs2 (osteosarcoma), SW403 (colorectal cancer) and H358 (lung cancer) xenografts with strong tumor growth inhibition when administered weekly at a dose of 1 mg/kg. [nd1] ADGN-903 combines a proprietary BRCA-1 derived mRNA complexed with a tumor selective peptide nanocarrier to rescue of BRCA-1 tumor suppressor function in BRCA-1 mutated cancers. ADGN-903 markedly inhibits the proliferation of BRCA-1 deficient cell lines depending on the type of BRCA-1 alterations. Systemic IV-administration ADGN-903 (2.0 mg/kg) prevented HCC-1937 breast cancer tumor growth. and resulted in strong tumor regressions. ADGN-903 was also highly effective, on large tumors of about 500-600 mm^3, in preventing further progression and achieving shrinkage of large tumors. Rescue of lost function of mutated tumor suppressors p53 or BRCA-1 by ADGN-531 and ADGN-903 respectively were well tolerated, with no sign of clinical toxicity detected after single or repeated administrations. ADGN nanoparticles constitute a safe and effective platform for tissue-specific delivery of functional mRNAs with potential clinical applications. Our study provides a proof-of-concept that restoration of tumor suppressor function by ADGN-531 or ADGN-903 could be used as a single agent therapy in P53 or BRCA-1 altered tumors independent of other driver mutations or with other therapies for potent combinatorial cancer treatment.

762. Exosomes as a Stromal-Targeting Extension of Oncolytic Virus Therapeutics

Kate R. J. Friesen[1], Dhanu Gupta[2,3], Kerry D. Fisher[1], Matthew J. Wood[2], Leonard W. Seymour[1]

[1]Oncology, University of Oxford, Oxford, United Kingdom,[2]Paediatrics, University of Oxford, Oxford, United Kingdom,[3]Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden

Background: Oncolytic viruses (OVs) selectively replicate in and kill cancer cells. OVs can be used as a gene delivery vector for selective expression of therapeutics in the tumour microenvironment (TME). One current barrier of OV-mediated therapy in solid tumours is the poor viral spread due to the extracellular matrix produced from stromal cells such as cancer-associated fibroblasts (CAFs). Exosomes are naturally derived lipid-coated nanoparticles that transport various molecular payloads between donor and recipient cells. They range between 30 nm - 150 nm, are secreted by most cell types, have the same topology as the plasma membrane, and are generated from the inward budding of late endosome membranes forming multivesicular bodies (MVBs). Additionally, exosomes can be engineered to transport therapeutics between donor and recipient cells. This property makes them a promising nanoparticle-based delivery system for targeted drug and gene therapy. Aim: To enhance the spread of therapeutics *in situ*, we are developing an exosome-targeting system where DNA coding for a tripartite fusion protein (consisting of a targeting protein (anti-FAP scFv), a scaffold protein, and a therapeutic protein (cytosine deaminase)) is delivered by an oncolytic adenovirus. The fusion protein is synthesized and loaded into exosomes for delivery to surrounding potentially non-infected tumour and stromal cells such as CAFs.

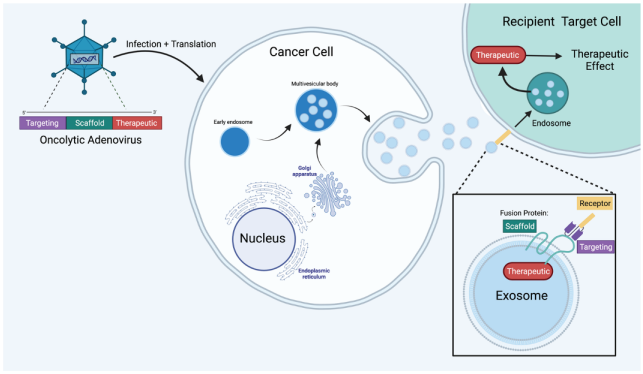


Figure 1. Project hypothesis: An oncolytic adenovirus can be used to deliver DNA coding for a tripartite fusion protein (consisting of a receptor-binding targeting protein, a scaffold protein, and a therapeutic protein) that will be synthesized and packaged into exosomes which will deliver the cargo to uninfected cancer and stromal cells.

Methods: Exosomes were isolated via size exclusion chromatography from adenocarcinoma cells stably transduced with the tripartite fusion protein and characterized according to MISEV guidelines. Real-time exosome uptake into cancer cells and CAFs was monitored using a split nanoluciferase reporter system. Nano-flow cytometry and a FlipGFP-reporter system were used to evaluate the functionality and targeting potential of the anti-FAP scFv exosmes. Cell viability assays were used to quantify the ability of exosomes to deliver cytosine deaminase to cancer cells and CAFs. Results: Exosomes containing a subunit of split nanoluciferase were capable of being taken up in real-time and delivering functional cargo into the cytoplasm of recipient tumour cells expressing the other subunit. A functional anti-FAP scFv was capable of being expressed on exosomes, and treatment with exosomes armed with cytosine deaminase resulted in a significant decrease in tumour cell viability following the addition of 5-fluorocytosine. Conclusions: Exosomes can be engineered to selectively package therapeutics and deliver cargo to recipient cells resulting in target cell death. The cytotoxicity of the oncolytic virus expressing the tripartite fusion protein is currently being evaluated using these methods *in vitro* and *ex vivo*. Concentrating the production of bioengineered EVs directly in the TME provides a novel approach to overcome the biodistribution and rapid EV clearance challenges that limit the clinical potential of therapeutic exosomes/EVs.

763. *In Vivo* Gene Editing of Non-Small Cell Lung Tumors Directed by CRISPR/Cas Reduces Tumor Growth and Extends Survival

Katelynn Owens[1], Kelly Banas[1], Nicole Haas[1], Emily Gielda[1], Kristen Pisarcik[1], Komal Khan[1], Hannah Pachalis[1], Laura Flanagan[2], Eric B. Kmiec[1]

[1]Gene Editing Institute, Christiana Care, Newark, DE,[2]Office of Laboratory Animal Medicine, University of Delaware, Newark, DE

Lung cancer is the leading cause of cancer-related mortality in the United States, with chemotherapies serving as the main line of treatment. While chemotherapies sometimes halt tumor progression, repeated or prolonged treatments often cause patients to become resistant. One gene that has been implicated in this resistance pathway is nuclear factor erythroid 2-related factor (NRF2). We hypothesized that targeting NRF2 with CRISPR Cas9 in patients with non-small cell lung cancer, will reverse chemo-resistance and allow the current standard of care to work better. To do this, we used athymic nude mice implanted with either an engineered H1703 cell line or patient derived tumor fragment as a model. Subcutaneous tumors were directly injected with lipid nano particles (LNP) encapsulating CRISPR Cas9 mRNA and gRNA. Data show high editing in tumors injected with LNP. Additionally, data show little to no biodistribution of the mRNA after injection, suggesting that delivery of the drug remains in the tumor after injection. Knockdown of NRF2 with CRISPR Cas9 at the genomic level is sufficient to affect the protein's function, as shown by altered target gene expression. LNP delivery of CRISPR Cas9 intra-tumorally, combined with chemotherapy, showed delayed tumor growth and increased survival when compared to controls. These data hold promise for overcoming chemotherapy resistance in patients with NSCLC and other diseases.

764. Engineered Ribonucleoproteins (eRNPs) for Non-Viral *In Vivo* Delivery of Gene Editors to Treat Ophthalmic Disorders

Rina Mepani[1], Aaron Cantor[1], Pawan Shahi[2,3], Spencer Wei[1], Vihasi Jani[1], Kory Melton[1], Prachi Khekare[1], Suhani Gupta[1], Jonathan Wang[1], Peter Otoupal[1], Jack Walleshauser[1], Benjamin Gowen[1], Weng In Leong[1], Krishanu Saha[2,4], Adam Silverman[1], Bikash Pattnaik[2,3], Mary Haak-Frendscho[1]

[1]Spotlight Therapeutics, Hayward, CA,[2]McPherson Eye Research Institute, University of Wisconsin-Madison, Madison, WI,[3]Department of Pediatrics, University of Wisconsin-Madison, Madison, WI,[4]Wisconsin Institute for Discovery, University of Wisconsin-Madison, Madison, WI

Genome editing technologies offer great potential as durable therapies for a myriad of diseases, including many inherited disorders previously deemed untreatable. The recent approval of ex vivo gene editing therapy for blood disorders showcases the immense promise of this transformative technology while underscoring the need to develop in vivo gene editors to enable application expansion. Current in vivo delivery approaches for gene editors, such as AAV, face multiple challenges, including pre-existing immunity, capsid-induced inflammation, persistent expression of nucleases, and complexity of manufacturing. Here we describe a new class of single administration genomic medicine, engineered RNPs (eRNPs). eRNPs are non-viral, non-encapsulated biologics designed for safe localized in vivo gene editing. These protein-RNA constructs are a scaffold of Cas nuclease fused with cell-penetrating and nuclear-localizing peptide sequences, with or without a deaminase domain, and complexed with a single guide RNA. Highlighting the modular nature of the protein scaffold, we illustrate the ability to generate bespoke eRNPs tailored to introduce indels, excise exons, or perform base editing for selected genetic lesions. Inherited retinal disorders (IRDs) represent a class of genetic disorders with high unmet need. eRNPs are particularly well suited for subretinal administration, which enables direct delivery to retinal pigmented epithelial (RPE) cells or photoreceptors. We demonstrate that eRNPs delivered by subretinal injection are highly efficient at introducing indels in RPE and retina in wild-type, transgenic reporter, and functional mouse models. In an Ai14 tdTomato reporter model we observed high focal tdTomato signal in the retinal pigment epithelium (RPE). We demonstrate editing efficiencies are biologically functional by targeting the wild-type allele in KCNJ13+/W53X mice, which resulted in near complete ablation of electroretinogram c-wave. We further show that eRNPs with engineered adenine deaminase domains mediate efficient base editing in the retina and RPE of wild-type mice. Taken together, these data suggest the highly modular biologic approach of eRNPs opens a new path for treating a wide range of IRDs and ocular diseases with tremendous unmet medical need.

765. Optimization of Fusogenix Proteolipid Vehicles for Extra-Hepatic Delivery of mRNA

Douglas Brown[1], Arun Raturi[1], Ping Wee[1], Prakash Bhandari[2], Amirali Bukhari[2], Maryam Hejazi[3], Jitendra Kumar[4], John Lewis[2]

[1]Entos Pharmaceuticals, Edmonton, AB, Canada,[2]Entos Pharmaceuticals, San Diego, CA,[3]Entos, Edmonton, AB, Canada,[4]Entos Pharmaceuticals Inc, San Diego, CA

The promise of genetic medicines for the treatment of a wide range of diseases remains limited by viral and non-viral delivery platforms. These legacy platforms face challenges in the effective and tolerable delivery of nucleic acids. While non-viral lipid nanoparticle (LNP) delivery platforms have undergone incremental improvements, they still suffer from a narrow biodistribution profile, predominantly hepatic cells, and substantial dose-limiting toxicities. To overcome these issues, we developed an alternative all-encompassing platform capable of body wide delivery of nucleic acids including mRNA, called Fusogenix. The Fusogenix proteolipid vehicle (PLV) platform leverages a proprietary fusion-associated small transmembrane (FAST) protein incorporated into well-tolerated lipid formulations. The Fusogenix platform achieves extra-hepatic delivery and tropism through manipulation of manufacturing parameters such as charge ratio, PEGylation, microfluidic flow rate, and lipid composition. Using this approach, we achieved dramatic shifts in surface properties of the PLVs, independent of the encapsulated cargo. Modulating these parameters altered the affinity of FAST-PLVs to a wide array of extra-hepatic tissues, such as muscle, lungs, and kidneys and increased the efficacy of mRNA-mediated gene expression in target tissues as well. Direct comparison of FAST-PLV mediated delivery of mRNA-FLuc to three conventional clinical LNP formulations (MC3-LNPs, Pfizer-LNPs, and Moderna-LNPs) via intramuscular, intrathecal, intracerebroventricular, and intravitreal injection show the dramatically improved biodistribution of FAST-PLVs. Intramuscular, intracerebroventricular, and intravitreal injection of FAST-PLVs resulted in robust localized expression of FLuc in the intended tissue. Intrathecally administered FAST-PLVs distribute throughout the central nervous system and achieved robust FLuc expression in multiple brain regions. All three clinical LNP formulations displayed predominant liver-tropism, resulting in saturating levels of FLuc expression in the liver regardless of the injection route. Conversely, no off-target FLuc expression was detected with FAST-PLVs by any route of injection. Precise delivery of nucleic acid cargoes associated with a favorable safety profile, as circulating cytokine levels were lower in mice administered FAST-PLVs, and histological analysis indicated no signs of pathology. Intravenous administration of FAST-PLVs yielded notable mRNA expression in the lung, with a subsequent profile analysis confirming expression in both type I and type II alveolar cells. To assess therapeutic gene expression, we administered mRNA encoding erythropoietin (mRNA-EPO) through both intravenous and intramuscular routes, leading to a rapid increase in circulating erythropoietin levels in the hours following injection. These findings collectively underscore the feasibility and optimization of extra-hepatic mRNA delivery achieved by fine-tuning the biophysical attributes of FAST-PLVs. Importantly, the ability to target extra-hepatic tissues and reduced liver tropism establishes FAST-PLVs as a promising platform for advancing the clinical development of mRNA-based genetic medicines.

766. Development of *In Vitro* Transcribed mRNA Therapeutics for Cystic Fibrosis

Yiman Li[1], Ruhina Maeshima[1], Claire Smith[2], Stephen L. Hart[1]

[1]Department of Genetics and Genomic Medicine, UCL Great Ormond Street Institute of Child Health, London, United Kingdom,[2]Department of Infection, Immunity and Inflammation, UCL Great Ormond Street Institute of Child Health, London, United Kingdom

Introduction: Cystic fibrosis (CF) is an autosomal recessive disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The dysfunctional CFTR protein leads to production of a thick sticky mucus, chronic bacterial infection and loss of lung function. Modulator drugs provide effective therapies for some CFTR genotypes but approximately 20% of patients remain untreatable. **Aim**: We propose to develop in vitro transcribed CFTR messenger RNA (mRNA) as a novel therapy, which has the potential to work in any CF patient. The aim of this study was to first optimize the structural elements of in vitro transcribed mRNA, including the design of the 5' and 3' untranslated regions (UTRs), regulating luciferase activity in epithelial cell models, as well as a novel non-viral nanoparticle formulation for their delivery. **Methods**: We have developed an epithelial cell targeting nanoparticle to improve mRNA delivery comprising targeting peptides and lipids. Different lipids and peptides were compared. The in vitro transcription protocol was optimized with the CleanCap cap analog added during in vitro transcription to allow co-transcriptional capping. Unmodified uridine-5'-triphosphates (UTPs) were replaced by modified UTPs, N1-methylpseudouridine-5'-triphosphate. Double-stranded RNA contaminants were removed by cellulose-base purification. The mRNA structural elements were optimized with various 5' Untranslated regions (UTR) and 3'UTRs cloned into the DNA template including known UTRs and novel UTRs from transcripts highly expressed in the airway epithelium. **Results**: The optimal design for transfection of 16HBE epithelial cells and CF bronchial epithelial cells comprised the cationic lipid, ditetradecyl trimethyl ammonium propane (DTDTMA), the neutral helper lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and the epithelial targeting peptide Y (K_{16}GACYGLPHKFCG) with luciferase mRNA which led to high transfection efficiency. According to the optimised in vitro transcription protocol, up to 280 micrograms (μg) of RNA could be produced from 1 μg of DNA. The luciferase mRNA produced transfected 16HBE and CFBE cells with similar efficiency to commercially available luciferase mRNA. The presence of certain UTRs from genes that are expected to give high epithelial expression further increased the translation efficiency. Compared to mRNA with no UTR, messenger RNAs with either individual UTR (5' or 3' UTR) or double UTRs (5' and 3'UTR) showed improved translation efficiency. Both human-derived and viral UTRs were involved in this study. While the optimal UTRs were cell type-dependent, mRNA with optimal UTRs showed a two-fold increase in translation efficiency over mRNA with no UTR in both 16HBE and CFBE. **Conclusions**: The in vitro transcription process and mRNA structural elements were optimized to produce synthetic mRNA with high translation efficiency, stability, and low inflammatory potential. Novel non-viral nanoparticle formulation was developed specific for mRNA delivery to epithelial cells. Taken together, this work provides the basis for the development of targeted gene therapy for cystic fibrosis that precisely delivers mRNA to epithelial cells in the lung.

767. A Platform Approach for Producing Engineered Extracellular Vesicles

Lisa Prendergast[1], Ali Navaei[2], Davide Zocco[3], Devarshi Kapadia[4]

[1]Licensing, Lonza Cell and Gene, Slough, United Kingdom,[2]R&D, Lonza Cell and Gene, Rockville, MD,[3]R&D, Lonza Cell and Gene, Sienna, Italy,[4]Commercial Development, Lonza Cell and Gene, Houston, TX

Extracellular Vesicles (EVs), including exosomes, are emerging as promising natural nano-scale platforms for delivering nucleic acids, proteins, and small molecules. However, precision EV engineering has been challenging to date. Such technologies have also been largely inaccessible for a broader community due to technical complexities. Here, we discuss materials and methods to express biomolecules on EVs. Protein scaffolds were developed and clinically proven to effectively load therapeutic proteins on the surface or lumen of EVs. Chemical linkers were developed to link nucleic acids or small molecules to the EV membrane while preserving their bioactivity and potency. Case studies and clinical data for each EV loading and linking technology will be presented. Technologies described here will be available to Pharma, Biotech and Academic communities under a Research License for broader access. They will also be available to therapeutics companies via contract development and manufacturing services for producing their engineered EVs.

768. Implementing Custom Particle Swarm-Differential Evolution Optimization (PSODE) for GFP Plasmid DNA Transfection in Jurkat and Primary T Cells

Devin Reslow, Andrew Hallinan, John Zhao, Ta-Chun Hang

Kytopen, Cambridge, MA

Optimization of transfection processes is critical to successful cellular drug product manufacturing. A key requirement for efficient transfection is utilization of an optimized buffer. Transfection buffer formulation is a convoluted problem that can benefit from sophisticated optimization methods. After defining a base formulation via traditional experimental design principles (DOE), we implemented a custom, hybrid particle swarm optimization and differential evolution (PSODE) algorithm with archived memory was used to simultaneously screen and optimize efficacy of numerous buffer additives and transfection parameters. PSODE was implemented with adaptive social and cognitive parameters to balance design exploration and exploitation of information. Mutation and crossover of designs further leveraged the information gained from successful formulations. In this approach, additives were evaluated to improve delivery of plasmid DNA. Traditional DOE methods with 22 unique additives (i.e., 3^k factorial design) results in a high-dimension space requiring >300E6 experimental iterations to characterize. PSODE identified optimal formulations after 5 iterations when combined with multi-objective optimization (MOO) of transfection efficiency and

cell viability. PSODE formulations demonstrated >75% transfection efficiency with >80% viability in Jurkats, a 3-fold increase over base formulation alone. PSODE formulations were subsequently tested in primary T cells, demonstrating >75% viability and >50% transfection efficiency. Transfections with various Flowfect® profiles identified synergistic buffer and energy effects, indicating further optimization is possible. PSODE implementation in Jurkats and T cells demonstrate the valuable coaction of Flowfect® technology and algorithm-based study designs, yielding high-throughput discovery capabilities to screen, optimize, and characterize transfection processes and parameters. In tandem, algorithmic buffer optimization methods can benefit cell and gene therapy applications, enhancing future development of specialized, optimal transfection processes for other cell type and payload combinations.

769. Improving Endotoxin Determination in Complex Gene Delivery Systems: Challenges and Novel Workflow Modifications

Andrew Dix, Alexia Bachir, Sneh Ringwala
Thermo Fisher Scientific, Eugene, OR

Endotoxin contamination, a common problem with recombinant proteins and nucleic acids used in cell and gene therapy, can trigger toxic shock, activation of nonspecific immune responses, or premature cell death. While tools for endotoxin quantification are not new, the techniques used for gene delivery are becoming increasingly complex and can interfere with the assay readout. Specifically, lipid nanoparticles (LNPs) and adeno-associated virus particles (AAVs) are known to scatter light, which is the reporting mechanism for commonly used chromogenic Limulus amebocyte lysate (LAL) assays. These challenges lead to inaccurate endotoxin measurements, which can have negative impacts on downstream experiments and applications. Here, we utilize spike-testing of a control endotoxin amount into the AAV and LNP samples to demonstrate challenges with the current state of endotoxin testing. Additionally, we detail best practices on how different endotoxin quantification assays can be used to determine contamination levels in these sample types. Overall, the highlighted data seeks to enable proper endotoxin determination and preserve the integrity of downstream applications.

Wednesday Posters: Cancer - Immunotherapy and Cancer Vaccines

770. Tumor Vaccination by HAdV5 and HC-HAdV5 and Its Targeting *In Vitro* and *In Vivo* to APCs by Engineered Modular Adapters

Fabian Weiss[1], Jonas Kolibius[2], Patrick Freitag[1], Andreas Plückthun[1]
[1]University Zürich, Zürich, Switzerland,[2]University of Zürich, Zürich, Switzerland

The clinical significance of B- and T-cell vaccines is undisputed, with adenoviral vectors demonstrating encouraging outcomes. Nevertheless, the commonly used human adenoviral vector serotype C5 (HAdV5) exhibits limited transduction efficiency in dendritic cells (DC), necessitating therefore very high viral loads. Thereby, gene-based therapy in APCs (antigen presenting cells) remains challenging. In response, we have developed a versatile platform that employs modular retargeting adapters to enhance transduction into specific cell types, including traditionally challenging host cells. By rational design we demonstrate successful targeting of HAdV5 to human and murine DCs through DC-SIGN and CD11c receptors. Our *in vivo* characterization highlights the potential for improved and specific transduction of DCs in draining lymph nodes without off-targeting to B-, T-cell, nor macrophages. Moreover, a tumor vaccination efficacy study showcases the advantageous co-expression of T cell stimulatory cytokines (IL-2v or IL-21) locally in lymph nodes alongside a potent tumor antigen. Lymph node directed gene-therapy at significantly reduced viral loads circumvents potential systemic toxicity of stimulating payloads. Our proposed low dosage DC-targeted therapy not only offers a cost-effective solution for patients but also minimizes potential adenoviral related side-effects. The robust immunogenicity of HC-HAdV5, with its expansive encoding capacity up to 37 kbp DNA, opens up exciting possibilities for future therapeutic combination strategies. This approach has significant potential to advance the field of combination immunotherapy by enhancing the precision of safe and low-cost DC based gene-therapies

771. The Strategy to Reduce Vector Doses by AAV-Extracellular Vesicles Composite

Ester Molina[1], Marcos Tejero Ambrosio[1], Ozgun Firat Duzenli[2], Hisae Kuoch[1], Colin Caine[1], Karina Krotova[1], Michael Paulaitis[3], George Aslanidi[4]
[1]The Hormel Institute, Austin, MN,[2]The Hormel Institute, University of Minnesota, Austin, MN,[3]Johns Hopkins University School of Medicine, Baltimore, MD,[4]University of Minnesota, Austin, MN

We recently showed advantages of using capsid and payload modified AAV vectors to generate protective antigen specific immune response in animal model of melanoma. However, one of the biggest challenges remaining in the field is the production of high yield and highly efficient vaccines. Extracellular vesicles or EVs have been used for years as a delivery platform since they are natural nanoparticles skilled in delivering their content between cells and tissues. Several studies have proposed the encapsulation of AAV vectors during common packaging process inside EVs to increase therapeutical performance. The advantage of such production method will be a one-step purification process. The aim of this work is to increase the efficacy of treatment using AAV-based cancer vaccine inside EVs. First, we comprehensively characterized EV-AAV vesicles by their concentration, average diameter, size distribution, vesicle morphology and EV biomarkers, as well as by AAV titers. Second, we showed that mice vaccinated with EV-opt-OVA antigen showed an increased in cellular immune response measured by flow cytometry of OVA-specific $CD8^+$ T cell, compared with those animals injected with opt-OVA at the same vector dose. We also showed that the dose EV-opt-OVA can be reduced by 100 to 1000 times to achieve the similar number of IFN-γ produced OVA tetramers positive $CD8^+$ T cell compare with opt-AAV-OVA. Additionally, mice injected with B16F10-OVA melanoma cells were protected against metastatic nodules in the lungs with 100-times less dose of EV-opt-OVA compared with opt-OVA alone, 10^8 vg/mice vs.

10^{10} vg/mice correspondingly. Finally, we used a therapeutic tumor self-antigen to assess the effectivity of EV-AAV vaccine with a less immunogenic payload. We observed the effective dose for EV-opt-TRP-1 is 10-times less compared with opt-TRP-1 alone, 10^{9} vg/mice vs. 10^{10} vg/mice correspondingly, to generate $CD8^{+}$ T cell and protect animals from B16F10 metastatic cells spreading in the lungs. The results of our study showed that EV associated AAVs can elicit a stronger immune response in mice compare with AAV vaccine alone. More importantly, EV-AAV vaccine have demonstrated a stronger protection against metastatic melanoma tumors in mice lungs at much lower doses used in previous experiments. Additionally, we have observed that the reduction of the vaccine dose is dependent on the antigen carried by AAV which underlines necessity to identify appropriate unmodified or neo-antigen(s) for effective cancer treatment. The natural ability of exosomes to deliver their cargo to cells particularly immune cells, together with AAV vectors highly efficient transduction of the antigen they carry is an attractive combination that has potential to be explored for the future cancer vaccine design.

772. Interplay Between Local Irradiation and AAV-Based Gene Delivery for Cancer Therapy

Juan Dubrot[1,2], Sonia Marco[1], Nerea Juanarena[1], Beatriz Honorato[1], Myriam Fernandez[1], Jesus Prieto[1]

[1]Cima-Universidad de Navarra, Pamplona, Spain,[2]Cancer Center Clinica Universidad de Navarra (CCUN), Pamplona, Spain

Radiotherapy (RT) is a pilar of cancer treatment. More than half of all cancer patients receive one modality of RT throughout the course of the disease. Originally, the rationale for its use relied on the direct cytotoxic effects that ionizing radiation (IR) causes in tumor cells. It is now recognized the importance of the host immune system in mediating and modulating the therapeutic effects of IR. Radiation causes immunogenic cell death that release tumor-associated antigens and damage-associated molecules which activate the innate immune system, triggering cytokine production and immune cell recruitment. These inflammatory signals are able to induce effective anti-tumor responses. At the same time IR initiates immune-resistance mechanisms such as IR-induced expression of PD-L1 in cancer and stromal cells and increased influx of immune-inhibitory populations. Thus, combination of RT with local delivery of cytokines that enhance antitumor responses or revert immunosuppressive mechanisms represents a reasonable strategy. Adeno-associated virus (AAV) vectors have been engineered for its use as gene delivery vectors has been used for the treatment of a variety of human diseases. Yet, the value of AAV vectors for cancer treatment has not been explored in depth. To interrogate the therapeutic potential of the combination of RT and AAV-based gene therapy, we designed an AAV vector encoding interleukin-12 (IL12), a multifunctional cytokine that plays a pivotal role in the immune system particularly in the context of anti-tumor responses. Due to its strong and diverse immunomodulatory properties, systemic expression of IL-12 can lead to toxicity with fatal consequences. Therefore, we designed an AAV vector that contains two critical safety elements. First, we have included micro-RNA 122 binding sites within the vector genome to prevent expression in the liver. Second, IR is known to induce type I IFN production through cGAS-STING pathway. Thus, we have designed an expression cassette encoding IL12 under the control of an IFN-inducible promoter that restricts its expression to inflamed tissue. These regulatory elements restrain the expression of IL12 within the tumor microenvironment (TME). We found that RT could be used not only as an initiator of the immune response and activator of IL-12 expression, but also as facilitator of AAV-mediated gene transfer, as we found that IR enhanced transduction of murine and human cancer cell lines by AAV vectors both *in vitro* and *in vivo*. In a constitutive promoter system, irradiated tumor cells infected with an AAV-GFP expressed higher levels of the transgene and retained GFP expression for longer periods of time. Similarly, expression of IL12 was detected at higher levels in irradiated in a dose-dependent manner. Importantly, expression of IL12 was restricted to the TME and no significant levels of the cytokine were observed in serum of treated animals. Local RT and intratumor injection of AAV-IL12 cooperate to induce a strong remodeling of the TME with remarkable presence of proinflammatory and effector populations. Consequently, the combination led to complete tumor rejections in several preclinical cancer models. Notably, elimination of the treated tumor is accompanied by a robust abscopal effect in distal untreated tumors. Overall, our work demonstrates the potential of an unexplored strategy for cancer treatment. We have designed a therapeutic combination that exploits the multiple benefits of local IR and safely delivers IL12 to unleash the full capacity of the immune system, which has been demonstrated to be the best way to obtain durable antitumor responses. The combination of RT and AAV-based immunotherapy represents a novel strategy that can impact a large number of cancer patients and improve the clinical results of current therapies.

773. Adenovirus Mediated HSP70 Combined with GM-CSF Enhances HPV E6/E7 Antigen Immunotherapy for HPV Infection Related Diseases

Philip C. Sin[1], Dingyue Tang[2], Wei Jiang[2], Xia Wu[2], Xiao Xiao[2]

[1]Belief BioMed Limited, Hong Kong, Hong Kong,[2]Belief BioMed Limited, Shanghai, China

Introduction: In recent years, with the application of various immune activating factors, immunotherapy has developed rapidly, especially in the field of tumor treatment. HPV infection related diseases are common diseases that pose a serious threat to women's health, persistent HPV infection is the main cause of most cervical cancers1, with E6 and E7 being the main oncogenic genes of HPV2. The prevention and treatment of E6 and E7 targets have always been a focus of research on HPV infection related diseases3. In order to enhance the efficacy of therapeutic vaccines targeting E6/E7 for HPV related diseases, we used a combination of HSP70 from mycobacterium tuberculosis4 and human GM-CSF, and used adenovirus vectors to mediate the expression of GM-CSF and HPV antigens fused with HSP70 to enhance the efficacy of immunotherapy. The research results indicated that the combination of HSP70 and GM-CSF could induce stronger T cell immunity and had a good synergistic effect. On this basis, after multiple efficacy studies and toxicological safety evaluations, the BBM-C101 adenovirus product was finally developed, demonstrating good preventive and therapeutic advantages for HPV positive diseases. **Methods and Materials:** Constructed relevant plasmids using adenovirus vectors, and obtained different adenovirus

products through transfection, cell culture, and purification. Evaluated the efficacy of different carriers through immunological experiments such as ELISPOT detection of splenic CTL lymphocyte production. After completing vector screening, further design optimization was carried out on the expression box and vector, and large-scale cell fermentation production and purification were carried out to obtain the BBM-C101 adenovirus drug product. Its effectiveness and safety were further verified in tumor monitoring experiments on TC-1 cell line tumor bearing mice and monkey toxicology experiments. (The final genetic basis of BBM-C101 product is Promoter-HPV E6/E7-HSP70-Promoter-GM-CSF). **Conclusions:** BBM-C101 is a novel immunotherapy drug for HPV infected diseases. It delivered the HPV 16/18 antigens, E6 and E7, into tissues and tumors by adenovirus 5, and activated T cell and B cell immuno-response of anti-HPV 16/18.BBM-C101 could inhibit the HPV 16/18 induced tumors, and established long-term immune memory.BBM-C101 specifically targeted the HPV 16/18 infected tissues and tumors, but it did not affect the tissues and tumors that were infected by other types of HPVDuring toxicology study, BBM-C101 did not induce the evident toxicity in rodent and non-human primatesIn clinic, BBM-C101 could clear away the cells that was infected with HPV 16/18, and turned subjects negative for HPV.In clinic, BBM-C101 did not induce SAE. Fever was common AE after BBM-C101 infusion.



Figure 1. BBM-C101 Gene Expression Cassette

Summary of treated cervical HPV 16/18 positive patients

Subject profile	Turning negative time	age	Type of HPV chronic infection	Number of injections	HPV16/18 (-/+)
Participant#1	3w	53ys	HPV16 positive	3	HPV16 -
Participant#2	20w	34ys	HPV16/51/68 positive	4	HPV16 -
Participant#3	6w	41ys	HPV18 positive	4	HPV18 -
Participant#4	12w	39ys	HPV16 positive	4	HPV16 -
Participant#5	10w	40ys	HPV16 positive &CIN1	2	HPV16 -

774. INTASYL PH-762: PD-1 Intratumoral Immunotherapy

Melissa Maxwell, Dingxue Yan, Andrew Boone, Brianna Rivest, Linda Mahoney, Mary Spellman

Phio Pharmaceuticals, Marlborough, MA

PH-762 is an INTASYL compound designed to precisely silence PD-1 mRNA. INTASYL™ is a patented, self-delivering RNAi technology platform designed to impart specific properties to small interfering RNAs, providing extremely efficient delivery to a broad range of cell types and tissues without the need for specialized formulations or drug delivery systems. INTASYL's innovative approach features a simplified chemical composition, potentially reducing toxicity and enhancing tolerability and efficacy. PH-762 can be administered via intratumoral (IT) injection, which is anticipated to diminish the systemic side effects associated with conventional systemic antibody treatments. PH-762's unique structural and chemical modifications ensure an optimized cell and tissue uptake profile with IT administration. In vitro investigations have demonstrated efficient uptake of PH-762 by human T cells, silencing of PD-1 mRNA and subsequent protein reduction. Preclinical studies have shown that IT injections of murine targeted PH-762 (mPH-762) can silence the PD-1 mRNA in the T cells within the tumor and increase the secretion of IFN-γ. mPH-762 was well tolerated at the maximum administered dose and treatment with mPH-762 provided robust and statistically significant inhibition of tumor growth. Toxicokinetic studies conducted in marmoset monkeys demonstrated that PH-762, when administered intravenously at doses of up to 147 mg/kg, is well-tolerated. Importantly, no detectable cytokine-release associated cytokines were found in the plasma of treated monkeys at this dose. PH-762 is currently being investigated in an open-label clinical study (NCT 06014086) to evaluate the safety and tolerability of neoadjuvant use of IT PH-762 in cutaneous squamous cell carcinoma (cSCC), melanoma, or Merkel cell carcinoma, to determine the pharmacokinetic profile of PH-762 after IT injection, to observe pathologic and immunologic tumor responses, and to determine the recommended dose for continued clinical development. Tumor changes are evaluated per iRECIST criteria and pathological response. Immunological response in tumor tissue and blood samples are assessed as secondary endpoints. This clinical study will establish the basis for continued clinical development of PH-762.

776. Sequence-Optimized Circular RNA E7 Cancer Vaccine Mediates Robust Protective T-Cell Immunity and Profound HPV+ Mouse Tumor Regression

Edo Kon[1], Xiaoliang Shi[2], Zhihao Chen[2], Fei Lin[2], Yang He[2], Xiaowen Zou[2], Jian Ye[2], Mengyao Zheng[2], Siqi Li[2], Roi Mashiach[1], Emma Pan[2], Weiyi Zhang[2]

[1]RiboX Therapeutics (Israel) Ltd, Ness Ziona, Israel,[2]RiboX Therapeutics, Shanghai, China

Human Papilloma Virus (HPV)-related cancers are estimated to account for 5% of all global cancer cases worldwide, including the majority of cervical and oropharyngeal cancers. "High-Risk" HPV infections can cause cancer by disrupting basic cellular mechanisms responsible for proliferation and communication. The E6 and E7 viral oncoproteins are consistently expressed in tumors and are required for both their initiation and maintenance. Therefore, vaccines targeting E6 and E7 as antigens represent a promising option for HPV positive tumors and have been explored using different modalities including mRNA, and have advanced to various stages of clinical studies. Different from mRNA, the covalently-closed structure of circular RNA (circRNA) prevents the degradation by exoribonucleases, therefore, leading to intrinsic stability. In addition, the translation of circRNA is in a cap-independent mechanism where the internal ribosome entry site (IRES) drives protein translation. To explore the potential of circRNA as a therapeutic vaccine, circular E7 RNA (circ-E7) constructs have been generated and evaluated for their *in vitro* antigen expression, *in vivo* immunogenicity and anti-tumor efficacy. Growing evidence shows that durable and specific cellular immunity against HPV antigens including E7 is key to achieving long-term disease control. Therefore, the optimization of circ-E7 was centered around tuning the expression

level of E7 to establish an understanding of the relationship between antigen expression and anti-tumor immunity driven by circular RNA vaccines. A library of 406 IRESs has been screened and 10 selected IRESs were constructed with an E7 antigen coding sequence. The circE7 RNAs were formulated in proprietary lipid nanoparticles (LNP) and the antigen expression was determined by the percentage of E7 positive cells, measured by FACS. To measure the antigen-specific immunity, the 10 formulated circ-E7 vaccines were intramuscularly administered into C57BL/6 mice, weekly, for 3 weeks. The percentage of E7 specific $CD8^+$ T cells in PBMCs was determined and ranged from 3.14% to 24.48%. In addition, the E7-specific $CD8^+$ T cells entered various stages of differentiation, including robustly expanded $KLRG1^+CD127^-$ short-lived effector cells (SLEC) and a pronounced portion of $KLRG1^-CD127^+$ memory precursor cell (MPEC) phenotypes. This indicates that a specific and durable immune response is elicited by the circ-E7 vaccine. The two circ-E7 vaccines that elicited the most antigen-specific responses were further evaluated in a subcutaneous E7 expressing TC-1 tumor bearing model. When tumors reached a mean volume of ~23 mm^3, mice were treated intramuscularly with 3 weekly doses. The immunization led to a strong infiltration of $CD8^+$ T cell into the tumor, with over 80% being specific for E7. Furthermore, this led to a profound tumor regression in 9/12 and 12/12 of circ-E7 vaccines treated animals. In conclusion, we demonstrate that optimized circRNA has the potential to be harnessed as therapeutic cancer vaccines. The circRNA vaccines elicit a robust antigen-specific immunogenicity with a significant portion of memory cytotoxic $CD8^+$ T-cells and a pronounced anti-tumor efficacy.

777. Identification of a Novel Antigen Presenting Cell Targeted Lipid Nanoparticle for mRNA Cancer Vaccine Therapies

Xi Zhu[1], Chenxi Hou[1], Jingwei Sun[2], Zhao Xu[2], Hong Chen[1], Shin-Shay Tian[1], Yarong Liu[2], Xiaoping Zhao[1]

[1]Shanghai Vitalgen Biopharma, Shanghai, China,[2]Shanghai Grit Biotechnology Co Ltd., Shanghai, China

Current lipid nanoparticle (LNP) based mRNA vaccines have been reported to induce side effects in the liver, such as reversible hepatic damages and T cell-mediated hepatitis, which are likely caused by the strong expression of the mRNA payload in hepatocytes following intravenous (IV) or intramuscular (IM) administration. Therefore, developing an antigen presenting cell (APC)-specific LNP formulation could be a promising strategy for next-generation mRNA cancer vaccines with better efficacy and fewer side effects. Targeted delivery and expression of mRNA-encoding tumor antigens in APCs could facilitate the processing and presentation of antigens to the major histocompatibility complex (MHC), particularly MHC class I, thereby achieving higher activation of specific CD8+ T cells and better anti-tumor effects. Through iterative *in vitro* and *in vivo* screening, we have identified an APC-specific targeting LNP (APC-LNP) formulation. In *in vitro* transfection studies, when human peripheral blood mononuclear cells (PBMCs) were exposed to APC-LNP carrying GFP mRNA at a concentration of 1 μg/mL, 45% of DCs and 73% of monocytes were positive, while T cells and NK cells had positive rates of less than 0.1%. To evaluate in vivo tropism, C57BL/6 mice were intravenously injected with APC-LNP loaded with tdTomato mRNA. Flow cytometry analysis of different cell subsets in various organs 24 hours later showed high and specific tdTomato expression in DCs and macrophages. The positivity for DCs and macrophages ranged from 13% to 47% in the blood and spleen, and 5% to 43% in the liver, lung, and lymph nodes. Importantly, no expression was detected in the parenchymal cells of the liver and lung. Next, immune activation and antigen-specific responses were evaluated by intravenous administration of APC-LNP encapsulating mRNA expressing a tumor-associated antigen, TRP-1 (APC-LNP-TRP-1) in C57BL/6 mice. Significant upregulation of activation markers on DC and macrophages, T cells, B cells, and NK cells was observed in the spleen, lymph nodes, liver and peripheral blood following vaccination. In contrast, no obvious immune activation was observed with empty APC-LNP. Finally, the anti-tumor efficacy was evaluated in a B16F10 melanoma model. Compared to the control group, the APC-LNP-TRP-1 group showed significantly enhanced anti-tumor effects with specific T cell response confirmed by TRP-1 tetramer staining. The above data collectively indicate that our novel APC-LNP can achieve specific delivery and robust mRNA expression in APC cells in different tissues, leading to specific immune activation and potent anti-tumor effects, demonstrating the immense potential of this novel APC-LNP for the development of next-generation mRNA cancer vaccines.

778. Refining Chimeric Antigen Receptors via Barcoded Protein Domain Combination Pooled Screening

Xavier Rios, Osmay Pardias, Marc A. Morales, Pradyot Bhattacharya, Yibin Chen, Linjie Guo, Chunchao Zhang, Erica J. Di Pierro, Gengwen Tian, Gabriel A. Barragan, Pavel Sumazin, Leonid S. Metelitsa

Department of Pediatrics, Baylor College of Medicine, Houston, TX

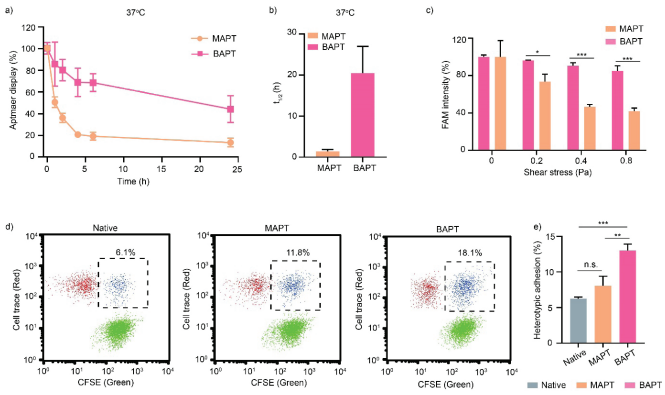
Chimeric antigen receptor (CAR)-T cells represent a promising frontier in cancer immunotherapy. However, the current process for developing new CAR constructs is time-consuming and inefficient. To address this challenge and expedite the evaluation and comparison of full-length CAR designs, we have devised a novel cloning strategy. This strategy involves the sequential assembly of individual CAR domains using blunt ligation, with each domain being assigned a unique DNA barcode. Applying this method, we successfully generated 360 CAR constructs that specifically target clinically validated tumor antigens CD19 and GD2. By quantifying changes in barcode frequencies through next-generation sequencing, we characterize CARs that best mediate proliferation and expansion of transduced T cells. The screening revealed a crucial role for the hinge domain in CAR functionality, with CD8a and IgG4 hinges having opposite effects in the surface expression, cytokine production and antitumor activity in CD19- versus GD2-based CARs. Importantly, we discovered two novel CD19-CAR architectures containing the IgG4 hinge domain that mediate superior in vivo antitumor activity compared to the construct used in Kymriah, an FDA approved therapy. This novel screening approach represents a major advance in CAR engineering, enabling accelerated development of cell-based cancer immunotherapies.

779. Biomimetic Surface Coating of Living Cells for Enhanced Aptamer Display and Cell Recognition

Xuelin Wang, Yong Wang

Department of Biomedical Engineering, The Pennsylvania State University, State College, PA

Introduction: Cell surface engineering with aptamers holds great promise for various applications ranging from cancer immunotherapy to tissue engineering. Traditional methods for aptamer modification primarily rely on lipid insertion. However, this approach suffers from the low stability of aptamers. The purpose of this study is to develop a surface coating for enhanced aptamer display. In this study, NK cells were used as a model. The surface of NK cells was functionalized with a DNA-alginate template. Aptamers were immobilized within this template via molecular interactions. Aptamer stability and cell recognition were evaluated via numerous assays such as flow cytometry. **Methods:** Cholesterol-conjugated DNA initiator (DI) was incorporated into the cell membrane through the incubation of cells in the DI solution. DNA monomer (DM) 1 and DM2-alginate were added to form DNA-alginate template via hybridization chain reaction (HCR). The template was sequentially treated with biotinylated-polylysine (PLL-bio), biotinylated-alginate (Alg-bio), streptavidin, and biotinylated-aptamer (aptamer-bio) to form an aptamer-functionalized polymer matrix (BAPT). Cholesterol-conjugated complementary DNA (cDNA) was inserted into the cell membrane for displaying monovalent aptamers (MAPT, used as control). For stability assessment, the coated cells were incubated at 37°C with or without shear stress. At predetermined timepoints, DNA molecules remaining on cell surface were labeled with FAM-cDNA and the fluorescence intensity of the cells was measured using flow cytometry. For the binding analysis, a model aptamer (i.e., Sgc8c) was used to treat CCRF cells. The CellTrace Far Red prelabeled coated cells were mixed with CFSE prelabeled target cancer (CCRF) cells to examine cell recognition. The binding percentage was measured via flow cytometry. **Results:** The kinetic analysis revealed that the half-life ($t_{1/2}$) of the aptamer on MAPT-engineered cells was approximately 1.4 h, while the $t_{1/2}$ of the aptamer on BAPT-coated cells was approximately 20 h. When coated cells were subjected to shear flow, over 85% of the aptamers remained attached to the cell surface at a shear stress of 0.8 Pa. These data demonstrate that BAPT exhibited a high stability on the cell surface. For binding assay, at a NK to CCRF ratio of 1 to 3, the percentages of NK-CCRF complexes were 6.1%, 11.8%, and 18.1% in the native NK group, the MAPT-engineered NK group, and the BAPT-coated NK group, respectively. This difference can be attributed to the increased density of aptamers on the surface of BAPT-coated cells. The binding of BAPT-coated cells to CCRF cells remained strong 4 h after the coating formation. The percentages of NK-CCRF complexes in the native NK, MAPT-engineered NK, and BAPT-coated NK groups were 6.2%, 8.3%, and 13.1% respectively. These results reveal that BAPT-coated cells exhibited a high ability to bind target cells. **Conclusions:** The biomimetic coating can enhance the efficiency and stability of aptamer display on the cell surface. The coated cells can acquire an enhanced ability to recognize target cells. This work has demonstrated a promising method for the display of ligands on the cell surface for targeted cell-cell recognition.



Wednesday Posters: Cancer - Targeted Gene and Cell Therapy

780. Development and Preclinical Characterization of an Engineered T-Cell Therapy Targeting PRAME-Expressing Solid Tumors

George Reyner Pope[1], Rebecca Burton[1], Sebastian Laycock[1], Chloe Tayler[1], Terri Cornforth[1], Karolina Wikiert[1], Guy Wiedermann[1], Vera Kmonickova[1], Agata Patel[1], Philip Simister[1], Rachel Abbott[1], Sarah Loftus[1], Sofie Van Rossom[2], Robert Connacher[3], Marco Della Peruta[1], Phillip Debnam[1], Joseph Sanderson[1], Barbara Tavano[1]

[1]Adaptimmune, Abingdon, United Kingdom,[2]CellCarta, Antwerp, Belgium,[3]Adaptimmune, Philadelphia, PA

Introduction PRAME (PReferentially expressed Antigen in MElanoma) is a cancer testis antigen (CTA) expressed in many cancers, including solid tumors and leukemia, but has restricted expression in somatic tissues, making it an attractive target for immunotherapy.[1] Here, we describe the development of ADP-600, a preclinical autologous T-cell product targeting PRAME for use in the treatment of solid tumors. ADP-600 comprises T cells transduced to express an affinity-enhanced, human leukocyte antigen (HLA)restricted T-cell receptor (TCR) engineered to specifically target a PRAME antigen (peptide: SLLQHLIGL) presented on the surface of tumor cells by HLA-A*02:01. ***Methods and Results*** In agreement with the literature,[1, 2] our own target validation studies confirmed that protein expression of PRAME is limited outside of the reproductive tissues but is upregulated with high frequency in a range of solid tumors, including melanoma, endometrial cancer, ovarian cancer, synovial sarcoma, lung squamous cell carcinoma, and triple-negative breast cancer. To develop a target-specific TCR, two parental TCR candidates were selected for engineering to enhance their affinity for the PRAME antigen. From the resulting panel of mutants generated, the ADP-600 TCR was ultimately selected for preclinical testing based on its potency and specificity. ADP-600 showed good potency toward a panel of PRAME-positive cell lines, as determined by interferon gamma (IFNγ)

and granzyme B ELISA, with cytokine levels broadly in keeping with the level of expression of PRAME protein in HLA-A*02-positive target cells. ADP-600 also displayed cytotoxic behavior towards primary melanoma tumor, non-small cell lung carcinoma patient-derived xenograft tissue, and PRAME-positive 3D spheroids, both in terms of IFNγ and granzyme B production and cytotoxicity. ADP-600 has demonstrated good target specificity in a variety of in vitro assays, including screening of a panel of primary cell lines (selected for their optimal coverage of the protein coding transcriptome), an alloscreen in which potential cross-reactivity toward off-target HLA allelic variants was assessed, and an evaluation of the cytokine response following addition of ADP-600 to whole blood. ***Conclusions*** PRAME is an attractive target for adoptive T-cell therapy using affinity-enhanced TCRs. We have generated and characterized an affinity-enhanced TCR and subjected it to our extensive preclinical testing package, where it demonstrated potency toward PRAME-positive tumor targets while maintaining stringent specificity. These data describing ADP-600 may be used to support an investigational new drug application to enable the use of this product in clinical trials. 1. Al-Khadairi G, Decock J. *Cancers (Basel)* 2019;11:984. 2. Gonzaga Almeida L et al. *Nucleic Acids Res* 2009; 37:D816.

781. Engineering Chimeric Antigen Receptor T Cells Against Protease-Activated Receptor 1 for Treating Pancreatic Cancer

Chao Lien Liu[1,2], Hao-Chien Hung[3], Ming-Huei Fan[1], Daniel Wang[1], Carol H. Miao[4], Pong Su[1]

[1]School of Medical Laboratory Science and Biotechnology, Taipei Medical University, New Taipei, Taiwan,[2]Ph.D. Program in Medical Biotechnology, Taipei Medical University, New Taipei, Taiwan,[3]Department of General Surgery, Chang-Gung Memorial Hospital, Linkou, Taoyuan, Taiwan,[4]Seattle Children's Research Institute, Seattle, WA

Background Pancreatic ductal adenocarcinoma (PDAC) is a devastating malignancy with a 5-year survival rate of 6% following a diagnosis, and novel therapeutic modalities are needed. Protease-activated receptor 1 (PAR1) is abundantly overexpressed by both tumor cells and multiple stroma cell subsets in the tumor microenvironment (TME), thereby offering a suitable immunotherapy target. **Methods** A chimeric antigen receptor (CAR) strategy was applied to target PAR1 using a human anti-PAR1 scFv antibody fused to the transmembrane region with two co-stimulatory intracellular signaling domains of cluster of differentiation 28 (CD28) and CD137 (4-1BB), added to CD3ζ in tandem. **Results** The engineered PAR1CAR-T cells eliminated PAR1 overexpression and transforming growth factor (TGF)-β-mediated PAR1-upregulated cancer cells by approximately 80% in vitro. The adoptive transfer of PAR1CAR-T cells was persistently enhanced and induced the specific regression of established MIA PaCa-2 cancer cells by > 80% in xenograft models. Accordingly, proinflammatory cytokines/chemokines increased in CAR-T-cell-treated mouse sera, whereas Ki67 expression in tumors decreased. Furthermore, the targeted elimination of PAR1-expressing tumors reduced matrix metalloproteinase 1 (MMP1) levels, suggesting that the blocking of the PAR1/MMP1 pathway constitutes a new therapeutic option for PDAC treatment. **Conclusions** Third-generation PAR1CAR-T cells have antitumor activity in the TME, providing innovative CAR-T-cell immunotherapy against PDAC.

782. Development of a Microscopy-Based IF/ISH Assay for Detection of ICT Cells in Patients Treated with AB-1015

Nickolas Attanasio, Xinyan Tang, Hongruo Yun, Lionel Berthoin, Muamong Y., Amanda Fearon, Ryan Fong, Samuel A. Williams, W. Nicholas Haining, Stephen Santoro

Arsenal Biosciences, South San Francisco, CA

AB-1015 is an autologous, genetically modified integrated circuit T (ICT) cell product in clinical development for the treatment of ovarian cancer. AB-1015 integrates an "AND" logic gate designed to limit off-tumor toxicity through the requirement for dual tumor antigen recognition. It is also engineered with a dual shRNA-miR to resist TME suppression and improve ICT cell function and persistence. The AB-1015 logic gate consists of a synthetic priming receptor that, upon engagement of its target ALPG/P (alkaline phosphatase germ-line/placental), induces expression of an anti-mesothelin (MSLN) CAR that mediates tumor-cell killing. Methods to enable the detection of CAR and PrimeR in primary tumor specimens would be useful to examine the performance of various features of the AB-1015 logic gate. In order to visually identify AB-1015 ICT cells in human tissue independent of host immune cells, and understand the presence of ICTs in patient tumor samples, a custom multiplex immunofluorescence (mIF) assay was developed using the RNAScope(™) platform. The mIF panel was developed to detect CD3 protein by immunofluorescence (IF) and mRNA transcripts for both the PrimeR and CAR portion of the circuit by in situ hybridization (ISH), enabling detection of AB-1015 engineered T cells and PrimeR/CAR expression status. PrimeR and CAR probe specificity was established with positive and negative control cell lines under brightfield IHC as single stains, and additionally in multiplexed IF format. Further, probeset utility was demonstrated in a xenograft model to verify the usability of the mIF assay in solid tissues, incorporating an additional marker in order to visualize tumor cells. To date, the assay has been used to detect ICTs in one on-trial tumor biopsy sample of an AB-1015-101 patient. In conclusion, a mIF panel capable of detecting AB-1015 product in tissue was developed, with the intention of deploying this assay in future on-treatment samples for the AB-1015-101 clinical trial in ovarian cancer.

783. Optimizing the Generation of Antigen-Specific T Cells for the Treatment of High-Risk Malignancies

Samantha Murphy[1], Samuel Couto[1], Gage Paul[1], Monica Gerber[2], Kajal Chaudhry[1], Nethaji Muniraj[1], Christopher A. Lazarski[1], Sandeep Kumar Srivastava[1], Conrad Russell Y. Cruz[1], Catherine M. Bollard[1], Amy Hont[1]

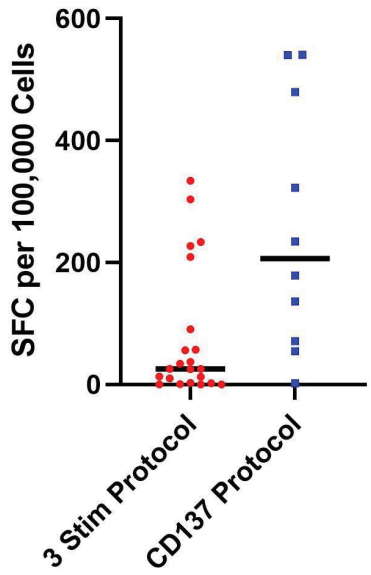
[1]Children's National Hospital, Washington, DC,[2]Virginia Tech Carilion School of Medicine, Roanoke, VA

Background: Tumor-associated antigen-specific cytotoxic T cells (TAA-Ts) have therapeutic potential in high-risk cancer patients. However, products can be difficult to manufacture, with widely variable specificity and phenotype that can be donor- and technician-dependent. Standard protocols for TAA-T generation use peptide-pulsed antigen presenting cells (APCs) to stimulate peripheral blood mononuclear

cells (PBMCs) in multiple cycles, a time consuming and inconsistent method. This has been previously addressed through isolation and culture of cells positive for CD137, a surface receptor expressed on T cells after antigen-induced activation, with a CD4+ predominant product. Utilizing CD137 selection with standard cytokines and APCs may optimize the generation of antigen-specific T cells for clinical use. **Objective:** This study aims to characterize TAA-Ts generated via an optimized protocol utilizing CD137 selection in terms of specificity and phenotype, comparing them to those generated by the standard protocol. **Methods:** Peripheral blood mononuclear cells (PBMCs) were pulsed with PRAME and anti-CD28 antibody and incubated for 16-24 hours. CD137+ cells were isolated via magnetic bead selection and plated with irradiated CD137- cells in the presence of cytokines. Cells were restimulated on day 11 with PRAME-pulsed PHA blasts and cytokines and harvested on day 18 for characterization. Specificity was measured via IFNy ELISpot and phenotype via flow cytometry. Final results for specificity were compared to those generated using a traditional 3 stim protocol. **Results:** Using this CD137 protocol, we generated 10 distinct products. Phenotypically, products generated using the CD137 protocol were CD3/CD45RO enriched with variable proportions of CD4+ (3.2% - 88.1%) and CD8+ (3.1% - 91.8%) T cells. CD137 products demonstrated higher median PRAME specificity (Figure 1) compared to the standard protocol (206.5 vs 25.5 spot forming cells above background (SFCs); $p<0.05$). 90% of CD137 products were specific (≥20 SFCs above background) and 70% exceptionally specific (≥100 SFCs above background) as compared to only 57% and 24% of standard products. Preliminary data suggests that these specific CD137 products demonstrate cytolytic activity against pediatric tumor lines in vitro. **Conclusion:** Overall, this modified CD137 selection protocol enhanced antigen specificity and phenotype in shorter culture time compared to the standard method. Future directions will further investigate cytotoxicity, alternative targets, and genetic modifications.

Figure 1

Median Prame Specificity Between Protocols



784. Metabolic Conditioning During *Ex Vivo* Stimulation of Naïve Tumor Antigen-Specific CD8 T Cells

Joseph Choy, Sydney Shannon, Si-Sim Kang, Shuyi Li, Kenneth Adusei, Shaelyn R. Marx, Hai-Quan Mao, Jonathan Schneck

Johns Hopkins University, Baltimore, MD

Enrichment and expansion of antigen-specific cytotoxic T lymphocytes from a naïve population offers the ability to generate allogeneic cell therapies capable of treating malignancies. This approach is amenable to the current state-of-art cell therapies, as it can be produced without genetic manipulation, mobilizes a broad polyclonal response, and is derived from functional, non-exhausted naïve precursors. Further, seminal work in computational prediction of neoepitopes and antigen immunogenicity has made this strategy increasingly feasible for a broader range of cancers. However, current expansion processes to generate a relevant number of antigen-specific cells results an effector-dominant phenotype, which are suboptimal compared to less-differentiated counterparts. To address this, we characterized the effect of the broad glutamine antagonist 6-diazo-5-oxo-L- norleucine (DON) during ex vivo expansion of tumor-antigen specific CD8 T cells from healthy C57BL/6J mice. We conjugated recombinant MHC-Ig fusion proteins and co-stimulatory agonist antibodies onto magnetic nanoparticles as artificial antigen-presenting cells (aAPCs) to facilitate enrichment and expansion of cells in a dendritic cell-free manner. aAPCs were used to expand for cells recognizing H-2Kb:SIY or H-2Kb:Trp2, antigens from the B16SIY melanoma line. Our results show that the proportion of antigen-specific T cells is not significantly

reduced in the presence of DON, despite glutaminolysis being an integral part of metabolic reprogramming during T cell activation. In addition, DON skews these cells towards a central-memory like CD44+CD62L+ phenotype, while reducing the expression of PD-1 and TIM3. These results were accompanied by a shift towards a naïve CD25-CD44-CD62L+ signature in the non-cognate population. Next, we probed the activity of DON to influence phenotype and cytokine production upon restimulation by performing dose-response experiments and found that initial dosing in the first 24 h of activation is essential, while sustained exposure is beneficial but non- critical. We further mechanistically studied early activation events across a range of antigen affinities and doses by using H-2Kb-restricted SIINFEKL altered peptide ligands SIIQFEKL and SIIVFEKL conjugated on aAPCs to stimulate OT-I transgenic TCR cells. We observed that DON restrains TCR downregulation and CD25 expression, while upregulating CD45RB levels in high- affinity primed cells but does not demonstrate differences in intermediate or low affinity primed cells. Overall, we demonstrate that expansion of rare, tumor-antigen specific CD8 T cells from healthy, naïve T cells with DON generates a cell product with a memory-like, less exhausted phenotype. Early application of DON during high-affinity priming show the most potent effect compared to vehicle controls, where the pharmacological agent leads to changes in the kinetics of activation events. We show that phenotypical conditional with DON is homologous in an endogenous-like settings beyond previous TCR transgenic or CAR studies. This study highlights that metabolic conditioning of T cells is best suited to take advantage of the broad glutamine antagonism to inform development of future ACT products.

785. Loss of Heterozygosity is Low for Specific HLA Alleles in Cancer Patients with Driver Mutations

Christian Roy[1], Tomasz Sewastianik[1], Patrick Halvey[1], Meagan Montesion[2], Aastha Jindal[1], Markus Vallaster[1], Hubert Lam[1], Sean Garrity[1], Gary Shapiro[1], Christopher A. Klebanoff[3], Loic Vincent[1], Greg Opiteck[1], Dirk Nagorsen[1]

[1]Affini-T Therapeutics, Watertown, MA,[2]Foundation Medicine, Cambridge, MA,[3]Memorial Sloan Kettering Cancer Center, New York, NY

Immunotherapies have revolutionized cancer treatment, yet the limited predictability of response and/or resistance mechanisms continues to present novel therapeutic developmental challenges, especially for solid tumors. Many of these use a sensitive and specific T cell receptor (TCR)-HLA:peptide complex interaction as a mechanism of action and can be impacted by HLA loss of heterozygosity (LOH). Allele-agnostic HLA LOH analyses assessing loss of any MHC class I allele are relevant for HLA-agnostic approaches such as immune checkpoint inhibitors; however, studies in the context of specific HLA alleles, mutations, and indications remain limited. Such context-specific HLA LOH frequencies are especially relevant to adoptive T cell therapies engineered with TCRs and bispecific TCR T cell engagers targeting neoantigen peptides derived from oncogenic driver mutations, such as those of KRAS and TP53, which are presented by a specific HLA allele. Thus, existing allele-agnostic analyses may overestimate the frequency and relevance of HLA LOH for allele-specific modalities. A comprehensive genomic profiling dataset consisting of 78,418 cancer patient samples (Montesion M. et al., PMID: 33127846), germline heterozygous for one or more HLA class I locus, was analyzed for HLA LOH in the context of frequent driver mutations (KRAS G12C, G12D, G12V and TP53 R175H), corresponding HLA alleles (HLA-A*02:01, HLA-A*03:01, and HLA-A*11:01) and within specific tumor subtypes. Allele-agnostic HLA LOH was observed in 16.6% of all samples and varied between indications and HLA alleles (Montesion 2021). However, allele-specific loss of HLAs A*02:01, A*03:01, and A*11:01 across all samples was much lower than allele-agnostic loss (6.3%, 6.6%, and 6.3%, respectively). Although, a trend for increased allele-specific HLA LOH was observed when analyzed by driver mutation, the frequency remained low. Specifically, across all cases, A*02:01, A*03:01, and A*11:01 allele-specific LOH ranged between 5.9%-9.7% in relation to KRAS G12C, G12D, and G12V compared to 5.9%-6.2% for wild-type (WT). Moreover, it did not achieve statistical significance for any HLA-KRAS mutation pairs in colorectal (CRC), non-small cell lung (NSCLC), and pancreatic (PDAC) cancers, except for HLA-A*11:01 and KRAS G12C in NSCLC, which was lower in mutated cases (5.3% for G12C vs 10.1% for WT KRAS, p=0.045). Both allele-agnostic and A*02:01-specific HLA LOH in TP53 WT samples were lower than average which may be related to the role of TP53 in genomic stability. The TP53 R175H mutation was associated with limited increase in HLA-A*02:01 LOH (from 4.4% for WT to 8.1% for TP53 R175H) and varied between indications, e.g. no significant increase was observed in R175H-positive breast cancer and NSCLC cases contrary to CRC and PDAC. Based on this real-world dataset, over 90% of cases retain the TCR-targeted allele and, therefore, preserve the molecular complex necessary for HLA/driver mutation-based therapies. Notably, the analyzed database is enriched for advanced cancer patients. Further studies are warranted to test the hypothesis whether tumors in earlier lines of treatment have an even lower rate of HLA LOH and if correlations with treatments change longitudinally.

786. Pooled *In Vivo* Screening of Hundreds of T Cell Therapy Designs Using Spatial Multi-Omics Identifies Novel Designs with Superior TME Performance

Cassandra Kontur, Gundula Povysil, Megha Sah, Mariya London, Nan Chen, Annie Dyatel, Stephen Pu, David Phizicky, Xinchen Wang

Waypoint Bio, New York, NY

Background: Adoptive cell therapies such as CAR T have been curative for many liquid tumors, yet this success has struggled to translate to solid tumors due to the challenges posed by the solid tumor microenvironment (TME). Pooled CAR screens (e.g. CAR-POOLING) have recently emerged as a powerful approach for testing many designs for improving CAR T cell performance. However, these screens are typically restricted to univariate readouts (e.g. abundance) and cannot measure the cell-cell and cell-TME interactions that are critical for CAR T success in solid tumors. Here, we present a platform for performing pooled screens in vivo using spatial biology, facilitating simultaneous measurement of cell autonomous phenotypes, cell-cell interactions, and cell-environment interactions for hundreds of distinct cell therapy designs in a single mouse. **Methods:** We developed a custom spatial biology workflow to determine the identity of specific CAR constructs, armorings, or combinations in specific individual cells

from a microscope image ("design deconvolution"). We combined this design deconvolution with simultaneous multiplexed spatial biology phenotyping in the same cells & samples, followed by computer vision interpretation to quantify each design's performance across functional categories important for efficacy in solid tumors such as: tumor infiltration, tumor cell killing, proliferation, response to antigen heterogeneity & escape, exhaustion, persistence, and activity in immunosuppressive tumor microenvironments. In multiple mouse cancer models, we quantified the performance of hundreds of different cell therapy designs. **Results:** Using our pooled screens, we identified designs with superior performance in solid tumors across one or more key functional categories (e.g. infiltration, activity in immunosuppressive TMEs, etc.). We also mapped the landscape of cell therapy design principles by comparing dozens of existing designs derived from the literature head-to-head in the same animal, and we compared these benchmarked designs to novel cell therapies designed to improve CAR T function. We used these data to identify common rules that govern the design-phenotype relationship for CAR Ts in solid tumors and to identify CAR T designs with desired properties in solid tumors. **Conclusions:** Using a pooled in vivo screening platform, we discovered cell therapies that outperform existing designs across a variety of clinically-relevant metrics. Our spatial multi-omics measurements are critical for identifying designs that can effectively traffic to, infiltrate, and function in the solid tumor microenvironment. We are now using our pooled in vivo screens to identify highly efficacious designs in multiple solid tumor models.

787. Fueling Immunotherapy- Selective Targeting of Glutamine Metabolism to Enhance Metabolically Engineered iPSC-NK Cell Resilience Against Solid Tumors

Shambhavi S. Borde

Purdue University, West Lafayette, IN

Natural Killer (NK) cells, characterized by their intrinsic recognition and targeting capabilities against aberrant cells, have emerged as promising candidates for safe and effective cancer immunotherapies. Essential to the effector responses of NK cells are nutrients such as glucose and glutamine. Notably, glutamine and its metabolic enzyme, glutaminase, play pivotal roles in NK cell proliferation, expansion, and anti-tumor activity. However, the heightened glutamine consumption by tumor cells results in a glutamine-deprived tumor microenvironment (TME), challenging the survival of NK cells. In this study, we investigated the dependency of NK cells on glutamine in supporting effector functions and found its uptake indispensable for NK cell activity. The application of glutaminase inhibitors significantly diminished the proliferation and anti-tumor activity of NK cells. Inhibitors targeting glutamine metabolism exhibited a considerable impact on NK cell viability, anti-tumor cytotoxicity, activation marker expression, and mitochondrial functions. Notably, the blockade of glutamine transporters, including SLC1A5, significantly impaired NK cell cytotoxicity, proliferation, and mitochondrial functions against glioblastoma (GBM) targets, emphasizing the critical role of glutamine in sustaining NK cell processes within the TME. To address the need for targeted inhibition of glutamine in GBM while sustaining NK cell metabolic activity, we are genetically engineering NK cells to selectively enhance their metabolic resilience, by enabling normal function even in the absence of glutamine. Critical to immunotherapeutic responses is the availability of functionally mature and metabolically active NK cells. Previous findings underscore the clinical utility of induced pluripotent stem cell-derived NK cells (iPSC-NK cells) within the context of glutamine modulation. To do so, we have used genetic modifications aiming to elevate the expression of glutamine transporters (SLC1A5) on iPSC-NK cells, amplifying their glutamine uptake within the TME. The functions of modified NK cells are assessed using phenotypic and cytotoxicity analyses, as well as metabolic assays for mitochondrial activity. Transcriptional gene expression analysis further elucidates the impact of targeting glutamine metabolism on the genotype of NK cells in the TME. The goal is to generate metabolically resilient and functionally robust engineered iPSC-NK cells, thus enabling targeted and selective depletion of the metabolic behavior of cancer cells.

788. TGFβ-Conditioned K-NK Cells Exhibit Strong Cytotoxicity and Tumor Organoid Infiltration Against a Wide Range of Tumor Types

Anna Oja, Ieva Cesonyte, Robert Igarashi, Andre Kunert

Oncology Research, Sanofi, Amsterdam, Netherlands

NK cells possess multiple attributes that make them suitable as an "off-the-shelf" product for adoptive cell therapy (ACT) in oncology. The use of ACT for solid tumor settings face challenges due to the immunosuppressive tumor microenvironment, in particular impeded migratory capacity to the tumor site. TGFβ-conditioned (TGFβ-c) K-NK cells, NK cells expanded using plasma membrane particles (PM21) from K562-mb21-41BBL cell line in the presence of TGFβ, display features of resistance to TGFβ-mediated immunosuppression. To increase our understanding of the functionality of TGFβ-c K-NK cells in a physiologically relevant model, TGFβ-c K-NK cells were co-cultured with 60 unique, patient-derived tumor organoids from a range of cancer settings. TGFβ-c K-NK cells show dose-dependent killing of tumor organoids and production of cytotoxic molecules that can manipulate the TME and other immune cells, such as dendritic cells and T cells. Interestingly, while the degree of tumor organoid infiltration differs between the unique organoids, TGFβ-c K-NK cells display infiltration into all organoids. Furthermore, there was a strong positive correlation between the level of organoid infiltration and organoid killing. To discern functional properties between tumor infiltrating and non-infiltrating TGFβ-c K-NK cells, we developed tumor cell line spheroid models, enabling us to isolate these population of cells and differentially characterize them. In conclusion, TGFβ-c K-NK cells display several advantageous features making them a promising platform for ACT against a range of solid tumor malignancies.

789. Efficacy of B7H3-Targeting CAR T Cells Derived from Novel Monoclonal Antibodies in Solid Tumor Treatment

Thananya Inthanachai[1], Chatikorn Boonkrai[2], Tanapati Phakham[2], Trairak Pisitkun[2], Norihiro Watanabe[3], Nattiya Hirankarn[4,5], Koramit Suppipat[5,6,7], Supannikar Tawinwung[5,6,8]

[1]Medical Microbiology, Interdisciplinary and International Program, Graduate School, Chulalongkorn University, Bangkok, Thailand,[2]Center of Excellence in Systems Biology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand,[3]Baylor College of Medicine, Houston, TX,[4]Cancer Immunotherapy Excellence Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand,[5]Thailand Hub of Talents in Cancer Immunotherapy (TTCI), Bangkok, Thailand,[6]Cellular Immunotherapy Research Unit, Chulalongkorn University, Bangkok, Thailand,[7]Department of Research Affairs, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand,[8]Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

B7 homolog 3 (B7H3) is overexpressed across multiple cancers with restricted expression on normal tissues, rendering it a potential target for CAR T therapy. To redirect T cells response against common solid cancers, we developed B7H3-targeting CAR T cells from monoclonal antibodies (mAbs) generated in-house. These mAbs were produced by immunizing mice with human B7H3 protein. Their binding kinetics were determined using surface plasmon resonance. Among the candidates, mAb clones 12G5 (K_D 6.01×10^{-9}), 11G5 (K_D 1.28×10^{-9}) and A2H4 (K_D 9.23×10^{-10}) were confirmed to be specific to recombinant human B7H3 protein and B7H3$^+$ cancer cell lines. Subsequently, we constructed second-generation CARs using these clones. These B7H3. CAR Ts exhibited dose-dependent cytotoxicity and cytokine response specifically against B7H3$^+$ targets (MDA-MB-231 and LN229). We next investigated their anti-tumor activity in a spheroid co-culture assay to simulate the tumor microenvironment. On day 7 of co-culture, B7H3 CAR T cells from all clones effectively eliminated the tumor spheroids. Notably, the clone 12G5, possessing the lowest affinity, demonstrated rapid tumor engagement. To verify the anti-tumor activity of our B7H3 CAR T cells *in vivo*, NOD-*scid* IL2Rg^{null} (NSG) mice subcutaneously engrafted with LN229 were treated with the CAR T cells. All B7H3 CAR T cells effectively eliminated and controlled the primary tumors, with a durable response observed in B7H3 CAR T clone 11G5 which has the intermediate affinity among the three clones. In summary, our B7H3 CAR T cells derived from novel mAbs demonstrated efficacy against B7H3$^+$ tumors. The study underscores the importance of selecting an scFv with optimal affinity for achieving durable anti-tumor efficacy.

790. Driving a Novel Platform of Cancer-Activated Genetic Expression for the Diagnosis and Treatment of Non-Small Cell Lung Cancer (NSCLC) Using Highly Selective and Active Transcription Factors

Elizabeth Stroebele, Dariusz Wodziak, Wendy Zhang, Ishan Podar, Ajda Rojc, Chloe Xia, Maggie Louie, David Suhy

Earli, Inc, Redwood City, CA

Early detection of cancer is an important for increased survival, quality of life and reduced healthcare costs. Earli is developing a highly sensitive, orthogonal approach that uses a genetic construct to usurp dysregulated molecular pathways and actively force cancer cells to drive the expression of a detectable 'synthetic' biomarker for the purposes of diagnostic imaging or expression of an engineered therapeutic protein. Identification of transcription factors (TFs) with abnormally elevated activity in cancer is crucial when developing a cancer-activated expression platform. Evaluating TF function in a genome-wide scale is challenging, especially since TF activity is not solely reflected by just its RNA expression or even by protein abundance, but also its post-translational modification including phosphorylation. Multi-omics Factor Analysis (MOFA) was performed on publicly available datasets from Clinical Proteomic Tumor Analysis Consortium (CPTAC) which includes RNA-seq based transcriptional profiling, MS based protein abundance and phosphorylation from 211 paired tumor and normal adjacent NSCLC samples. We identified 34 dysregulated TFs in NSCLC that displayed high expression levels in at least two of the three -omics platforms compared to normal adjacent tissues, and six exhibited high levels across all three. Expanding our TF analysis to benign lung lesions, we generated independent datasets for RNA-seq and protein abundance. Our findings revealed a distinct genetic signature that can be leveraged for the differentiation of malignant from benign and normal adjacent tissue. This suggests that differential activation of transcription could be used for selective gene expression in the context of diagnostics or therapeutics. Using the dysregulated TFs identified by the MOFA, we designed more than 2,000 unique synthetic promoter sequences containing arrays of homotypic or heterotypic TF binding sites with diverse motifs and spacing. We use a Massively Parallel Reporter Assay (MPRA) high-throughput pooled screening (HTS) method to assess cancer-specific activity in 8 NSCLC cell lines. We identified 30 strong candidate sequences for validation and found that 28 of 30 induced the expected expression, with the majority of response elements (n=20) showing expression 5-10X higher than background. Many of the tested synthetic promoters drove expression levels significantly higher than the activity of the constitutively active CAG promoter. In order to ensure sensitivity across a broad genetic background, the synthetic promoter sequences were empirically tested against 4 dozen primary tumor models - collected from individual NSCLC patients - that have been adapted for growth in vitro. Thus each primary tumor model exhibits a unique genetic background and a distinct dysregulated gene expression program responsible for the cancer development. Data from the first generation construct, with expression driven by sequences from the endogenous Survivin promoter, was only able to detect 5 of the 40 of the human primary tumors or a sensitivity of 13% when using 10% of CAG expression levels as a benchmark for expression. Conversely, one of our 4th

generation cancer-activated synthetic promoters produced signal against the same CAG benchmark, in 42 of the 43 samples or nearly 98% sensitivity, remarkable considering the different genetic background of each sample. Continued work towards clinical development is under way to use these synthetically engineered promoters in conjunction with a wide variety of expressed proteins and proprietary non-viral LNPs for application ofthe platform for therapeutics and diagnostic programs. These novel combinations being evaluated in a number of in vivo models including murine lung tumor models and larger animal models such as oncopigs.

791. Applification of CRISPR/Cas9-Mediated Gene Editing and Patient-Derived Organoids for Target Validation

Jinkyu Park

Gradiant Bioconvergence, Seoul, Korea, Republic of

Non-small cell lung cancer (NSCLC) is a major contributor to cancer-related deaths globally, with adenocarcinoma being the most prevalent subtype. Somatic mutations in the epidermal growth factor receptor (EGFR) are found in a significant proportion of lung adenocarcinomas. EGFR tyrosine kinase inhibitors (TKIs) have shown improved patient outcomes compared to conventional chemotherapy. However, tumors inevitably acquire resistance to TKIs, underscoring the imperative for a more profound comprehension of resistance mechanisms and the exploration of advanced combination therapies. In this study, we aimed to identify targets associated with drug resistance using patient-derived organoids (PDOs) and gene editing. We generated two sets of PDOs from pleural effusion at distinct time points for a patient with NSCLC. The divergent drug-responsive outcomes observed in these two organoids sets were consistent with the clinically observed resistance to the TKIs. Moreover, we identified genes correlated with drug resistance via transcriptome analysis. To validate their functional relevance, we conducted CRISPR/Cas9-mediated knockouts. Our results revealed a notable reduction in the IC_{50}, signifying a discernible alteration in drug response. Through these outcomes, the combined approach of PDOs and CRISPR/Cas9 not only elucidates molecular mechanisms underlying drug resistance but also provides valuable insights for developing targeted therapeutics.

792. Genomic Mechanisms of Interspecies Differences in Stromal Invasibility: Implications for Placental Invasion and Cancer Dissemination Using Gene-Edited Fibroblasts

Ashkan Novin, Yasir Suhail, Kshitiz .

UConn Health Center, Farmington, CT

Cellular invasion into surrounding tissues is crucial in both pregnancy and cancer metastasis. Placental invasion varies widely among eutherian mammals, from deep invasion in humans to noninvasiveness in cattle, and correlates with cancer malignancy. We previously demonstrated that fibroblasts from different species exhibit varying resistance to invasion by trophoblasts and cancer cells. In this study, we identified elements responsible for interspecies differences in invasibility and the key transcription factors governing proinvasibility and antiinvasibility genes in stromal cells. Using CRISPR-Cas9 gene editing, we found a set of genes significantly influences the invasibility of endometrial and skin fibroblasts. These findings provide insights into genomic mechanisms explaining species-specific disparities in stromal invasibility, with potential applications in regulating placental invasion, wound healing, and cancer dissemination. Additionally, we established a direct link between human vulnerability to placental invasion and the low resistance of human fibroblasts to cancer cell invasion, shedding light on therapeutic targets for modulating these processes.

793. NRF2 Functional Domains Dictate the Tolerance of Exon Skipping as a Result of CRISPR-Directed Gene Editing on the Phenotypic Outcomes

Natalia Rivera-Torres[1], John Anthony Rogowskyj[1], Guadalaupe Aguilar[2], Pawel Bialk[1], Eric B. Kmiec[1]

[1]Gene Editing Institute, ChristianaCare, Newark, DE,[2]Medical and Molecular Sciences, University of Delaware, Newark, DE

We are advancing a novel strategy for the treatment of solid tumors by employing CRISPR-directed gene editing to reduce levels of standard of care required to halt or reverse the progression of tumor growth. Our laboratory has been advancing a CRISPR-directed gene-editing platform technology as an augmentative therapeutic strategy for squamous cell carcinoma of the lung with follow-on applications to esophageal and head and neck cancer. Treatment for patients with these cancers remains diverse and can become ineffective over time due to the side effects of chemotherapy and the development of resistance for which both remain a serious barrier to successful treatment. Our genetic target is NRF2 a global transcription factor that regulates genes involved in response to cellular stress. It has been linked to chemo-resistance since its expression is regularly elevated in response to chemotherapy. Our objective has been to use CRISPR-directed gene editing to disable NRF2 functions so that the cancer cell becomes sensitive to the standard of care therapy. We have demonstrated that partial and complete knockout of this gene in human lung cancer cells increases chemo-sensitivity in cell systems and demonstrably retards tumor growth in animal models. We have successfully extended the approach to esophageal (KYSE 410) and head and neck (FADU) cancer cell lines. We have also identified two parameters that are crucial points to take into consideration when designing gene editing therapeutics. (1) The effect of exon skipping as a result of CRISPR-directed gene editing and (2) the effect of targeting specific functional domains of a gene. These parameters affect the phenotypic outcome that is intended to be achieved as a therapeutic effect. When these parameters are coupled and paired properly, we achieve an optimal gain of chemosensitivity and reduced cell proliferation. We have once again demonstrated that this gene is a viable target for gene editing to be used in therapeutic critical endpoints in solid tumors including halting the progression of tumor growth, inducing tumor cell death, and reducing tumor size to facilitate surgical resection. It is possible that gene editing and chemotherapy will act synergistically to reduce tumor growth. This treatment strategy could be used effectively in patients with locally advanced or even metastatic cancer or patients who have developed resistance to chemotherapy.

794. Living Anticancer *Clostridium Sporogenes* Colonizes *In Vitro* 3D Necrotic Spheroids

Merve Yildiz[1], Jella van de Laak[1], Alexandra M. Mowday[2], Ludwig J. Dubois[1], Jan Theys[3]

[1]Precision Medicine, University Maastricht, Maastricht, Netherlands,[2]Maastrict University, Maastricht, Netherlands,[3]University Maastricht, Maastricht, Netherlands

Background: Most solid cancers contain areas of necrosis resulting from chronic hypoxic injury. These high-risk tumour characteristics are associated with poor prognosis due to increased risk of metastasis and resistance to chemotherapy and radiotherapy. Strikingly, this necrotic environment also provides an opportunity for targeted therapy, such as our alternative anti-cancer approach that uses the harmless anaerobic bacteria *Clostridium sporogenes (C. sporogenes)*. Upon injection as spores, this species selectively penetrates necrotic areas and germinates into metabolically active vegetative bacteria that can produce anticancer effects inside the tumour. We have developed innovative genetic tools to further enhance these effects by "arming" *C. sporogenes* with therapeutic genes such as immune checkpoint inhibitors and cytokines. Novel therapeutic strains can be generated effectively in a short period of time. Therefore, it would be essential to evaluate and validate them in robust systems prior to in vivo studies because considering the Three Rs principle of animal research, the use of animals should be reduced or replaced if possible. It would thereby be beneficial to develop an in vitro validation method that can mimic tumour necrosis as an intermediate step.Methods: The Lewis Lung Carcinoma (LLC) cell line was used to generate 3D spheroids. Spheroids were first investigated on the development of necrotic fractions. Next, 5,000 colony-forming unit per millilitre (CFU/mL) of spores were added on different days to determine and optimize the experimental time frame of our spheroid model. The CFU/mL in spheroids was determined for each time point. Microscopic images were taken every day to follow-up spheroid growth. Furthermore, a *C. sporogenes* strain expressing the fluorescent reporter gene unaG was investigated as a live imaging tool in the spheroids.Results: LLC spheroids developed a necrotic core on day 5 after seeding. When spores were added on this day, *C. sporogenes* colonized the spheroids for 24, 48, and 72 hours, with 48 and 72 hours showing the highest number of bacteria inside the spheroids. Colonized spheroids started to disintegrate around 96 hours due to the antitumour effects of *C. sporogenes*. The fluorescent unaG strain was live imaged in the spheroid model. Fluorescent intensity positively correlated to CFU/mL and fluorescent signal was diminished after spheroid disintegration.Conclusion: These promising results show the high potential of 3D *in vitro* models that can potentially serve as important intermediate validation step before embarking in vivo studies. These models will be applied for testing therapeutic efficacy and antibiotic sensitivity of our *C. sporogenes* strains.

796. Enhancing the Efficacy of Retroviral Replicating Vectors-Mediated Suicide Gene Therapy in a Peritoneal Dissemination Cancer Model through Mesenchymal Stem Cell-Mediated Delivery

Shuji Kubo[1], Yuya Takeuchi[1,2], Nao Ogawa[1,2], Noriyuki Kasahara[3], Emiko Sonoda-Fukuda[1]

[1]Laboratory of Molecular and Genetic Therapeutics, Institute for Advanced Medical Sciences, Hyogo Medical University, Nishinomiya, Japan,[2]Departments of Biomedical Chemistry, School of Science and Technology, Kwansei Gakuin University, Sanda, Japan,[3]Departments of Neurological Surgery and Radiation Oncology, University of California, San Francisco, San Francisco, CA

Retroviral replicating vectors (RRVs) have demonstrated significant promise in cancer gene therapy through preclinical and clinical studies. Since retrovirus-based vectors are produced only a low titer in vitro, direct injection into local tumor tissue has been the preferred route of administration. However, inaccessible or metastatic tumors make this in vivo delivery feasible, necessitating the development of alternative methods for efficient delivery of RRV to tumor sites in vivo. To address this challenge, we are developing a novel technology that utilizes mesenchymal stem cells (MSCs) as carrier cells for RRV transport, leveraging their migratory and accumulative capabilities in cancer tissues. Previous investigations have established the ability of MSCs to acquire an RRV infection, to produce RRVs, to migrate toward tumor cells, and to transmit RRVs to tumor cells. In this study, we assessed the transmission efficacy and antitumor effect of RRV in peritoneally disseminated tumor models. First, RRV transmission efficacy was evaluated by in vivo imaging using a peritoneally disseminated tumor model in which nude mice were inoculated with humanMSTO-211H mesothelioma cells. In the non-ascites model, higher RRV transmission to tumors was observed in the RRV-infected MSC group (MSC/RRV group) than in the RRV-alone group (RRV group). On the other hand, in the ascites model prepared using human serum, the RRV group showed no RRV transmission to tumors, whereas the MSC/RRV group showed high RRV tumor-transmitting ability. Next, we evaluated the anti-tumor effect in the peritoneal disseminated tumor model and found that both the RRV and MSC/RRV groups showed a high anti-tumor effect in the non-ascites model. On the other hand, in the ascites model, the MSC/RRV group showed significantly higher efficacy than the RRV group in terms of tumor growth and survival. These findings suggest that using MSCs as carrier cells, RRV-mediated suicide gene therapy may be a novel treatment for peritoneally disseminated cancer, especially in cases of ascites.

Wednesday Posters: Challenges to Immunological Responses to Therapeutic Interventions

797. The Impact of Pre-Existing Antibodies and Complement on Immunological Safety of Adenoviral Vector Gene Therapy

Svetlana Atasheva, Jia Yao, Dmitry M. Shayakhmetov
Emory University, Atlanta, GA

Immunological safety is a one of the major concerns with the systemic intravascular administration of viral vectors in gene therapy. Targeting specific organs and cell types while surpassing uptake by the liver requires infusion of substantial doses of viral vectors. Introducing large quantities of viral vectors into the bloodstream poses the risk of eliciting severe and potentially fatal cytokine responses in some patients. The unpredictability and severity of systemic inflammatory response, coupled with a limited understanding of the molecular mechanisms behind the innate immune and cytokine responses to viral vectors, present significant obstacles to the development of new treatments, pushing the field to consider non-viral delivery methods. Nevertheless, viruses have evolved mechanisms allowing for a highly efficient gene transfer and can further be rationally engineered to provide targeted, safe, and efficient platforms for therapeutic gene delivery to patients. We hypothesize that in response to systemic administration of viral vectors, pre-existing neutralizing and non-neutralizing antibodies, together with the complement system, influence the activation of phagocytic myeloid cells, potentially inducing a hyperactivation state and the release of pro-inflammatory cytokines. To test this hypothesis, we immunized groups of mice with human adenovirus serotypes from species B (HAdv-B3, HAdv-B11), C (HAdv-C2, HAdv-C6), and D (HAdv-D28), followed by intravenous administration of HAdv-C5-based adenovirus vectors. Whereas the antibodies generated in these mice did not have any direct neutralizing activity to HAdv-C5, the amounts of inflammatory cytokines released into the blood 6 hours post HAdv-C5 vector administration varied dramatically between mouse groups pre-immunized with different virus serotypes. Further, mice immunized with adenoviruses phylogenetically distant from HAdv-C5 produced antibodies that could bind to HAdv-C5 viral particles and trigger C3 complement fixation. We termed these antibodies cryptic non-neutralizing opsonizing antibodies (CON-Abs). Importantly, CON-Abs-mediated C3 opsonization of HAdv-C5 viral particles potentiated inflammatory cytokine release from human PBMCs in vitro. While immunization with HAdv-C5 led to the generation of virus-specific neutralizing immunity in both wild-type and C3-KO mice, the intensity of the cytokine storm following HAdv-C5 vector administration was markedly greater in C3-KO mice, indicating that complement C3 in vivo plays a role in suppressing the inflammatory response. We found that in the absence of C3 the virus is accumulating in larger quantities in CD169 positive macrophages in the spleen, suggesting that C3 opsonization targets adenovirus-immune complexes away from immune phagocytic cells to non-responding cell compartments. Our findings underscore the unanticipated role of CON-Abs in potentiating the severity of cytokine storm responses and highlight the need for novel approaches to pre-screen patients for pre-existing humoral factors that could amplify inflammatory reactions to therapeutic vectors.Conflict of interest: DSH is a founder and a shareholder of AdCure Bio, which develops adenovirus technologies for therapeutic use.

798. Immune Silent Circular RNA (oRNA™) in TLR7 and TLR8 Expressing Transdifferentiated Monocytes

Blake Cohen[1], Yessica Wiryawan[1], Dovile Strimaityte[1], Justin Lee[1], Tobi Wuraola[1], Corey Ciullo[1], Subiksha Parthasarathy[1], Subiksha Parthasarathy[1], Ian Langer[1], Tracy DiMezzo[1], Genevive Bondaryk[1], Dylan Hoots[1], R. Alexander Wesselhoeft[2], Isin Dalkilic[1], Allen Horhota[1], Nelson Chau[1], Robert Mabry[1], Thomas M. Barnes[1], Thomas Lee[1]
[1]Orna Therapeutics, Watertown, MA,[2]Gene and Cell Therapy Institute, Mass General Brigham, Cambridge, MA

Orna Therapeutics is developing circular RNA (oRNA™) therapies offering advantages in magnitude and duration of protein expression over linear mRNA. Orna's proprietary autocatalytic circularization procedure and purification processes provide a simple, yet robust, method to generate immunoquiescent oRNA. Nucleobase modification is traditionally required to mask impurity driven immunostimulatory effects of delivered mRNA therapies. Absent modification, mRNA impurities trigger multiple immunostimulatory pathways (RIG-I, MDA5, TLR3, TLR7, TLR8), resulting in cytokine secretion and tolerability limitations. Orna's circular RNA architecture, coupled with optimized production r processes render oRNA immunoquiescent, abrogating the need for modified nucleotides. In vitro transcription and purification processes significantly impact RNA therapeutic immunostimulatory impurity levels. Optimized oRNA processing conditions generating highly translated with greatly reducing the immunostimulation properties. Efficient co-transcriptional circularization, elimination of capping reagents and steps, coupled with optimized purification processes, generate highly pure circular RNA. Demonstrating oRNA purity and translation to the human setting have been limited by available assays. Traditional ELISA, cell-based assays (A549), and in vivo murine models do not entirely recapitulate the suite of RNA sensing immune receptors reported to play key roles in human responses to mRNA , specifically TLR-7 and TLR-8. These TLR receptors sense single strand RNA degradation products in the endolysosome and are essential for detecting conserved RNAs from pathogens. Immortalized myeloid cell lines are widely utilized to investigate human innate immune pathways. Transdifferentiated BlaER1 cells have been shown to closely resemble primary human monocytes based on their PRR signaling pathways, from TLRs to inflammasomes. By inducing the myeloid transcription factor C/EBPα supplemented with IL-3 and M-CSF, the Blaer1 B cell precursors can transdifferentiate into a non-proliferative monocytic state. Phenotypic changes are observed where CD19, a canonical B cell marker, is reduced, while CD11b and CD14 expression upregulate during the differentiation process. Furthermore, transdifferentiated monocytic BlaER1s respond to agonist that stimulate TLR7, TLR8, and the RLR pathway, supporting the expression & functionality of their RNA-sensing PRRs. Functional TLR7 & TLR8 were validated by the R848 agonist in transdifferentiated BlaER1s cells which were reactogenic to unmodified mRNA controls. High levels of IL-6 and TNFα secretion,

cytokines associated with RNA-induced reactogenicity were observed. Utilizing the standard production process for oRNAs (Process A), we observed stepwise reduction of reactogenic responses at 200 to 50 ng of oRNA transfection per 100k BlaER1s, but undetectable levels at 10 ng. Cytokine responses observed with the standard oRNA process were not comparable to that of unmodified mRNA, demonstrating the improved purity and immunoquiescence afforded by circular oRNA, even in the absence of nucleotide modifications. Additional improvements to oRNA processing (Process B) removed immunostimulatory elements to levels that were not stimulatory to Blaer1s at any concentration. oRNA's high protein expression and purity, longer duration than mRNA, and simple manufacturing procedure, make it an ideal protein coding modality. Minimal reactogenicity in cells expressing both TLR7 and TLR8 further support oRNA's therapeutic potential.

799. Quantifying Alloreactive T-Cells in Recipients of Allogeneic Cell Therapies

Saida G. Wiltz, Rafael Fracasso, Sandhya Sharma, Mariana Mazzi, Lisa A. Rollins, David H. Quach, Cliona M. Rooney

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Banked therapeutic T-cells derived from healthy donors for use in allogeneic recipients could overcome many of the problems associated with the use of customized autologous cell therapies. However, alloreactive T-cells (ARTs) in both the donor cell product and in the recipient cause graft versus host disease and rapid graft rejection respectively. We have used banked EBV-specific T-cells (EBVSTs) modified with a chimeric antigen receptor for CD30 (CD30.CAR) as a solution to both problems in patients with multiply relapsed CD30+ lymphoma. With their limited TCR repertoire, EBVSTs have not caused GVHD in hundreds of allogeneic recipients, while CD30 is upregulated on activated ARTs that then become susceptible to killing via the CAR *in vitro*. We have treated 20 lymphoma patients with CD30.CAR-EBVSTs with promising clinical results. No patients developed GVHD and CD30.CAR EBVSTs produced objective tumor responses in 78% of patients, and complete responses in 39%. However, tumor responses were of short duration and after a brief expansion, circulating CD30.CAR EBVSTs were almost undetectable by day 7. To test the hypothesis that ARTs, which comprise up to 10% of all T-cells, overwhelm the infused CD30.CAR EBVSTs, and to determine if the CD30.CAR is able to prevent allorejection, we are developing quantitative assays to measure ARTs in patients. This will allow us to compare the pre- and post- infusion frequencies of ARTs in patients that received allogeneic EBVSTs alone or expressing the CD30.CAR. Since there is no means to identify or quantify ARTs, we have used a stimulation assay to identify T-cells that proliferate in response to irradiated CD3- and CD28-activated allogeneic activated T-cells (ATCs) that upregulate costimulatory molecules after activation and are effective alloantigen presenting cells. We then quantified responder T-cells using CFSE assays, TCR Vbeta family analysis, TCR sequencing and cell cycle profiling before and after 7 days of stimulation. Since most ARTs reside in the naïve fraction of PBMCs, we expect to see an increased frequency of ARTs in the memory population in patients that received unmodified EBVSTs. So far, we are validating our quantitative assays in healthy donors. Alloactivated "recipient" T-cells showed 4 to 9 fold bulk expansion over 6 to 7 days of stimulation. We observed no marked changes in TCR Vbeta usage after stimulation, indicating that the response was polyclonal. TCR analysis identified clonotypes that proliferated in response to stimulation. To confirm that the expanded cells are alloreactive, we will sort the CFSE-negative fraction and evaluate their ability to kill "donor" ATCs and 3rd party HLA-mismatched allogeneic ATCs. We now plan to quantify alloreactive T-cells in patients that received allogeneic cell therapy products. A quantitative assay for ARTs will be useful for the evaluation of strategies designed to prevent rejection in recipients of allogeneic cell therapies.

800. Use of Immunological Adjuvants in Combination with Multiple Modes of Vaccination: Next-Generation Boosting of RNA and Protein Vaccines

Jiho Kim, Jenn Davis, Sean Gray, Darrick Carter

PAI Life Sciences, Seattle, WA

Immunological adjuvants are often included with vaccinations to boost the response of the various components of the immune system to raise an enhanced response against the target antigen. While certain adjuvants have been used since the advent of modern vaccines, recent RNA vaccines do not typically include adjuvants as RNA molecules are perceived to be self-adjuvanting. However, traditional immunological adjuvants such as those targeting TLR-4 are still powerful, relatively cheap additions to vaccines resulting in a clear boost in response. A next generation of TLR4 targeting adjuvants in differing formulations (EmT4; Oil-in-water emulsion, LiT4Q; Liposomal, MiT4; Micellar, AlT4; Alum-adsorbed) were developed to be alternatives and successors to some of the already approved adjuvants available for use in commercial vaccines, such as alum, AS-01, AS-04, or MF59. We have tested these adjuvants in boosting the immune response in multiple vaccines including those against influenza, tuberculosis, other diseases of global significance. Recently, these adjuvants were tested in combination with an RNA vaccine against SARS-CoV2. The adjuvants boosted the strength and breadth of the immune response against several variants of SARS-CoV2 such as D614G, Delta and Omicron. An interesting finding was that the duration of the response was also increased, with a significantly higher titer being maintained up to day 56 post-immunization. This addresses a common problem with mRNA vaccines: a quickly fading immunity which necessitates the frequent use of booster doses. These indicate that the combination of immunological adjuvants in combination with RNA vaccines holds much potential in enhancing a relatively novel mode of immunization. In addition, the adjuvant Em-T4 is currently being tested for use in combination with a vaccine against schistosomiasis, a neglected tropical disease prevalent in much of the tropics. The combination of the Sm-P80 antigen and Em-T4 has been previously tested in multiple animal models including mice, baboons, resulting in an elevated immune response and enhanced worm egg killing, thus reducing disease burden. We present recent results of using these next-generation adjuvants in the context of both new and old modes of vaccination, RNA and protein respectively. The adjuvants boost the responses in both modalities, resulting in gains in strength and breadth of response. The use of these adjuvants represents a rapidly deployable, economical option to implement in future vaccines, for use in emergency pandemic situations or conventional vaccine design and trials.

801. Fc-Modification of Anti-PcrV Gene-Encoded Antibodies Modulates Complement-Mediated Killing of *Pseudomonas aeruginosa*

Jillian Eisenhauer[1,2], Abigail Tracthman[2], Spencer Dublin[3], Jihae Choi[2], William T. Witt[3], Suman Bharti[2], Maria de la Paz Gutierrez[3], Sarah Miller[3], Elizabeth Parzych[2], Jacqueline D. Chu[2], Kaitlyn Flowers[2], Michaela Helble[1], Daniel Kulp[2], Trevor Smith[4], Mariette Barbier[3], David B. Weiner[2], Ami Patel[2]

[1]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[2]Vaccine & Immunotherapy Center, Wistar Institue, Philadelphia, PA,[3]Microbiology, Immunology, and Cell Biology, West Virginia University, Morgantown, WV,[4]Inovio Pharmaceuticals, Plymouth, PA

Prior to the SARS-CoV-2 pandemic, growing antimicrobial resistance (AMR) was a critical global health concern due to a rise in broad-spectrum antibiotic usage. During the pandemic, majority of COVD-19 patients received one or more antibiotics at the time of hospital admission which has led to an increase in multi-drug resistant *Pseudomonas aeruginosa*. With a lack of new antibiotic therapeutics or successful vaccine candidates, monoclonal antibody (mAb) approaches are emerging as potential strategies to combat AMR pathogens. Synthetic DNA plasmid vectors can be engineered to encode immunoglobulin heavy and light chains, followed by direct delivery *in vivo* by IM injection and electroporation. DNA-encoded antibodies (DMAb) enable transient expression and secretion of mAb directly by skeletal muscles resulting in biologically relevant levels for months. Previously, we described both protection and long-term expression of anti-type IIII secretion system component PcrV DMAb-V2L2. It has been shown that control and clearance of *P. aeruginosa* depends on phagocyte recognition, engulfment, and degradation of bacteria. Here we extend this work to hypothesize that *P. aeruginosa* mAb-mediated protection in high-risk patients like those with Cystic Fibrosis could benefit from enhanced recruitment of immune components that trigger the classical complement system to augment bacteria clearance. Building on previous work evaluating Fc-modified DMAbs in a vaginal *Neisseria gonorrhoeae* challenge, we designed 7 V2L2 DMAb variants encoding modifications in the human IgG1 heavy chain constant region with the goal of modulating binding to complement protein C1q and complement deposition. BALB/c mice (n=5/group) administered Fc-complement variant V2L2 DMAbs transiently express antibody for months post-administration. Mice (n=10/group) were either administered WT-fc or Fc-mod DMAb-V2L2 and infected after 28 days in an acute *Pseudomonas aeruginosa* PAO1 infection model. Here significantly lower bacterial load was detected in the lung and nasal washes in the mice administered complement enhanced Fc-DMAb compared with WT-Fc DMAb. Overall, we demonstrated that gene-encoded antibodies can be sequence modified to modulate complement engagement as a strategy to increase antibody potency as determined by bacterial clearance. To date, we have also expanded on this work to modulate the potency of DMAbs targeting both *Streptococcus pneumoniae* and *MRSA*. Overall, we have demonstrated an advantage of plasmid engineering and sequencing optimization to improve downstream complement engagement for improved bacterial clearance. Together these studies show implications for gene-delivered antibody development as a potential strategy for the treatment or prevention of AMR infections in larger animals and humans.

802. Delivery of an Fc-Modified DNA-Encoded Monoclonal Antibody Targeting the Pneumococcal Histidine Triad Protein D (PhtD) Improves Protection During Fatal Pneumococcal Infection via Engagement with Complement Proteins

Jillian Eisenhauer[1,2], Aaron D. Gingerich[3], Elizabeth Parzych[2], Anna L. McCormick[3], Yue Li[2], Bhavya Bhardwaj[2], Jarrod J. Mousa[3], David B. Weiner[2]

[1]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[2]Vaccine & Immunotherapy Center, Wistar Institue, Philadelphia, PA,[3]Center for Vaccines and Immunology, University of Georgia, Athens, GA

Streptococcus pneumoniae (pneumococcus, *Spn*) infection remains a leading cause of death globally despite widespread use of current capsular polysaccharide vaccines. Despite a reduction of pneumococcal disease, these vaccines are limited by serotype specificity with disease persisting for some vaccine serotypes due to immune system evasion capability (i.e., capsule shedding, electronegative cell surface). Therefore, there is a growing need for serotype-independent approaches to combat *Spn* infection. One promising approach is utilizing antibodies targeting conserved surface antigens thereby bypassing serotype limitations of current vaccines. We have previously characterized a candidate human monoclonal antibody against the conserved surface antigen, pneumococcal histidine triad protein D (PhtD), and demonstrated protective efficacy in pneumococcal pneumonia challenge model. While recombinant antibody therapies are constrained by cold storage and high dose requirements, we have utilized DNA-delivered monoclonal antibodies (DMAbs), produced by muscle cells, for long-term systemic circulation of fully functional Abs by a single injection. Furthermore, pneumococcal vaccines are reported to be protective via opsonophagocytosis, which involves the complement system. To build on this prior work, we developed DMAb variants for direct *in vivo* expression to drive improved antibody durability, and secondly, we engineered an Fc-point mutation to enhance complement binding activity to improve the *in vivo* potency of these antibodies compared to DNA-derived mAb without the Fc-complement mutation and recombinant mAb control. Importantly, *in vivo* expressed anti-PhtD DMAbs retain binding to recombinant antigen and serotype 3 pneumococci. We compared, positive control protein mAb delivered at the time of challenge to IM delivered WT-Fc DMAb, spanning 25 ug to 200 ug, at day 28-post administration. At the lowest dose of 25 ug, mice showed 50-58% survival while protection by higher dose groups maxed out at 75% survival which was not statistically significant from recombinant PhtD mAb control. We hypothesized that single point mutations within the Fc-region may improve binding efficacy for effector proteins, like complement, to improve antibody potency and decrease protective dose. We then compared complement enhanced anti-PhtD DMAb (200 ug, 10 ug) versus non-complement enhanced DMAb (200 ug, 10 ug) and protein control (as above). Mice administered low dose (10 ug, 30-fold lower) of the complement enhanced anti-PhtD DMAb exhibit similar protection to mice administered high dose WT-Fc DMAb or recombinant PhtD mAb control in pneumococcal pneumonia challenge model. Furthermore, mice administered anti-PhtD DMAbs show persistent expression at protective levels suggesting the application of our DMAbs

as a single dose injection for protection lasting over one year. Overall, these studies support our previous work on enhanced complement-mediated membrane attack complex (MAC) anti-*N. gonorrhea* DMAbs and show further application of Fc-engineering to enhance complement mediated phagocytosis by anti-*S. pneumoniae* DMAbs at the lung mucosa in a pneumococcal pneumonia challenge model.

803. Impaired Anti-Toxin Memory B Cell and Serum Anti-Toxin B IgG Responses to *C. difficile* Vaccination Increase Susceptibility to CD196 Spore Challenge in Aged Mice

Emily Konopka, Matthew Bell, Elias Haddad, Michele A. Kutzler

Drexel University College of Medicine, Philadelphia, PA

Clostridioides difficile is a spore-forming gastrointestinal pathogen responsible for nearly half a million infections and up to 30,000 deaths in the U.S. annually. Aged individuals (>65) are among those with the highest susceptibility to severe infection, recurrence, and death. Current treatments for primary *C. difficile* infection (CDI) predominantly involve antibiotic use, but these may increase the risk for recurrent infection. To further understand the age-associated impairments in the immune response to *C. difficile* for the development of effective therapeutics, aging mouse models of CDI are critical. We hypothesized that immune dysfunction in the elderly will make them more susceptible to infection with *C. difficile*. To test this, we first established the lethal CD196 spore dose in aged (72-80 weeks) mice. After identification of this lethal dose, vaccination followed by challenge was performed to determine vaccine efficacy and impairments in immune response associated with elevated mortality in young versus aged mice. Mice were immunized twice over 30 days with a DNA vaccine expressing the receptor binding domain of toxin A (RBDA) and B(RBDB) (5ug/plasmid/mouse) followed by orogastric challenge with 200 CD196 spores at thirty days post boost. Serum analyses showed that while young and aged mice developed similar anti-toxin (TcdA/B) IgG responses following two doses of the RBDA/RBDB vaccine, aged mice that succumbed to CD196 infection trended to lower anti-toxin B IgG responses. Additionally, post-challenge, aged mice developed significantly less toxin A-specific memory B cells compared to young unvaccinated mice and significantly lower toxin B-specific memory B cells compared to young RBD vaccinated mice. While these age-related defects in the humoral response are associated with increased morbidity and mortality following CDI, we also show that these defects can be restored with the addition of the molecular adjuvant Adenosine Deaminase -1 (ADA-1) during vaccination of aged mice with RBDA/RBDB-encoding DNA. When ADA-1 is used as an adjuvant in aged mice, the anti-TcdA/B antibody response is restored to levels observed in young adult mice. Additionally, the use of ADA-1 as an adjuvant boosts the frequency of T follicular helper cells in aged mice following vaccination, suggesting that ADA-1 targets the germinal center and restores the development of protective immunity following vaccination. Together, these results indicate that increased susceptibility to CDI in the aged is associated with reduced vaccine-specific anti-toxin B IgG in sera and lower frequencies of toxin-specific memory B cells suggesting the necessity for adjuvanted vaccine formulations for improvement of aged immune responses to *C. difficile* infection.

804. A Genetically Engineered, Stem-Cell-Derived Cellular Vaccine

Tom Henley

Intima Bioscience, Cambridge, United Kingdom

Despite rapid clinical translation of COVID-19 vaccines in response to the global pandemic, an opportunity remains for vaccine technology innovation to address current limitations and meet challenges of inevitable future pandemics. We describe a universal vaccine cell (UVC) genetically engineered to mimic natural physiological immunity induced upon viral infection of host cells. Cells engineered to express the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike as a representative viral antigen induce robust neutralizing antibodies in immunized non-human primates. Similar titers generated in this established nonhuman primate (NHP) model have translated into protective human neutralizing antibody levels in SARS-CoV-2-vaccinated individuals. Animals vaccinated with ancestral spike antigens and subsequently challenged with SARS-CoV-2 Delta variant in a heterologous challenge have an approximately 3 log decrease in viral subgenomic RNA in the lungs. The universal vaccine cell has been improved further to not only release protein antigen upon lysis post-vaccination, but to also deliver a payload of antigen-encoding, self-amplifying mRNA, through microvesicle release into the immune microenvironment. CRISPR engineering of the UVC enables the tethering of mRNA molecules to the structural proteins of microvesicles that are formed within the cells and released upon vaccination, thus protecting the mRNA within these living nanoparticles. These microvesicles are additionally modified via genetic engineering to specifically target immune cells to promote delivery of the antigen-encoding mRNA to the adaptive immune system. This cellular vaccine is designed as a scalable cell line with a modular poly-antigenic payload, allowing for rapid, large-scale clinical manufacturing and use in an evolving viral variant environment.

805. T Cell Predominant Response to AAV-Spike Protects hACE2 Mice from SARS-CoV-2 Pneumonia

Coral M. Kasden[1], Christopher D. Greer[1], Tanvi Singh[1], Leon Morales[1], Cristhian Salas-Quinchucua[2], Benjamin Kozyak[3], Paul Bates[1], Jean Bennett[1], Hansell H. Stedman[4]

[1]University of Pennsylvania, Philadelphia, PA,[2]University of Wisconsin, Madison, WI,[3]University of Pennsylvania School of Medicine, Philadelphia, PA,[4]University of Pennsylvania Medical Center, Philadelphia, PA

Prevention of COVID-19 has been predominantly based on neutralization of SARS-CoV-2 by vaccine-induced humoral immunity, leading to emerging escape variants that have perpetuated the pandemic. Here we show that a single intramuscular injection of Adeno-Associated Virus-6 (AAV6) or AAV9 encoding a modified, N-terminal domain deleted (^NTD) spike protein induces robust cellular immunity and provides long-term protection in k18-hACE2 transgenic mice from lethal SARS-CoV-2 challenge, associated weight loss and pneumonia independent of vaccine-induced neutralizing humoral immunity. In both mice and macaques, vaccine-induced cellular immunity results in the clearance of transduced muscle fibers coincident with macrophage and CD8+ cytotoxic T cell infiltration

at the site of immunization. Additionally, mice demonstrate a strong Type-1 polarized cellular immunophenotype and equivalent *ex vivo* T cell reactivity to peptides of wildtype and alpha (B.1.1.7) variant spike. These studies demonstrate not only that AAV6 and AAV9 can function as effective vaccine platforms, but also that persistent antigen expression in muscle is associated with robust and long-term T cell immunity, and the immunobiological properties of skeletal muscle may be contributing to both the sustained expression of our Spike-M8B transgene product near the injection site 29 days post-immunization and the long-term T cell-mediated immunity induced by a single injection of our non-adjuvanted intramuscular AAV-Spike-M8B platform. These results may also be compatible with the retracted nature of the myositis seen in dystrophin-deficient patients receiving gene therapy for DMD. Taken together, these findings may have broader implications for the development of variant-agnostic universal vaccines against a wider range of pathogens, and demonstrate the critical transgene-dependent balance between immunogenic vaccine and non-immunogenic gene therapy on the AAV platform.

806. STING and Type-I Interferon Signaling Do Not Contribute to AAV Immunogenicity in Mice

Robert Junkins, Kelsey Higgins, Layth Awartani, Dylan Sourthers, Alan Curtis, Rachel Hedinger, Erik Wielechowski, Yash Shah, Audry Fernandez, Rohini Sheth, Ricardo Rodriguez, Angela Mote, Anna Tretiakova, Sherif Gabriel, Shari N. Gordon, R. Jude Samulski

Asklepios BioPharmaceutical, Inc., Morrisville, NC

Introduction: The host immune response remains a significant hurdle to adeno-associated virus (AAV) gene delivery, redosing, and long-term transgene expression. Efforts in the field have been heavily biased towards understanding the acquired immune mechanisms that limit AAV transduction such as neutralizing antibodies and T cell responses. However, generation of these acquired responses first requires viral sensing and induction of innate immune pathways. In contrast to the well-defined mechanisms of acquired immunity against AAV, the innate immune sensors and signaling pathways involved in viral sensing remain poorly defined. One such example is the cyclic GMP-AMP Synthase (cGAS) and Stimulator of Interferon Genes (STING) pathway, which is critical for antiviral immunity and is the focus of this study. It is a key regulator of anti-viral immunity that senses cytosolic DNA to initiate a potent type-I interferon response. **Methods:** Wild-type, Sting-deficient ($Sting^{-/-}$) and type-I interferon receptor-deficient ($Ifnar^{-/-}$) mice were injected with 1E11 vg/mouse of AAV-rh32.33 intramuscular (IM) or AAV8 intravenous (IV) expressing luciferase transgene or formulation buffer and monitored for 30 days. Acute inflammatory responses were assessed in the serum by cytokine bead array. T cell responses were assessed by interferon-γ enzyme-linked immunosorbent spot (ELISPOT) and flow cytometry (AAV capsid specific-dextramer). Binding antibodies were assessed in serum by enzyme-linked immunosorbent assay (ELISA). Transgene activity was assessed by *in vivo* imaging, while transgene expression and viral genomes were assessed by reverse transcription digital polymerase chain reaction (RT-dPCR). **Results:** No acute systemic inflammatory responses were detected at 2 hours post IV administration or 14 hours post IM administration of AAV. Intramuscular administration of AAV-rh32.33 induced significant capsid specific T cell responses in wild-type and Sting-deficient mice as assessed by both dextramer staining and ELISPOT compared to buffer controls. Interestingly, no significant differences in AAV specific T cell responses were observed between knockout strains. Both AAV8 and AAV-rh32.33 induced high titer antibody responses across all strains on day 30 relative to buffer controls. However, no significant differences in the magnitude of binding antibodies were observed between strains. Transgene activity peaked on day 8 and was maintained in all strains for the duration of the study. For IM delivered AAV-rh32.33, no differences in transgene activity, expression or vector genome copies in the tibialis anterior muscle were observed across knockout strains *in vivo* or *ex vivo*. For IV delivered AAV8, a moderate but significant increase in transgene activity in the liver was observed in Sting and Ifnar-deficient mice relative to wild-type controls in vivo and increased AAV genome copies were measured in the livers of type-I interferon receptor deficient animals. **Conclusions:** This study used a clinically relevant AAV8 capsid (frequently used for liver directed gene therapies) and the highly immunogenic T cell inducing AAV-rh32.33 capsid to investigate the contributions of the Sting/type-I interferon pathways on anti-AAV immunity in mice. Neither the frequency or functional capacity of the anti-AAV T cell response nor the magnitude of the antibody response was different in wild-type vs Sting/type1 interferon receptor deficient mice suggesting that these innate pathways are not major contributors to the development of the adaptive response in these murine models.

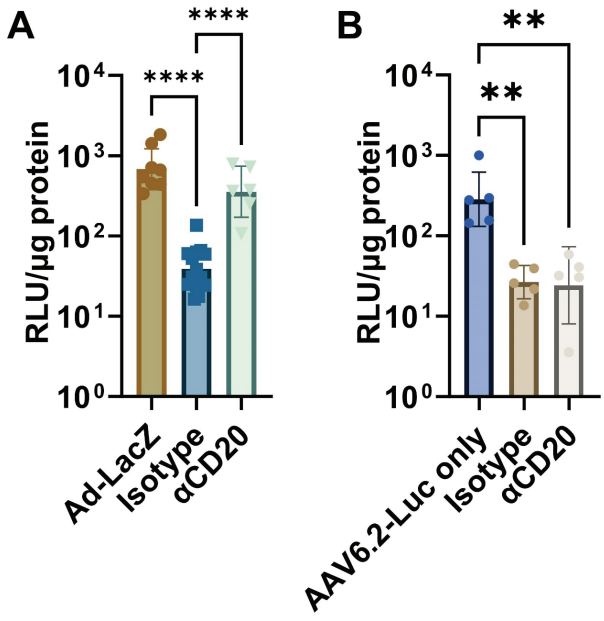
807. Evaluation of αCD20 for Repeated Delivery of Inhaled Gene Therapy Vectors

Robert David Edward Clark, Dora Pungan, Felix Rabito, Ferris Munyonho, T. Parks Remcho, Jay K. Kolls

Center for Translational Research in Infection and Inflammation, Tulane University Health Science Center, New Orleans, LA

RATIONALE Host immune responses to viral gene therapy vectors such as adenovirus (Ad) and adeno-associated viruses (AAV) have emerged as a primary impediment to effective treatment. While extensive work has been published on the nature of immune responses to systemically delivered gene therapy vectors, our understanding of immune responses to inhaled gene therapy vectors is less well-developed. In the systemic context, anti-AAV neutralizing antibodies and CD8+ T-cell responses have been observed to block repeated vector transduction and reduce the duration of transgene expression, respectively. However, the lung is a unique mucosal niche and site of frequent pathogen encounter that has evolved mechanisms such as resident memory B (B_{RM})- and T (T_{RM})- cells which may present unique challenges for gene therapy vector delivery. Furthermore, in addition to promoting mucosal humoral responses, B-cells in the lung also play critical roles as antigen-presenting cells to prime memory T-cell responses and promote the formation of T_{RM}. Therefore, we reasoned immunomodulation with depleting αCD20 might permit redelivery of inhaled viral vectors. **METHODS** We first studied Adenoviral (HAdV) vectors due to their well-known ability to elicit potent immune responses, and later sought to extend our results to clinically relevant adeno-associated viruses (AAV6.2). Mice were dosed with a depleting αCD20 antibody or isotype control 2 days before primary HAdV or AAV6.2 vector delivery. 4 weeks later, 6-week old female C57BL/6 (HAdV and AAV6.2) or BALB/c (AAV6.2) mice received a second

dose of a LacZ- (HAdV) or luciferase- (AAV6.2) expressing vector, followed by euthanasia after 3 days (HAdV) or 3 weeks (AAV6.2) to assess second-round gene transfer and anti-vector immune responses. Intravascular staining of lymphocytes and flow cytometry was used to evaluate the development of lung-resident memory cells, and ELISPOT and MHC-I tetramer staining were used to enumerate the number of vector-specific B- and T-cells. Second-round gene transfer was assessed by chemiluminescent activity assay and anti-vector antibody responses were evaluated by ELISA. **RESULTS** Delivery of HAdV and AAV6.2 vectors leads to the development of anti-vector humoral responses in serum and bronchoalveolar lavage fluid (BALF). For both vectors, pretreatment with αCD20 depletes both circulating and intraparenchymal B-cells but did not reduce the number of antigen-specific IFNy-secreting T-cells. However, αCD20 did reduce the number of airway infiltrating T-resident memory (T_{RM}) cells in BALF one month after HAdV delivery. As anticipated, HAdV delivery elicited substantially more T_{RM} and B_{RM} compared with AAV6.2. For HAdV, αCD20 treatment before first round gene transfer completely abrogated mucosal humoral and B-resident memory (B_{RM}) responses and rescued second-round gene transfer (figure 1A). For AAV, αCD20 similarly abrogated mucosal humoral responses; however, there was no benefit to transgene expression 3 weeks after second vector delivery (figure 1B). **CONCLUSIONS** Mucosal humoral immunity is critical in preventing re-administration of HAdV vectors, and αCD20 treatment before vector delivery rescues re-administration. However, for AAV6.2, despite a reduction in anti-vector humoral immune responses, transgene expression was not rescued by αCD20. Future work will elucidate the possible role of CD8+ T-cell mediated immunity in limiting AAV6.2 gene expression in the lung.



Wednesday Posters: Immune Targeting and Approaches with Genetically-Modified Cells and Cell Therapies

808. Fully Human B7-H3 CAR T Cells Exhibit Potent Preclinical Activity Against Solid Tumors

Pradip Bajgain
National Cancer Institute (NIH), Frederick, MD

B7-H3, a cell surface protein overexpressed in multiple types of solid tumors, has emerged as an attractive target for cancer immunotherapy. T cell therapies employing B7-H3 chimeric antigen receptors (CARs) are currently being tested against various cancer types in phase I/II clinical trials. To date, B7-H3 CARs predominantly utilize mouse derived monoclonal antibodies (mAbs) obtained from hybridomas. For instance, all B7-H3 CAR trials underway in the U.S. utilize CARs with scFvs derived from mAbs 376.96 or MGA271, both of murine origin. While humanization of the variable domain framework regions helps reduce the potential immunogenicity of these CARs, they still maintain murine complementarity-determining regions (CDRs) that can potentially elicit host mediated immunorejection. This obstacle can be evaded by constructing CARs using scFvs isolated from phase display libraries of human scFvs. In this study, we investigated if human scFv library derived "binders" can be used to generate potent fully human B7-H3 CARs with strong anti-tumor activity against aggressive solid tumors like pancreatic cancer and neuroblastoma. To accomplish this, we screened a human scFv library and identified ten unique binders that were then used to generate CARs with the same architecture including - CD28 derived hinge and transmembrane regions, a 4-1BB costimulatory domain, and a CD3ζ cytoplasmic tail. Based on superior *in vitro* performance in assays comparing CAR expression on T cell surface, antigen binding, and tumor-killing capacity, three CARs - Y111, Y117, and Y868 were selected for further evaluation *in vivo*. In orthotopic pancreatic ductal adenocarcinoma and metastatic neuroblastoma tumor models, the Y111 CAR consistently demonstrated the most robust and durable anti-tumor responses. To determine how the Y111 CAR activity compares to the B7-H3 CARs being pursued clinically, for a head-to-head assessment, we also constructed 376.96 and MGA271 CARs with architecture identical to that of Y111. While the tumor-killing ability of all three CARs was similar *in vitro*, the Y111 CAR exhibited similar or better anti-tumor efficacy compared to 376.96 and MGA271 derived CARs in mouse studies. Our data demonstrate that highly effective CARs against B7-H3 can be developed using scFvs obtained from human scFv phage display libraries. Additionally, fully human B7-H3 CARs like the Y111 CAR harbor the lowest potential for immunogenicity, eliminate the need for humanization, and help streamline the path to effective clinical translation.

809. Synthetic Gene Expression Regulatory Switches for CAR T Cells to Cure Solid Tumors

Leidy Diana Caraballo Galva

Pediatrics, Baylor College of Medicine, Houston, TX

Chimeric antigen receptor (CAR) T cells induce response rates of up to 90% and durable complete remission rates of ~50% in patients with lymphoid malignancies but have had limited efficacy in patients with solid tumors. The inhibitory tumor microenvironment (TME) of solid neoplasms lacks most survival factors needed for T cell homeostasis, contains inhibitory mediators, and together with chronic tumor antigen exposure induces CAR T cell dysfunction, ultimately leading to treatment failure. Our group and others have shown that modulation of positive, or knock out of negative, regulators of T cell function can improve the antitumor activity of CAR T cells against solid tumors. We hypothesize that simultaneous overexpression of T cell survival-related genes (*IL15, JUN, BACH2*, and *TCF7*) and downregulation of T cell dysfunction-associated genes (*TOX, BLIMP1, BTG1, RASA2*, and *REGNASE1*) using cell state-specific regulatory switches will be both safe and effective in improving CAR T cell expansion, persistence, and anti-tumor activity. We developed Synthetic Gene Expression Regulatory Switches (SynGERS) for tumor antigen-specific and cell state dependent bi-directional regulation of genes in CAR T cells. SynGERS contain a cell-state specific sensor unit acting on a minimal promoter that drives overexpression of genes associated with T cell survival (enhancer unit), and an RNA interference-based unit to repress T cell dysfunction-related genes (repressor unit). We developed a novel activation-specific sensor responding to nuclear receptor 4A2 (NR4A2) that outperformed previously described NFAT sensor unit by 10-fold (**Fig 1**). Furthermore, we generated a library of 50 SynGERS and controls and tested them in a bicistronic vector constitutively co-expressing our clinically validated glypican-3 (GPC3)-specific CAR. We targeted GPC3 as it is expressed in several pediatric solid tumors but not in non-malignant tissues. We screened the library of SynGERS-CAR for optimal combinations of positive and negative regulator genes resulting in superior expansion, persistence and killing function in repeat tumor challenge assays against GPC3+ tumor cells. Compared to T cells expressing the GPC3-CAR alone, eight SynGERS had increased cytotoxicity and/or proliferation. The top four performing SynGERS (IL15-TOX, IL15-BTG1, IL15-RASA2, and IL15-REGNASE1 had increased tumor killing capacity (fold change: 1.43, 1.27, 1.08, and 1.23, respectively), and had superior expansion (fold change: 2.75, 1.97, 1.88, and 1.58, respectively) at round four after repeat killing assay (**Fig 2**). These results demonstrate the potential for SynGERS to induce potent anti-tumor function by CAR T cells with improved persistence and proliferation in solid tumors.

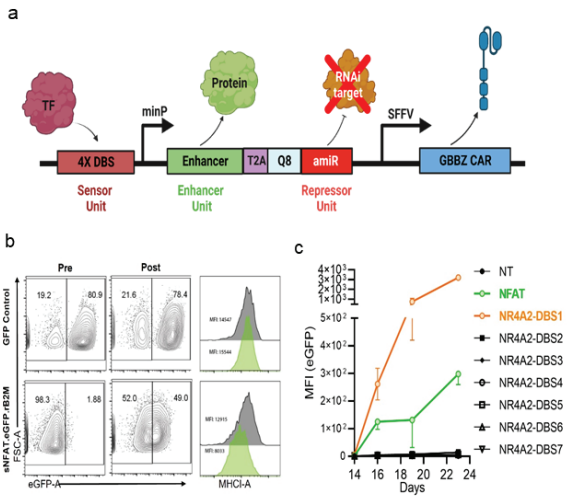


Figure 1. Development of SynGERS. a) Schematic of SynGERS: The expression cassette consists of a 5' sensor cell state specific sensor; acting on a minimal promoter (minP) followed by the enhancer and repressor units. An independent promoter (SFFV) drives the GPC3-CAR (GBBz, GPC3-specific second-generation CAR incorporating the 41BB endodomain). The enhancer unit incorporates the Q8 tag for induction detection, which is translated into a separate peptide with the use of a T2A sequence. b) Activation dependent induction and repression of target genes. T cells were transduced with indicated SynGERS and pre- and post- activation gene expression induction (eGFP) and repression (MHC Class I) was measured by FACS. One representative of three independent experiments. c) Generation of NR4a2 sensors. Transduced cells were stimulated via TCR and FACS measured induction of GFP expression. Combined results from three independent experiments.

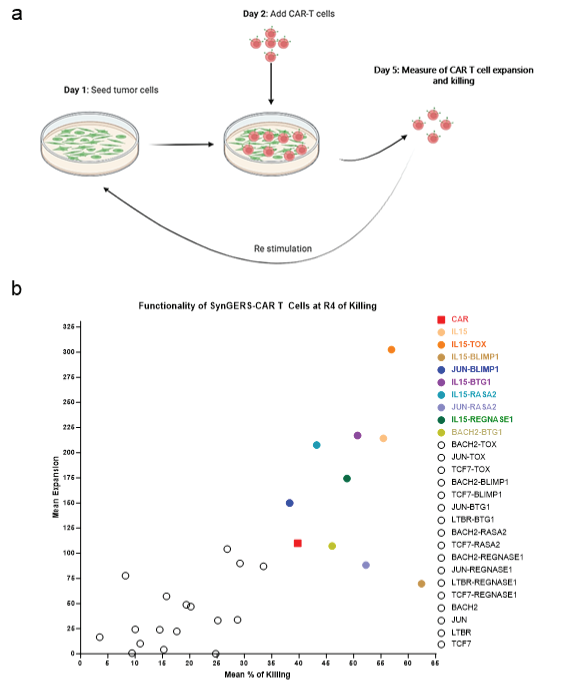
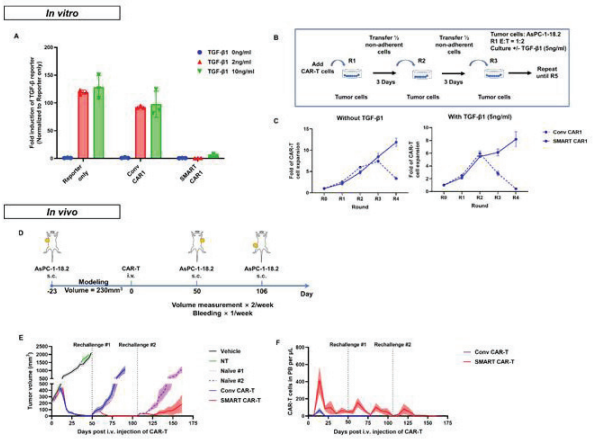


Figure 2. Figure 2. Unbiased screening of SynGERS-CAR T Cells. SynGERS-CAR T cells were evaluated in repeat tumor cell killing assay (a) in which SynGERS- CAR T cells were co-cultured with Huh7-eGFP cells at a 1:1 effector to target ratio. Cells were analyzed at the end of each round for expansion and killing. Functionality of SynGERS-CAR T Cells, based on ability to repeatedly kill tumor cells and proliferate, was assed at round four of repeat killing (b). Cell expansion was measured by flow cytometry using counting beads, and tumor cell killing was determined by IncuCyte. Data are presented as mean of two donors.

810. A Nanobody-Based and TGF-β-Resistant Claudin 18.2-Specific CAR-T Generates Effective and Durable Anti-Tumor Response: A Preclinical Study

Qi Dong, Wenjie Yin, Tao Wang, Manli Yin, Di Wu, Xinxin Wang, Wei William Cao, Lianjun Shen
Gracell Biotechnologies, Shanghai, China

Introduction: Claudin 18.2 (CLDN18.2), a member of the tight junction protein family with limited expression in normal tissues, is selectively overexpressed in solid tumors, such as gastric cancer/gastroesophageal junction cancer (GC/GEJ) and pancreatic cancer. CAR-T therapies targeting CLDN18.2 have been actively investigated in clinical trials. However, due to the immunosuppressive tumor microenvironment (TME), the overall efficacy of CAR-T therapy for solid tumor still needs improvement. Here, we generated nanobody-based CLDN18.2-targeted CAR-T cells armed with a SMART (Suppressive Molecule Activated and Rejuvenated T cells) module that combines the ectodomain of the dominant-negative TGF-β receptor 2 and a cytokine signaling domain. By converting immunosuppressive signals into pro-T cell signaling, SMART CAR-T cells can resist and persist in the TME with enhanced efficacy and prolonged durability. **Methods:** Candidate CLDN18.2-specific binders were screened from a camelid nanobody phage display library that was constructed from PBMCs isolated from an alpaca immunized with CLDN18.2 peptides and CLDN18.2-expressing cells. Binders that showed specificity to CLDN18.1 were eliminated during the initial screening process. Selected binders were further tested with membrane proteome array (MPA) to verify their specificity. Second-generation CAR-T cells expressing the CLDN18.2 binders with and without SMART were generated. CLDN18.2-targeted SMART CAR-T cells were compared with conventional CAR-T cells in terms of cell activation status, memory phenotype, short-term cytotoxicity and cytokine levels when co-cultured with tumor cells. Repeated antigen stimulation assays assessing long-term expansion and cytotoxicity in in the presence or absence of TGF-β were also performed. Immunodeficient mice bearing xenografts derived from CLDN18.2-expressing cell lines were used to study the *in vivo* potency and safety of CLDN18.2-targeted CAR-T cells. Tumor volumes, mouse body weights and survival, and CAR-T cell expansion were assessed. **Results:** Three binders (1, 2, 3) demonstrated high specificity to CLDN18.2 without any detectable off-target binding, as evidenced by MPA and/or toxicity to human primary cells in the form of CAR-T cells (CAR1, CAR2, CAR3). CAR1 cells displayed a higher proportion of naïve or stem-like memory T cells and non-activated ($CD69^{-}CD25^{-}$) cells compared to CAR2 and CAR3 cells. The short-term cytotoxicity to target cells, including those with low antigen densities, was similar among the three CARs, with CAR1 secreting the highest amount of IL-2. When armed with the SMART module, although all CARs showed elevated frequencies of T cells of a younger phenotype and enhanced resistance to TGF-β in long-term cytotoxicity assays by reducing Smad-mediated signaling, SMART CAR1 outperformed the others by effectively eradicating established TGF-β-expressing CLDN18.2-positive tumor xenografts and suppressing tumor rechallenges *in vivo* without causing significant toxicity. Significant amount of tumor infiltrating and peripheral CAR-T cells were detected. **Conclusions:** These data indicate that the camelid nanobody-derived, CLDN18.2-targeted SMART CAR-T cells are a promising immunotherapeutic approach to overcome the TGF-β-rich TME and elicit a deeper and more durable response. This CAR-T therapy warrants further clinical studies in patients with CLDN18.2-positive solid tumors, including GC/GEJ and pancreatic cancer.



Representative results of *in vitro* and *in vivo* studies. (A) Responsiveness to TGF-β1 by SMART or conventional (conv) CAR-expressing TGF-β reporter cell lines. (B) Schema of multi-round antigen stimulation assay. (C) Expansion of SMART or conv CAR-T cells in the presence or absence of TGF-β1. (D) Schema of *in vivo* rechallenge study. (E) Tumor volumes. (F) Peripheral blood CAR-T cells.

811. Development of AB-2100, a PSMA-Inducible Anti-CA9 Car T Cell Therapy Intended for the Treatment of ccRCC

Irene Scarfo, Mark Landon, Rakesh Sudhakah, Michelle Nguyen, Suchismita Mohanty, Jeremy Chen, Alma Gomez, Nickolas Attanasio, Amanda Fearon, Ivan Chan, Christine Nelson, Thomas J. Gardner, Darrian Moskowitz, Vince Thomas, Stanley Zhou, Angela Boroughs, Jennesa Smith, Jennifer McDevitt, Levi Gray-Rupp
ArsenalBio, South San Francisco, CA

Relapsed/refractory clear cell renal cell carcinoma (ccRCC) progressing after treatment with CPI and VEGF inhibitor remains an area of unmet need. We have developed AB-2100, an autologous integrated circuit T (ICT) cell product, generated via CRISPR-mediated site-specific integration of a single DNA cassette into a safe-harbor locus. AB-2100 is composed of three modules: a sequential "AND" logic gate that requires the presence of two antigens in the tumor microenvironment (TME) to trigger T cell killing; an shRNA-miR module to enhance resistance to suppressive TME via constitutive knockdown of FAS and TGFBR2; and a constitutive synthetic pathway activator (SPA) that enhances antitumor activity. The sequential "AND" logic gate consists of a priming receptor (PrimeR) targeting PSMA, and a CA9-targeted CAR that is upregulated upon PrimeR engagement with PSMA expressed on the tumor neovasculature. This design is intended to minimize the potential risk of on-target, off-tumor toxicity and to improve therapeutic index relative to a constitutive CA9 CAR T cell therapy. The dual-antigen specificity of the logic gate was tested in vitro and in vivo against CA9+ and PSMA+CA9+ tumors, showing that AB-2100 selectively kills tumors that express both antigens, and not tumors that express CA9 alone. Furthermore, we confirmed that co-culturing AB-2100 cells with PSMA-expressing endothelial cells was sufficient to upregulate CA9 CAR expression and enable tumor cell killing. Finally,

AB-2100 containing both shRNA-miR and SPA modules demonstrated enhanced anti-tumor activity in xenograft RCC models. These data demonstrate that AB-2100 selectively targets tumors expressing both PSMA and CA9, and can overcome multiple suppressive mechanisms in the TME. Based on these promising preclinical data, AB-2100 is being evaluated in a phase 1/2 clinical trial for the treatment of advanced or metastatic ccRCC.

812. Redirected Soluble Modulators (RSM) - A Novel Engineering Strategy to Enhance Immune Receptor Signaling

Nicholas G. Minutolo, Lauren C. Shaw, Kerri Ciccaglione, Tierra S. Tobin, Robert J. Saporito, Thomas Condamine, Michael Klichinsky

Carisma Therapeutics Inc, Philadelphia, PA

Immune cells require activation of primary and secondary signaling pathways to achieve maximum effect. To harness secondary signals in the design of synthetic immune receptors such as chimeric antigen receptors (CARs), costimulatory domains have been grafted directly into the membrane proximal intracellular space of these receptors. While direct incorporation of co-stimulatory domains such as 4-1BB and CD28 has been a successful strategy in CAR-T cell design, there are certain signaling domains for which membrane localization can lead to constitutive (tonic) signal propagation. This challenge is particularly notable for macrophages which signal through innate immune pathways such as MyD88 which activate from membrane proximity. To overcome the hurdle of tonic signaling from costimulatory domains, we developed redirected soluble modulators (RSMs). These chimeric soluble proteins combine the phospho-tyrosine binding capabilities of Src Homology 2 (SH2) domains from one protein with signal-transducing portions of another. Here we demonstrate an RSM that combines the phospho-ITAM binding SH2 domains from Syk with the signaling death domain of MyD88. The Syk-MyD88 RSM is capable of localizing to an ITAM domain that endogenously recruits Syk, but upon recruitment drives MyD88 signaling alongside endogenous Syk signaling. We co-expressed an ITAM-containing CAR together with the Syk-MyD88 RSM in macrophages. Upon antigen-induced CAR activation, the Syk-MyD88 RSM greatly enhanced proinflammatory cytokine output while simultaneously driving an expansion of the proinflammatory cytokine profile and enhancing M1 polarization compared to CAR activation alone. When the Syk SH2 domains of the RSM were mutated, the effect was lost, confirming the necessity for recruitment of the RSM to the ITAM to drive function. Additionally, the Syk-MyD88 RSM abrogated the tonic, antigen-independent release of proinflammatory cytokines seen with CARs that contain MyD88 death domain directly in the CAR construct. Syk-MyD88 RSMs enhanced the anti-tumor activity of CAR macrophages (CAR-M). Macrophages are critical effector cells of monoclonal antibodies. We evaluated the ability of Syk-MyD88 RSM to redirect endogenous Fc receptors to activate MyD88 in response to antibody signaling as a strategy to endow antibodies with multi-pronged innate immune signaling. When activated with antigen-specific monoclonal antibodies such as trastuzumab, macrophages expressing the Syk-MyD88 RSM exhibited a marked increase in antigen specific cytokine release, an expanded proinflammatory cytokine/chemokine output profile, and enhanced M1 polarization. RSM macrophages stimulated with antibodies activated MyD88 signaling, and thus offer a universal strategy to enhance monoclonal antibody-based therapy. In summary, these results demonstrate the development of a novel method to provide costimulation to macrophages. RSMs allow for the decoupling of costimulatory domains from the CAR construct, helping alleviate issues with constitutive signaling, while maintaining the antigen-dependent activation of costimulatory signals. Additionally, Syk-MyD88 RSMs provide a universal method to rewire monoclonal antibody Fc receptor signaling to boost macrophage activation and anti-tumor function. RSMs have cell type agnostic potential to rewire signaling downstream of TCRs, FcRs, CARs, and other immunoreceptors.

813. Development of Tunable Microenvironment-Responsive CAR T Cells Using Synthetic Genetic Circuits

Yannick Schreiber[1], Joshua N. Leonard[2]

[1]Chemical and Biological Engineering, Northwestern University, Chicago, IL,[2]Chemical and Biological Engineering, Northwestern University, Evanston, IL

Engineered cell therapies expressing a chimeric antigen receptor (CAR) in human T cells have demonstrated great promise for the treatment of hematological malignancies. However, unique challenges remain in translating the success of CAR T cell therapies to solid tumors, namely: (1) *specificity*: there are few truly unique antigens present on solid tumors, leading to off-target effects that damage normal tissue and pose safety risks; and (2) *suppression:* solid tumors develop microenvironments that are immunosuppressive and decrease T cell survival and tumor-directed cytotoxicity. To address these challenges and improve the safety and potency of solid tumor cell therapy, we engineered cells with synthetic genetic circuits containing a previously validated hypoxia biosensor (HBS) that responds to upregulation of the native HIF1α and HIF2α transcription factors by hypoxia, enabling the cells to sense and respond to the hypoxic tumor microenvironment ubiquitous among solid tumors. We evaluated CAR expression from circuit architectures containing the HBS promoter driving expression of both CAR and native HIF1α or HIF2α, as well as from circuit architectures containing the HBS promoter and our previously described toolkit of synthetic transcription factors and promoters, under normoxic and hypoxic conditions in HEK cells. We identified novel circuits providing fast reporter output with minimal background signal, and elucidated design rules that enable or restrict amplification of transgene in native HIF1α and HIF2α-containing circuits. We observed differences in CAR transgene expression between HIF1α and HIF2α-containing circuits, suggesting mechanistic differences in regulation of HIF1α and HIF2α when overexpressed. Additionally, we observed greatly amplified transgene expression in certain circuit topologies containing our synthetic transcription factor and promoter toolkit compared to native HIF1α and HIF2α-containing circuits. Ultimately, this project will provide a new therapeutic technology that enables the development of safer, more effective cell therapies for solid tumors, as well as provide new biological and mechanistic insights that can inform the future development of hypoxia-responsive cell therapies.

814. Development of Logic-Gated "Payload" Module in CAR T Cells; Aimed at Increasing Local Concentration of a Secreted Pro-Inflammatory Molecule in an Antigen-Dependent Manner

Gavin E. Shavey, Joseph H. Choe, Audrey Fung, Luke Cassereau, Marian Sandoval, Emily Ng, Suchi Mohanty, Jeremy Chen, Alma Gomez, Sofia Kyriazopoulou Panagiotopoulou, Alba Gonzalez, Aaron Cooper, Nick Haining

ArsenalBio, South San Francisco, CA

Background: CAR T cell therapies may require genetic modifications that enhance expansion and persistence in order to be clinically effective against solid tumors. We have previously deployed several modules in Integrated Circuit T cells (ICTs): engineered T cells that express a logic gate and are generated via non-viral, CRISPR-mediated transgene knock-in. The previous modules include ICT-intrinsic enhancements that provide increased antigen specificity (logic-gated CAR), resistance to the immunosuppressive tumor microenvironment (shRNA to FAS, TGFBR2), and constitutive signaling through the Janus-kinase signal transducer and activator of transcription (JAK/STAT) pathway (SPA: synthetic pathway activator) within each ICT cell. We hypothesized that logic-gated secretion of a pro-inflammatory molecule (or "Payload") could provide an ICT-extrinsic enhancement by supporting activation of the endogenous immune system to aid in the anti-tumor response. **Methods:** We evaluated the ability of a variety of supplemented cytokines to enhance both ICT and bystander immune cell anti-tumor function in vitro. From the top cytokine hits, we used synthetic biology approaches to engineer a library of over 30 cytokine variants (including tethered cytokines, muteins, and fusion proteins) to be secreted as a logic-gated Payload by ICT cells. We funneled down our library of cytokine variants by analyzing enhancements to desired anti-tumor activity in an assay modeling chronic antigen stimulation and by monitoring undesired antigen-independent T cell outgrowth in an assay modeling engineered T cell transformation potential. Payload leads were evaluated for antigen-dependent cytokine secretion, ability to induce activation of a bystander immune cell subset, and potency in a subcutaneous MSTO-211H in vivo model. **Results:** Incorporation of many distinct Payload designs into ICT cells resulted in significant augmentation of ICT anti-tumor activity without increasing cytokine-independent outgrowth. Additionally, we showed that incorporation of the Payload module into ICTs can boost bystander immune cell anti-tumor activity and is secreted from ICT cells in an antigen-dependent manner. Most importantly, incorporation of Payload into ICT cells resulted in a significantly lower required dose for maintained clearance in a MSTO-211H in vivo model, and desired ICT contraction after tumor clearance. **Conclusions:** We have developed an ICT-based Payload platform that allows for antigen-dependent expression of a user-defined secreted protein. The Payload module can enhance function of both ICT and bystander immune cells without signs of increased transformation risk in preclinical assays, and can increase capacity for tumor clearance in vivo at significantly lower dosage. Several lead Payload designs are being considered for inclusion in future ICT products.

815. Unleashing the Potential of NK Cell Therapy by Genetic Disruption of Key Immunoregulators Identified via ImmuT Finder® Platform

Jingwei Sun[1], Yiyang Tan[1], Jiahui Jin[1], Jingman Wang[1], Lingyun Chen[1], Ke Liu[1], Pin Wang[2], Yarong Liu[1]

[1]Grit Biotechnology, Shanghai, China,[2]University of Southern California, Los Angeles, CA

Natural killer (NK) cells, a distinct subset of innate lymphoid cells, play a pivotal role in cancer immunity. With multiple cytotoxicity mechanisms against tumors and a unique advantage for allogeneic therapeutic applications, NK cells have emerged as an attractive candidate for adoptive cellular therapy in recent years. Since early clinical trials have demonstrated a favorable safety profile and promising efficacy, strategies aimed at enhancing persistence and potency, as well as overcoming the immunosuppressive tumor microenvironment (TME), may lead to increased clinical benefits and expand the application of NK cells to solid tumors. To identify key immunoregulatory targets that could maximize the efficacy of NK cell therapy, we established a CRISPR/Cas9 screening platform, ImmuT Finder®, to pinpoint functional gene targets in primary human NK cells. NK cells transduced with a CAR construct carrying a designed sgRNA library were repeatedly stimulated by tumor cells, leading to an exhaustion profile and significantly diminished cytotoxicity by the end of the screening assay. Importantly, we achieved high and stable gene editing efficiency with subsequent rapid expansion in primary NK cells. Multiple quality control parameters were implemented to ensure the reliability of the screening system. Following several rounds of screening, we selected the top 25 targets and validated their function. Subsequently, the top targets that exhibited significantly enhanced cytotoxicity and/or persistence in CAR-NK cells were further subjected to comprehensive functional evaluation, resulting in the validation of several key functional targets that showed significantly improved cytotoxicity during tumor serial killing, cytokine release, proliferation and/or cytokine-independent long-term survival compared with CISH used as a benchmark in primary NK cells. In summary, the findings from our ImmuT Finder® platform demonstrates its capability to identify crucial immunoregulatory targets within primary NK cells. This underscores the potential of unleashing the power of NK cells through genetic engineering of identified targets, paving the way for the development of next-generation NK cell therapies for solid tumors.

816. Harnessing Single Cell Multiparametric Analysis for nS/MAR DNA Vector Based TCR-T Cell Therapy

Silvia Rizzato[1], Alice De Roia[1], Laura Rocchi[2], Paola Poggi[2], Rita Ruggiano[2], Silvia Bocchi[2], Andrea Faenza[2], Massimo Bocchi[2], Richard P. Harbottle[1]

[1]German Cancer Research Centre (DKFZ), Heidelberg, Germany,[2]Cellply S.r.l., Bologna, Italy

The potential of T cell therapies, which employ the use of transgenic T cell receptors (TCRs), hold great promise for advancing cancer treatment. Our research focuses on overcoming key challenges associated with TCR-T cell therapies while simultaneously developing

potent treatments utilizing high-potency TCR-T cells. TCR-T cells are modified with a novel S/MAR DNA NanoVector platform which offers a safe, efficient and fast system for generating recombinant T cells. nS/MAR vectors, in contrast to integrating systems, remain episomal, minimizing the risk of insertional mutagenesis and the potential for secondary malignancies. Moreover, they streamline the manufacturing process, offering a cost-effective and practical timeframe for the treatment of cancer patients. The identification of TCR-T cell products with enhanced potency and low exhaustion is crucial for prolonged and effective treatment. To characterize the effectiveness of our transgenic nS/MAR anti-MART-1 TCR-T cells we employed a novel automated single-cell level analysis platform, the VivaCyte®. This allows us to categorize TCR-T cells based on function and specific marker expression, providing a comprehensive understanding of the product's potency. In this study, nS/MAR vector features were refined, and the most efficient promoter for driving TCR expression (strong sEF1-α and weak hTCR) was investigated. Utilizing the novel VivaCyte® platform, we assessed single-cell cytotoxicity, focusing on the identification and frequency of serial killer cells capable of killing three or more target cells. Simultaneously, the expression of exhaustion markers (PD-1, LAG-3) and activation marker (CD137) was determined. To test functionality and fitness of the anti-MART-1 nS/MAR TCR-T cells, a 24-hour co-culture was performed with the K562 target cell line genetically modified to express HLA-A2, MelanA/MART-1 target epitope, and GFP reporter gene. High-throughput, multi-parametric analysis determined cytotoxicity and phenotype of individual T cells. Interestingly, an increase in serial killer cell frequency was observed after transfection. TCR-T cells consistently displayed a Potency Score higher than 1, suggesting each cell's capability to eliminate more than one target cell. Exhaustion and activation markers provided insights into serial killer population kinetics; an increase in CD137 marker expression occurred, while no signs of exhaustion were observed during the assay. In conclusion, we assessed the effectiveness of recombinant TCR-T cells genetically modified with the novel nS/MAR DNA vector platform, employing conventional assays and the innovative VivaCyte® system. Single-cell analysis underscored the capacity of the (nS/MAR) DNA Vector to increase the frequency of serial killer cells, linking this to specific phenotypic characteristics.

817. Investigating the Role of Immunological Synapse in Chimeric Antigen Receptor (CAR) Immunotherapy

Jong Hyun Cho, Xuening Wang, Maeve Byrne, Dongfang Liu

Rutgers University-NJMS, Newark, NJ

Chimeric antigen receptor (CAR) immunotherapy utilizes genetically engineered immune cells that express a unique cell surface receptor which combines tumor antigen specificity with immune cell activation in a single receptor. CAR immunotherapy has shown remarkable success in improving the overall survival of patients with hematological malignancies, yielding 70-90% complete remission rates for treatment of relapsed/refractory B-cell acute lymphoblastic leukemia using CD19-CAR-T cells. However, long-term follow-up revealed that 33%-50% of patients who achieved remission ultimately relapsed. With ongoing clinical trials, CARs with different target specificities and sequence modifications are being developed and tested by many laboratories to target different tumor antigens and to improve the safety and efficacy of the treatment. Despite the impressive progress, conventional ways to identify optimal CAR constructs are limited by costly, time-consuming, and labor-intensive tools. Furthermore, precisely predicting the efficacy of different CAR products and selecting clinical responders to the therapy remain a key challenge in clinical settings. Recently, our lab has developed novel CAR-natural killer (NK) cells expressing anti-CD147 against hepatocellular carcinoma (HCC), showing promising results in reducing HCC burden in vitro and in a xenograft mouse model. However, the CAR-NK cell cytotoxicity mechanisms and the ways to determine its efficacy in the tumor microenvironment are still under investigation. Our lab has also identified that the quality of immunological synapse (IS) between CAR-T or CAR-NK cells and tumor cells is important for optimal CAR-modified cell antitumor function. The IS is a highly organized interface between lymphocytes and their target cells and includes multiple adhesion, signaling, regulatory, and structural molecules. Previous studies have demonstrated that T or NK cell efficacy is not only controlled by the specificity and avidity to the tumor antigen, but also it depends on the spatially and temporally organized IS formation. Recognizing the limitations of conventional tools and the importance of IS in immune cell functions, we investigate a new strategy for assessing CAR-NK cell efficacy by quantifying CAR IS quality. Here, we evaluate CAR-NK IS quality by quantitation of F-actin, clustering of tumor antigen, polarization of lytic granules, and distribution of key signaling molecules within the IS using immunofluorescence confocal microscopy. We propose to address the basic biology of CAR-modified cell immunological synapse and its application to develop novel means to predict CAR-modified cell efficacy.

818. Persistent Genetic Modification of NK cells Using Non-Integrating S/MAR DNA Vectors for Cancer Immunotherapy

Luisa Burger[1], João S. Cardoso[1], Julia D. Peterson[1], Toros Tasgin[1], Patrick Schmidt[2], Richard P. Harbottle[1]

[1]German Cancer Research Centre (DKFZ), Heidelberg, Germany,[2]National Center for Tumor Diseases, Heidelberg, Germany

Achieving an optimal genetic modification of NK cells for enhanced cancer-fighting capabilities has posed significant challenges and relies mainly on retro- and lentiviral transduction in current clinical studies. NK cells play a crucial role in defending against viral infections. Accordingly, NK cells react strongly to viral vectors which leads to low transduction efficiencies. Additionally, the risk of insertional mutagenesis and constraints on transgene size limit the effectiveness of transduction methods. To address these challenges, we have created a DNA Vector platform which is devoid of viral components and comprises a minimal bacterial backbone. The vectors do not integrate into the target cell's genome but can replicate extrachromosomally in the nucleus of dividing cells using sequences derived from Scaffold/Matrix Attachment Regions (S/MAR). Therefore, they are promising candidates for long-term, stable modification of NK cells without compromising genomic integrity. We have developed electroporation protocols and modified our S/MAR vectors to determine optimal vector features to improve the genetic modification of NK cells as these cells are notoriously difficult to transfect. Using a feeder-based expansion protocol and electroporation we achieved transfection efficiencies of

up to 70 % in peripheral blood derived NK cells with reporter gene expressing S/MAR vectors and the transgene was detectable for three weeks. Furthermore, we studied our vectors in the NK-92 cell line which is used in preclinical and clinical studies to explore NK-cell based cancer immunotherapies and is suitable for off-the-shelf applications. We successfully generated an NK-92 cell line which stably expresses a chimeric antigen receptor (CAR) against the CEA antigen, a common antigen in solid tumours. The CAR expression was persistent for at least six months and was not influenced by cryogenic cell storage. The cell line shows enhanced and CAR specific killing against the CEA-expressing MCF7 breast cancer cell line while the molecular phenotype of the NK-92 line was unaltered. A key step to enable the modification of NK-92 cells with CAR-expressing S/MAR vectors was the treatment of NK-92 cells with small molecules inhibiting the TBK1/IKKε Kinase complex, a central node of cytosolic DNA sensing, before and after transfection which improved transfection efficiencies up to sevenfold for reporter gene vectors. These findings represent a significant stride toward the safer and stable modification of NK cells for clinical applications, laying a robust foundation to extend the utilization of our episomally maintained vectors to a diverse array of promising applications in the field of NK cell immunotherapy.

819. Outlast™ CAR-T Cells Exhibit Superior Effector Function Against Solid Tumors

Kevin Carbajal, Warren Anderson, Prasanna Ekambaram, Jessica Gorczynski, Matthew Esquivel, Adam Pecoraro, Max Richter, Matthias Schroff, Sumiti Jain

Inceptor Bio, Raleigh, NC

The success of CAR-T cell therapy in treating solid tumors has faced several limitations. Key factors limiting CAR-T efficacy in the hostile tumor microenvironment (TME) are lack of T cell persistence and poor durability due to the immunosuppressive and hypoxic milieu. To equip CAR-T cells with attributes that improve their function against solid tumors, we have developed a novel method to condition CAR-T cells during *ex vivo* expansion. Without the need for complex engineering, our conditioning regimen is a radically simple and effective way to epigenetically program CAR-T cells for long-term persistence and durable effector function, proven in *in vivo* models. Our proprietary conditioning regimen generates a unique brand of Outlast™ CAR-T cells (OTX), that exhibit superior effector function against solid tumors. Our first OTX CAR-T product is an autologous anti-CD70 CAR-T to treat clear cell renal cell carcinoma (ccRCC). When compared to conventional T cell (Tconv), we have shown that OTX CD70 CAR-T cells have 1) superior metabolic fitness, 2) superior activation state (by CD25 MFI) in the presence of target cells, 3) lower exhaustion state after repeated stimulation (by PD-1, LAG-3 and KLRG1 MFI), 4) superior resistance to TGFb suppression and 5) superior control of tumor cells *in vivo* in an aggressive repeat challenge model. Overall, we show that OTX CD70 CAR-T cells exhibit multiple attributes desired of CAR-T cells for effective and superior function in the solid tumor microenvironment, and that they 'outlast' conventional CAR-T cells leading to more durable control *in vivo*.

820. Constitutive IL-7 Signaling in Car-NK Cells is Associated with High AP-1 Activation and Effector Function Decline

Matthew Dysthe, Ishwar Navin, Dayenne van Leeuwen, Cliona M. Rooney, Robin Parihar

Baylor College of Medicine, Houston, TX

Background: The efficacy of CAR-NK cellular therapies is limited, in part, by insufficient survival and expansion within the tumor microenvironment (TME). To improve survival and anti-tumor function, CAR-NK cells can be augmented with cytokines or modified to express cytokine transgenes (e.g. IL-15/IL-15R-alpha) that converge on signal transducer and activator of transcription-5 (STAT5) activation. However, systemic administration or transgenic expression of cytokines is associated with toxicities, expansion of inhibitory T-regulatory (Treg) cell populations, and rejection of allogeneic CAR-NKs. Thus, novel cytokine approaches are needed to bypass the limitations of current strategies. IL-7 is a proto-typical NK-cell survival and expansion factor that does not activate Tregs or mediate allo-NK rejection, and thus is an attractive option for cytokine support. Here, we genetically modify CAR-NK cells to express a constitutively active IL-7 receptor complex, termed C7R (C7R.CAR-NK), that confers intrinsic, sustained STAT5 activity within CAR-NK cells. We hypothesized that C7R would provide a potent intrinsic activation signal that would promote NK cell survival and function within inhibitory TMEs typically devoid of such signals. **Results:** Activated and expanded primary human NK cells were co-transduced with retroviral constructs encoding C7R and a GD2-specific CAR. C7R.CAR-NK demonstrated a 3.4-fold increase in phosphorylated STAT5 ($p<0.01$) compared to CAR-NK without cytokine support [CAR-NK(-)]. In short-term co-cultures with GD2+ neuroblastoma, C7R.CAR-NK demonstrated superior intracellular IFN-gamma accumulation ($p<0.05$) compared to CAR-NK(-). We next used a novel *in vitro* TME that mimics a suppressive myeloid infiltrate. In this culture system, C7R.CAR-NK cells demonstrated superior cytotoxicity, survival, and IFN-gamma and TNF-alpha secretion ($p<0.05$) when compared to CAR-NK(-). Despite this promising *in vitro* activity, C7R.CAR-NK did not exhibit improvement in tumor control *in vivo* against neuroblastoma TME xenografts compared to non-treated mice and CAR-NK(-) treated mice, while CAR-NK augmented with systemic IL-15 administration (sIL-15+CAR-NK) demonstrated modest tumor control. *In vivo* bioluminescence imaging and intra-tumoral analysis by flow cytometry confirmed that C7R.CAR-NK cells were present in similar proportions to sIL-15+CAR-NK, both significantly increased over CAR-NK without cytokine support. To investigate the mechanisms behind the paradoxical lack of anti-tumor efficacy *in vivo* despite the C7R-mediated NK survival, we compared gene expression in C7R.CAR-NK and sIL-15+CAR-NK 14 days post cell generation in the absence of any antigen-mediated CAR signaling. C7R.CAR-NK exhibited significant increases in genes associated with AP-1 signaling, most notably *FOS*, *JUNB*, and *JUND*, as well as *NR4A1* and *DUSP1*, recently implicated in both NK- and T-cell dysfunction. This epigenetic phenotype was associated with significantly reduced cytotoxicity ($p<0.05$) compared to sIL-15+CAR-NK. **Conclusion:** While C7R enhanced short-term cytotoxicity and survival and expansion of CAR-NK cells both *in vitro* and *in vivo* TME models, these properties did not translate to enhanced *in vivo* anti-tumor efficacy. Long-term constitutive IL-7R signaling via C7R confers

a transcriptional signature associated with NK dysfunction. Our data inform consequences of continuous cytokine signals within CAR-NK cells and provide novel candidate genes related to NK dysfunction that represent targets for improving CAR-NK function with the solid TME.

821. Pooled Screening for CAR Signaling Optimizes Function in NK Cells

Khloe S. Gordon, Taeyoon Kyung, Aurelija Grignoyte, Siqi Zhao, Joshua Mace, Jai Raman, Michael Ledbetter, Narendra Maheshri, Shawdee Eshghi

Ginkgo Bioworks, Boston, MA

NK cells are an attractive alternative to T cell-based therapies because they are capable of producing similar levels of anti-tumor cytotoxicity and are a potentially universal source for "off-the-shelf" CAR therapies. However, exploration of activating signals used for CAR-NK cell architectures has been fairly limited, with the majority of inputs being derived from T cell or NK cell receptors. We hypothesized that a more systematic exploration of diversified CAR signaling in NK cells could enhance their antitumor function and expedite their utility beyond hematological malignancies. To address this, we evaluated 10,000 unique signaling domain combinations in CAR-NK cells. We expressed our previously reported library of anti-ROR1 2nd generation CARs with diversified signaling domains in human primary peripheral NK cells, which we then screened for cytotoxicity and cytokine release following endogenous NK cell receptor blockade and solid tumor challenge by sorting for degranulation markers and intracellular cytokines. We found hits that were enriched across multiple selection criteria, with most containing activating and costimulatory domains from various immune cell types. Notably, the membrane proximal position drove stronger enrichment of these signaling domains, while the membrane distal position showed overall lower domain preference with the exception of 5 costimulatory domains. Furthermore, screening for IFNy secretion was the most stringent selection criteria and produced the lowest levels of enrichment, while screening for CD107a was the least stringent. Polyfunctionally enriched hits were validated individually, where novel combinations that outperformed standard CARs in NK cell cytotoxicity and cytokine secretion were identified. Our platform is enabled by our expertise in molecular diversification and high-throughput screening, making it readily adaptable to antigen receptors designs, immune cell types, and disease contexts beyond CAR-T and NK cells for use as cancer therapies. For example, this approach could be applied to other structural elements such as the ligand binding or hinge domains or secreted payloads, other types of chimeric receptors like switch receptors, and emerging non-conventional immune cell types such as macrophages, γδ T, or NKT cells. Furthermore, it could be applied to optimize for a diverse range of functions, including immune suppression in the context of autoimmune disorders. Thus, our pooled screening approach is uniquely positioned to provide biological and preclinical insights in a broad range of therapeutic applications.

822. CAR T Cell Based Inducible Protein Delivery: Application and Analysis in Immunocompetent Mice

Sherri L. Newmyer[1], Harikrishnan Radhakrishnan[1], Harold S. Javitz[2], Parijat Bhatnagar[1]

[1]Biosciences Division, SRI International, Menlo Park, CA,[2]Education Division, SRI International, Menlo Park, CA

Recent advancements in CAR T cell therapy, primarily used to treat blood cancers, are now being explored for delivering therapeutic proteins. Such proteins could strategically counter the immunosuppressive tumor microenvironment associated with solid tumors that represents a barrier to immunotherapy. Preclinical CAR T cell therapy validation typically occurs in immunocompromised mice xenografted with human tumors, which inadequately assess potential toxicities, like cytokine release syndrome. Addressing this gap, our study introduces a novel CAR-triggered protein delivery system in murine T cells, enabling analysis in immunocompetent mice with intact immune systems. Lentivirus transduction efficiently generated engineered murine CD4 and CD8 T cell subsets, achieving 40% and 20% transduction efficiencies that expanded cells ~20- and 75-fold, respectively. The engineered cells' response to tumor antigen used a nanoluciferase (Nluc) reporter. In vitro, CD8 T cells demonstrated approximately double the Nluc expression of CD4 cells when exposed to MSLN-expressing A2780 tumor cells. Membrane-bound IL15 transgene was incorporated to enhance CAR-T cell persistence, comparing three anti-mesothelin (MSLN) CAR T cell variants: CAR alone, CAR with constitutive IL15 (cIL15), and CAR with inducible IL15 (inIL15). IL15 co-engineering did not significantly impact Nluc expression or cellular expansion upon coculture with MSLN expressing tumor cells. In vivo, anti-MSLN->Nluc CD4 and CD8 T cells were injected into C57BL/6 mice engrafted with pancreatic cancer model expressing MSLN. Here, membrane bound IL15 improved CAR T cell expansion within the tumor and was notably associated with Nluc activity measured in tumors treated with CAR + cIL15 with the CD8 T cell chassis. In contrast to inIL15, cIL15 increased CAR T cell expansion in peripheral tissues. These findings reveal that murine CD8 CAR T cells, in contrast to CD4 CAR T cells, demonstrate superior tumor-targeted reporter protein expression both in vitro and in vivo. The inclusion of IL15 enhances overall T cell persistence and targeted protein expression in vivo, improving cell therapy potency. Rendering IL15 expression inducible further drives tumor-specific activity, improving the precision of cell delivered therapy. This study highlights the potential of engineered CAR T cells for the delivery of therapeutic proteins in immunocompetent models, offering a more accurate assessment of therapeutic efficacy and safety.

823. Enhancing the Efficacy of Anti-HER2-JAK/STAT-CAR-T Cells through Shortening the Cell Production Period

Yasunori Amaishi, Ryosuke Nagata, Kumiko Uemura, Seina Inui, Yoko Kudo, Sachiko Okamoto

Takara Bio Inc., Kusatsu, Shiga, Japan

Human epidermal growth factor receptor 2 (HER2)-specific antibody or antibody-drug conjugate therapy is widely used for the treatment of HER2-positive tumors, including breast cancer and gastric cancer.

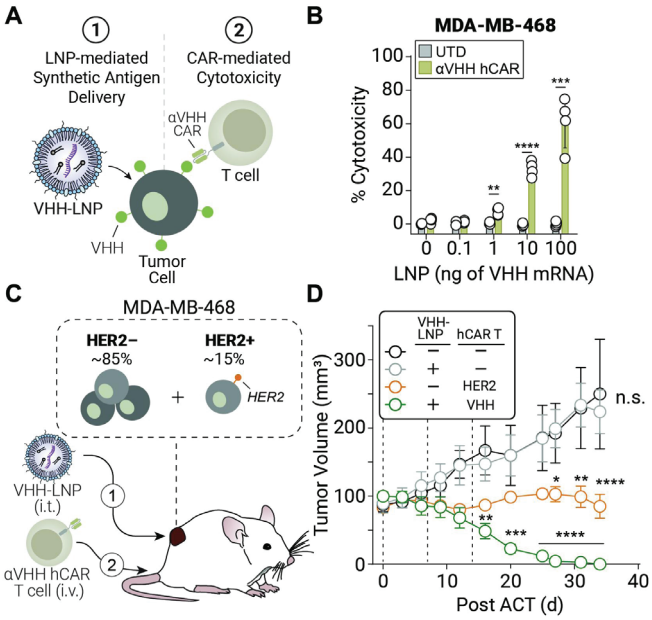
However, there is still a need for the development of alternative therapies due to the recurrence of tumors and the difficulty in achieving a cure for many patients. One promising solution is CAR-T therapy, which has demonstrated remarkable efficacy in targeting hematological tumors expressing antigens like CD19 and BCMA. However, its clinical effectiveness in solid tumors remains inadequate. Enhancing the persistence of CAR-T cells in the body is one of the challenges that needs to be addressed in CAR gene therapy. Our novel JAK/STAT CAR, a next-generation CAR, demonstrates antigen specific activation of the JAK/STAT signaling pathway through a truncated intracellular domain of the IL-2 receptor beta chain and a STAT3/5 binding motifs. Consequently, JAK/STAT CAR-T cells show lower expression of exhaustion markers compared to conventional CAR-T cells, exhibits antigen-specific high cell proliferation, and maintain long-term cytotoxic activity against continuous antigen stimulation. With these characteristics, JAK/STAT-CAR-T cells have sustained anti-tumor activity and are expected to be effective against solid tumors. In this study, we utilized three antibodies that recognize HER2 to construct anti-HER2-JAK/STAT-CAR expression vectors and Fc fusion proteins. The 4D5-8 and 2C4 clones were derived from antibodies already approved for clinical use, namely, Trastuzumab and Pertuzumab, respectively. The C6.5 clone is widely used in research targeting HER2. However, each clone exhibits different epitope recognition and affinity toward the HER2 molecule. We compared the reactivity of Fc fusion proteins and CAR-T cells against various HER2-positive tumor cell lines. The reactivity varied depending on the intensity of HER2 expression and scFv clone, but particularly strong correlation was observed between reactivity towards HER2-low-expressing PC-3 and Trastuzumab-resistant JIMT-1 cells and antibody affinity. CAR-T cells derived from the 4D5-8 and 2C4 antibodies demonstrated high antitumor activity in vivo, indicating their potential efficacy in clinical settings.Another challenge in CAR-T cell therapy is the cost of T cell manufacturing and the differentiation and exhaustion of T cells during the manufacturing process. Anti-HER2 (2C4)-JAK/STAT-CAR-T cells prepared using the Spo-T™(Short period operation for T-cell production) method showed higher cytotoxic activity and lower exhaustion than those using conventional methods. Furthermore, these cells demonstrated long-term survival and inhibited tumor engraftment in an in vivo experiment using a mouse model simulating tumor recurrence.Based on these results, anti-HER2-JAK/STAT-CAR-T cells prepared using the Spo-T™ method exhibited high antitumor activity, long-term survival, and the ability to suppress tumor recurrence. Therefore, anti-HER2-JAK/STAT-CAR-T cells have the potential to be a promising option for the treatment of HER2-positive tumors.

824. Sensitizing Solid Tumors to CAR-Mediated Immunity by Lipid Nanoparticle Delivery of Synthetic Antigens

Lena Gamboa[1], Ali Zamat[1], Chloé Thiveaud[1], Hee Jun Lee[1], Zizhen Zha[1], Elif Kulaksizoglu[1], Noah Campbell[1], Ching Shen Chan[1], Sydney Fábrega[1], Abbey Oliver[1], Fang-Yi Su[1], Hathaichanok Phuengkham[1], Daryll Vanover[1], Hannah Peck[1], Anirudh Sivakumar[1], Shreyas Dahotre[1], Adrian M. Harris[1], Philip Santangelo[2], Gabriel A. Kwong[1]

[1]Georgia Institute of Technology, Atlanta, GA,[2]Georgia Tech, Atlanta, GA

Despite the therapeutic efficacy of chimeric antigen receptor (CAR) T cells to treat certain hematological malignancies, their clinical success against solid tumors remains limited. Technical challenges associated with the identification of neoantigens characteristic of individual tumors - along with the need to engineer and validate CARs or TCRs against new targets - are significant bottlenecks to achieving therapeutic efficacy across tumor types. Additionally, identifying targetable tumor-associated antigens (TAA) remains challenging as heterogenous antigen expression, low antigen density, and antigen loss reduce CAR T cell efficacy and raise concerns with antigen escape. Expression of CAR antigens at low-levels in healthy tissue also pose safety concerns arising from on-target off-tumor toxicities. T cells targeting solid TAAs (e.g., HER2), for example, show cross-reactivity with healthy cells in vital organs that limit safe and effective clinical translation. Collectively, these limitations underscore the need to develop strategies that reduce the bottleneck of antigen selection and enable CAR T cell recognition of tumors otherwise lacking targetable antigens. Here, we developed synthetic antigens to sensitize solid tumors to CAR T cell mediated immunity agnostic to a tumor's endogenous antigen expression profile. We repurposed a xenogeneic protein, the antigen binding fragment of the heavy-chain-only camelid antibodies (VHH), as a synthetic antigen. We deliver VHH as an mRNA-encoded synthetic antigen using lipid nanoparticles (LNPs) and demonstrate stable expression on the surface of various tumor types - including breast, colon, lung, prostate, and brain cancer - which subsequently led to recognition and killing by αVHH CAR T cells (**Fig. 1A-B**). Moreover, we show that αVHH CARs are well tolerated based on the measurement of cytokine release in a coculture of human αVHH CARs T cells with PBMCs as well as serum chemistry analysis in naïve C57BL/6J mice up to 100 days following adoptive transfer of murine αVHH CARs T cells. Adoptive transfer of αVHH CAR T cells to mice bearing VHH-treated tumors reduced tumor burden in multiple syngeneic and xenograft models of cancer, improved survival, induced epitope spread, prevented tumor growth after rechallenge, and mitigated antigen escape in heterogenous tumors (**Fig. 1C-D**). Our data show that sensitizing tumors to CAR-mediated cytotoxicity using synthetic antigens can potentiate antitumor immunity and improve responses against solid tumors. **Caption**: **Fig. 1** | (**A**) Following LNP-mediated synthetic antigen delivery, tumor cells express VHH and become sensitized to killing by αVHH CAR T cells. (**B**) Cytotoxicity of MDA-MB-468 tumor cells following coincubation with untransduced (UTD) or αVHH CAR T cells at various VHH-LNP doses. 2-way ANOVA, mean ± s.e.m., n=4. (**C**) Mice bearing a heterogenous tumor mixture comprised of 85% HER2- and 15% HER2+ tumor cells were treated with i.t. with either saline or VHH-LNP followed by transfer of αVHH or αHER2 CAR T cells (**D**) Tumor growth curves of following i.t. treatment with with saline or VHH-LNP and systemically with indicated T cells. 2-way ANOVA, mean ± s.e.m. n= 4-5.



825. Manufacturing CAR T Cells with DNA Methylation Inhibitors Enhances Their Fitness in Immunocompetent Solid and Brain Tumor Models

Zhongzhen Yi[1], Elton VanNoy[1], Abigail Lee[2], Katherine Chiappinelli[2], Dalia Haydar[1]

[1]Center for Cancer and Immunology Research, Children's National Hospital, Washington, DC,[2]Department of Microbiology, Immunology, and Tropical Medicine, George Washington University, Washington, DC

Epigenetic reprogramming and T cell exhaustion emerge as critical factors significantly impacting the persistence and effectiveness of chimeric antigen receptor (CAR) T cell adoptive immunotherapies in solid and brain tumors. Several of these processes are regulated by DNA methylation, an epigenetic modification that is crucial for gene regulation and function. Therefore, this study investigates whether targeting the dysregulated DNA methylome in T cells can improve immune activation and anti-tumor efficacy of engineered adoptive immune cells. We optimized a transduction protocol that incorporates adding DNA methyltransferase inhibitors (DNMTi) early during the manufacturing process of murine CAR T cells targeting B7-H3. We then used standard *in vitro* assays and immunocompetent solid and brain tumor models to evaluate whether DNMTi treatment improved CAR T cell effector functions by regulating activation, memory differentiation, and cytokine responses in adoptive T cells. In repeated-stimulation assays, CAR T cells generated in the presence of DNMTi exhibited significantly higher levels of expansion and persistence compared to CAR T cells without DNMTi exposure. Notably, CAR T cells manufactured in the presence of DNMTi persisted up to 12 repeat stimulations, in contrast to a maximum of 7 stimulations for CAR T cells generated in the absence of DNMTi treatment. Moreover, DNMTi-treated CAR T cells expanded up to 55-fold higher than T cells generated without DNMTi treatment upon repeated stimulation with B7-H3-positive tumor cells. This was associated with remarkably enhanced secretion of immune-stimulatory cytokines that persisted across repetitive stimulations. Furthermore, DNMTi-treated CAR T cells exhibited greater cytotoxicity at lower effector-to-target ratios compared to untreated CAR-T cells, with sustained differential cytotoxicity even after the 4th stimulation. Preliminary *in vivo* data from ovarian and brain tumor immunocompetent models further support the potential of DNMTi pre-treatment, showing improved T cell persistence and reduced dose requirements. Mechanistic studies suggest that DNMT inhibition enhances B7-H3 CAR T cell anti-tumor activity by regulating activation and memory differentiation of CAR T cells early in the production phase. This study proposes that DNMTi pre-treatment may offer a promising approach for optimizing CAR-T cell manufacturing, improving product quality, and enhancing its performance. Our findings hold potential for addressing challenges related to CAR T cell exhaustion and improving the overall efficacy of adoptive cellular therapies in the treatment of solid and brain cancers, warranting further exploration.

826. *In Vivo* Pooled Screening Platform for the Discovery of Optimized Chimeric Antigen Receptor (CAR) Design in T cells

Taeyoon Kyung[1], Joshua Mace[1], Matthew Forsberg[2], Nina La Vonne Denne[3,4], Siqi Zhao[1], Khloe Gordon Wei[1], Jai Raman[1], Narendra Maheshri[1], Christian Capitini[2], Krishanu Saha[3,4], Shawdee Eshghi[1]

[1]Ginkgo Bioworks, Boston, MA,[2]University of Wisconsin School of Medicine and Public Health, Madison, WI,[3]Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, WI,[4]Wisconsin Institute for Discovery, University of Wisconsin-Madison, Madison, WI

While Chimeric Antigen Receptor expressing T cells (CAR-T cells) have shown high response rates against hematological cancers in the clinic, they have shown limited efficacy against solid tumors due to lack of persistence and susceptibility to various immunosuppressive factors in the tumor microenvironment (TME). To address the suppressive TME, recent efforts to systemically discover novel intracellular signaling domains (ICDs) at scale have demonstrated success in engineering CAR designs with enhanced solid tumor efficacy. However, in vitro screens are inherently limited in mimicking the TME and other physiologically relevant aspects of adoptive cell therapy (ACT). Here, we developed an in vivo pooled screen approach to identify potent CAR-T designs in a CHLA-20 neuroblastoma xenograft model. We utilized a DNA-barcoded 10,000-member CAR ICD library that we previously used to identify novel ROR1 CAR ICDs with enhanced anti-tumor function. This library was cloned into a GD2 CAR-T backbone, GD2 CAR library-expressing primary T cells were injected into tumor-bearing mice at >100x coverage of our library size, and 2 weeks post-ACT, spleens and tumors were collected. Sequencing genomic DNA quantified the abundance of barcodes linked to each CAR ICD combination. Based on previous literature, we hypothesized that any remaining CAR-T cells at the tumor site and spleen would represent T cells with long-term persistence elicited by antigen-specific CAR ICD signaling. Novel ICD combinations were preferentially enriched across multiple replicate mice. We selected the top 100 shared hits and validated their anti-tumor function in an arrayed screen, assessing cytotoxicity, cytokine secretion profiles, proliferation, and memory and exhaustion phenotypes in a tumor rechallenge setting.

These functional datasets allowed us to dissect the effects of particular signaling components in T cells that endow long-term persistence and potentially resist immunosuppressive TME factors. In vivo pooled screen approach is readily applicable to identifying signaling modules driving favorable phenotypes of T cells against solid tumors. Further development of an in vivo screening platform in humanized mice could be a promising next step to discovering cell therapy products with strong translational potential.

827. Innovative This TCRT: Revolutionizing TCR-T Therapy for Solid Tumors without Gene Editing

Kaichun Liu, Yinhang Zhang, Shanshan Chen, Guanggui Xu, Jun Li

Fundamenta Therapeutics, Suzhou, China

Background: TCR-T therapy shows promise for treating solid tumors by targeting neoantigen peptides. However, its efficacy and individualized production are limiting factors. Our study introduces ThisTCRT, an innovative allogeneic TCR-T platform that bypasses gene editing. ThisTCRT employs a novel intracellular retention mechanism for endogenous TCRab, significantly enhancing efficacy against difficult targets like KRAS G12V/HLA-A*11:01. Methods: We developed ThisTCRT cells using a specialized vector encoding TCR chains and a KDEL-tagged anti-TCR scFv. Comprehensive in vitro and in vivo analyses assessed surface TCR expression, tumor cytotoxicity, and safety in a GvHD model. Results: ThisTCRT cells demonstrated a unique expression profile of exogenous TCRab with endogenous retention. They showed potent tumor-specific cytotoxicity, eradicating tumors in the Panc01 model without inducing GvHD, signifying a major advance in solid tumor therapy. Conclusions: ThisTCRT marks a breakthrough in TCR-T therapies, offering effective, off-the-shelf solutions for treating solid tumors. Its remarkable preclinical results warrant immediate clinical trials.

828. Antitumor Activities of Chimeric Antigen Receptor T cells Targeting Mucin-1 with Self-Secreting Anti PD-1 IgG Antibody

Nattarika Khuisangeam[1], Koramit Suppipat[2,3], Nattiya Hirankarn[2,4], Supanikar Tawinwung[2,5]

[1]Graduate Program in Medical Microbiology, Graduate School, Chulalongkorn University, Bangkok, Thailand,[2]Cancer Immunotherapy Excellence Center, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand,[3]Department of Research Affairs, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand,[4]Center of Excellence in Immunology and Immune-Mediated Diseases, Department of Microbiology, Chulalongkorn University, Bangkok, Thailand,[5]Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand

In various types of solid tumors, mucin-1 (MUC1) is overexpressed in hypoglysylated form which can be targeted using cellular immunotherapy. Chimeric antigen receptor (CAR) T cells targeting MUC1 have shown potential efficacy against multiple solid tumors. However, treatment using CAR T cells faces challenges in solid tumor due to the immunosuppressive microenvironment, particularly the overexpression of programmed death-ligand 1 (PD-L1) in solid tumors that can induce T cell exhaustion and dysfunction. To overcome immunosuppression and enhance the efficacy of MUC1 CAR T cells, we successfully generated MUC1 CAR T cells secreting anti PD-1 IgG antibody derived from nivolumab (MUC1.PD1) using retroviral system. The transduction level of MUC1.PD1 CAR was maintained at day 11 after transduction but was statistically lower than MUC1 CAR T cells (63.7% ± 3.96% and 82.8% ± 3.68%, n = 5). T cell expansion and cellular composition showed major subset was CD3+ T cell with a higher percentage of CD8+ than CD4+ T cells. To determine the cytolytic activities of the CAR Ts, the MUC1(-) 293T cells and two cholangiocarcinoma cell lines endogenously expressing MUC1 and PD-L1, HuCCT-1 and KKU-213, were used as targets. Both MUC1 and MUC1.PD1 CAR T cells showed anti-tumor activity against HuCCT-1 and KKU-213 in a dose-dependent manner but not 293T cell, indicating a specific antigenic response of the two CARs. However, the cytolytic activities of MUC1 and MUC1.PD1 CAR T cells were comparable. To explore the effects of the secreted anti PD-1 antibody, we used the repeated antigen stimulation assay, which determine the effector functions of T cells throughout multiple presentations of tumor cells. We found that MUC1.PD1 CAR T cells exhibited higher proliferation and lower expression of PD-1 compared with MUC1 CAR T cell after co-culture with MUC1+PD-L1+ tumor cells in vitro. Moreover, MUC1.PD1 CAR T cells decreased the tumor growth when compared with MUC1 CAR T cell in NOD-SCID mice bearing HuCCT-1 cholangiocarcinoma cell line. In conclusion, our work suggests that engineering CAR T cells to secrete anti PD-1 antibody tends to be more effective than second generation CAR T cells, partly owing to the enhanced T cell proliferation and the mitigated PD-1 expression after repeated antigen stimulation. Our study presents a strategy to improve the efficacy of CAR T cells.

829. Virus-Free CRISPR Knock-In of a Chimeric Antigen Receptor into *KLRC1* Generates Potent Target-Specific Natural Killer Cells

Keerthana Shankar[1,2], Isabella Zingler-Hoslet[1], Lei Shi[3,4], Varun Katta[5], Brittany Russell[1], Shengdar Q. Tsai[5], Christian Capitini[3,4], Krishanu Saha[1,2,4]

[1]Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, WI,[2]Wisconsin Institute for Discovery, University of Wisconsin-Madison, Madison, WI,[3]Department of Pediatrics, University of Wisconsin-Madison, Madison, WI,[4]Carbone Cancer Center, University of Wisconsin School of Medicine and Public Health, Madison, WI,[5]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN

Natural killer (NK) cells are strong candidates for "off-the-shelf" immunotherapy products due to their high cytotoxicity profile. However, their success against solid tumors remains suboptimal when compared to hematological malignancies due to the upregulation of NK-inhibitory ligands, such as HLA-E, within the tumor microenvironment. Moreover, the manufacturing of NK cell therapies largely relies on viral vectors, risking insertional mutagenesis. Here, we describe a robust gene editing platform utilizing CRISPR-Cas9 to disrupt the *KLRC1* gene, encoding for the HLA-E binding NKG2A inhibitory receptor, and virus-free insertion of large transgenes (>2.7

kb), including a GD2-targeting chimeric antigen receptor (CAR) within human primary NK cells. The use of Cas9 ribonucleoprotein (RNP) complexes yields high protein level knock-out of the *KLRC1* gene with 70% efficiency and specific insertion of the CAR transgene as high as 22% (*Figure A*). *KLRC1*-CAR NK cells display robust and specific cytotoxicity and cytokine release against GD2$^+$ human melanoma cells (*Figure B*). Moreover, deep sequencing analysis of the *KLRC1*-CAR NK cells shows minimal off-target activity, highlighting the specificity of our edit. Using a completely non-viral workflow, this work provides compelling evidence for the use of the CRISPR-Cas9 system to manufacture potent and effective allogeneic NK cell products from routine peripheral blood donation and collection.

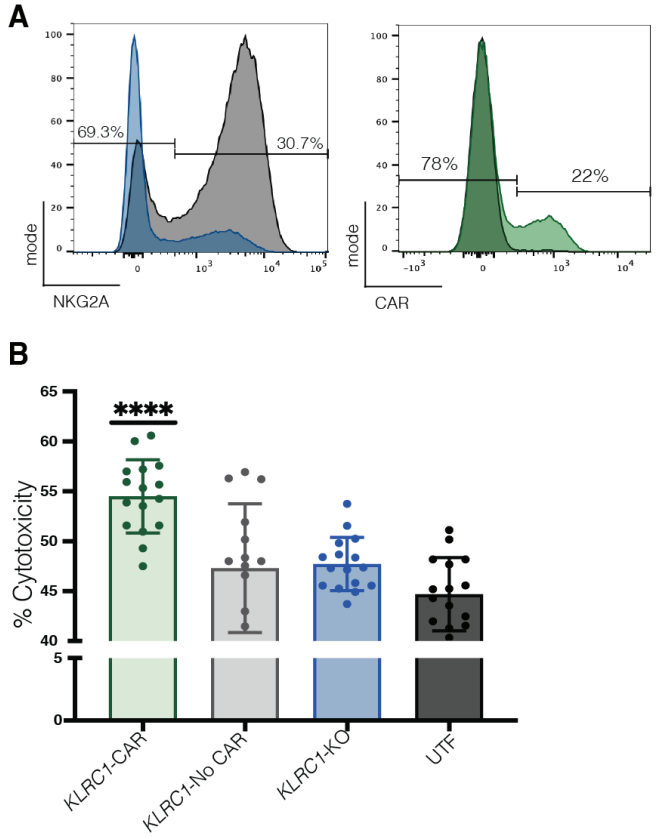


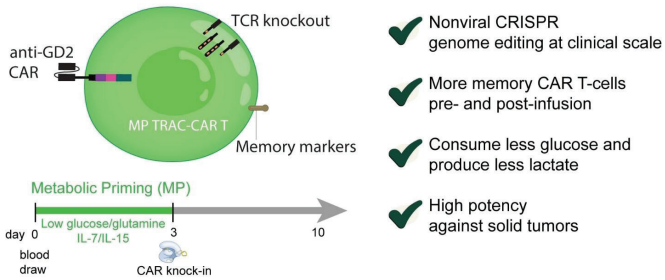
Figure. CRISPR-engineered anti-GD2 CAR NK cells show improved potency against melanoma target cells. **(A)** Representative histogram plots showing knockout of NKG2A and knock-in of CAR one week after nucleofection. X-axis describes protein expression levels. Y-axis describes frequency after normalization to the mode. UTF samples are shown as black histograms in both plots. The percent shown for each gate represents protein levels in the *KLRC1*-KO (left) and *KLRC1*-CAR (right) samples. **(B)** Following FACS, NK cells were co-cultured with M21 melanoma cells at a 5:1 E:T ratio for 24 hours, after which secreted LDH was measured with the CyQuant LDH Cytotoxicity Assay kit. Data is shown as the mean ± SD across three donors, with 5-6 replicates per donor (n = 3).

830. Metabolic Priming of GD2 *TRAC*-CAR T Cells During Manufacturing Promotes Memory Phenotypes While Enhancing Persistence

Dan Cappabianca[1], Dan L. Pham[1], Matthew H. Forsberg[1], Anna Tommasi[1], Tony Lauer[2], Jolanta Vidugiriene[2], Brooke Hrdlicka[1], Alexandria McHale[1], Quaovi Sodji[1], Melissa C. Skala[3], Christian M. Capitini[1], Krishanu Saha[1]

[1]University of Wisconsin-Madison, Madison, WI,[2]Promega Corporation, Fitchburg, WI,[3]Morgridge Institute for Research, Madison, WI

Manufacturing Chimeric Antigen Receptor (CAR) T-cell therapies is complex, with a limited understanding of how media composition impacts T cell phenotypes. CRISPR/Cas9 ribonucleoproteins can precisely insert a CAR sequence while disrupting the endogenous T cell receptor alpha constant (*TRAC*) gene, resulting in *TRAC*-CAR T cells with an enriched stem cell memory T-cell population, a process that could be further optimized through modifications to the media composition. In this study we generated anti-GD2 *TRAC*-CAR T cells using "metabolic priming" (MP), where the cells were activated in glucose/glutamine low media and then expanded in glucose/glutamine high media. T cell products were evaluated using spectral flow cytometry, metabolic assays, cytokine production, cytotoxicity assays *in vitro* and potency against human GD2+ xenograft neuroblastoma models *in vivo*. Compared to standard *TRAC*-CAR T cells, MP *TRAC*-CAR T cells showed less glycolysis, higher CCR7/CD62L expression, more bound NAD(P)H activity and reduced IFN-γ, IL-2, IP-10, IL-1β, IL-17, and TGFβ production at the end of manufacturing *ex vivo*, with increased central memory CAR T cells and better persistence observed *in vivo*. Metabolic priming with media during CAR T cell biomanufacturing can minimize glycolysis and enrich memory phenotypes *ex vivo*, which could lead to better responses against solid tumors *in vivo*.



831. Exploiting the Phosphocreatine Energy Pathway to Enhance CAR T Memory Differentiation and Cytolytic Activity

Zhiyang Gan, Alexander A. Shestov, Saba Ghassemi, Roddy S. O'Connor

Center for Cellular Immunotherapies, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA

What fuels CAR T cell serial killing is unknown. T cells rely on their spare respiratory capacity (SRC), a finite energy reserve, to support persistence following adoptive transfer. The SRC depletes following antigen stimulation in vivo, rendering it obsolete. Given this inevitable decline, we explored alternate energy systems to support CAR T cell

growth and differentiation. Competition for nutrients provides a barrier to adoptive immunotherapies, a feature exacerbated in solid tumors. We developed a "crossover" model to exploit the most efficient fuel source that can be stored prior to adoptive transfer. As the phosphocreatine (PCr) energy system supports energy production independent of oxygen, we hypothesized that "loading" CAR T cells during their ex-vivo expansion will enhance their functional competence in hostile conditions. Using a gain-of-function approach, overexpression (OE) of key creatine kinases (CK), CKM and CKMT2, led to enhanced cytotoxicity, reduced terminal differentiation, and exhaustion-resistant phenotype following repeat antigen encounter. Using metabolic analysis and electron microscopy, we showed that CK-OE CAR T cells preserve intact mitochondrial structure and higher levels of citrate as well ATP, suggesting our model where the PCr/Creatine energy system can overcome energy deficits and sustain mitochondrial function in tumor environments. We are currently exploring the impact of creatine/CK on CAR T cell function in xenograft models of leukemia and neuroblastoma. Our findings reveal a novel conditioning strategy and genetic approach to overcome CAR T cell metabolic dysfunction in hostile tumor environments.

832. Engineering a Bispecific Claudin 18.2/TGF-β CAR to Target Claudin 18.2 Positive Tumors and Overcome the Tumor Microenvironment

Jessica Reyes, Ethan BenDavid, Orit Foord, Ximin Chen, Michael Weist, Giulia Parisi, Jiajia Cui, Melanie L. Munguia

Research, ImmPACT Bio, West Hills, CA

Gastric cancer (GC) is among the top three deadliest cancers and has the fifth-highest global cancer incidence (1). Claudin 18.2 (CLDN 18.2) is an isoform of the tight junction protein Claudin 18 and is emerging as a promising target for CAR T cell therapies against GC. Limited expression of CLDN 18.2 is observed in normal tissues but is increased during malignant transformation as seen in GC, making this molecule an attractive target. (2). Recently, a clinical trial used CLDN 18.2 CAR T cells to treat patients with GC and pancreatic cancer. For the GC population, the overall response rate and disease control rate was 57.1% and 75.0%, respectively (3). The median progression-free survival rate was 4.2 months and 39% of patients required repeat infusions of the CAR T product, which speaks to the potential need to enhance T cell persistence and function in solid tumors. Studies have shown that GC patients have elevated levels of transforming growth factor-β (TGF-β) in their gastric mucosa (4). This highly pleiotropic cytokine has profound immunosuppressive effects that limit T cell persistence while also promoting tumor survival, immune evasion, and tumor extravasation into the TME. Moreover, TGF-β induces the differentiation of CD4+ T cells into Treg cells that in turn attenuate immune activation and production of proinflammatory mediators (5). Therefore, we intend to convert the TGF-β anti-inflammatory signal into a pro-inflammatory signal to ameliorate its inhibitory effects by enhancing T-cell function, proliferation, and infiltration of CAR T cells in gastric tumors. We designed a bispecific CAR to target both CLDN 18.2 and TGF-β. The CLDN 18.2/TGF-β bispecific CAR T cells were co-cultured with either CLDN 18.2 positive gastric cancer cell lines or with different isoforms of TGF-β. When co-cultured with CLDN 18.2 cell lines, both the single input (CLDN 18.2 only) and bispecific CAR T cells, showed high cytotoxicity with similar levels of T cell activation, evaluated by IFN-ɣ secretion. In addition, the bispecific CAR T cells were challenged with a CLDN 18.2 positive cell line, that endogenously secretes TGF-β, in a repeat antigen challenge (RAC) assay. The bispecific CAR T cells demonstrated reduced exhaustion markers and fewer Treg cells compared to the single input CAR T cells and the benchmark CLDN 18.2 CAR. In summary, we show that the CLDN 18.2/TGF-β bispecific CAR converts the immunosuppressive TGF-β signal into an activation signal, thereby enhancing T cell fitness, reducing Treg cell conversion, and decreasing phenotypic exhaustion.

833. Transforming Tumor Research: Engineering MHCs as Versatile Reagents

Sherry Weems

KACTUS, Waltham, MA

When dealing with solid tumors lacking specific biomarkers, the effectiveness of adoptive T cell therapy using CAR-T treatments is limited. The absence of dependable, multifaceted MHC reagents has been a hindrance in the research and development of treatments for solid tumors. To tackle this challenge, we have engineered MHC molecules in several formats with diverse applications. Our chimeric MHC format can be used for MHC immunization, leading to the discovery of TCR-like monoclonal antibodies that target peptides derived from solid tumor antigens. Our peptide-ready MHC format enables researchers to load peptides conveniently and with ease, whereas the single-chain MHC format can be used for enhanced sensitivity. Our VLP MHC format can be a viable alternative to the conventional tetrameric MHC format, potentially with increased avidity. These improved MHC molecules have the potential to facilitate solid tumor research and development. Form 1: Mammalian-Expressed SCT (Single-Chain Trimer) MHC for High sensitivity. The SCT MHCs incorporate linkers strategically, maintaining MHC integrity and antigen presentation. The SCT design, on the other hand, has been facing scrutiny over worries that its linker might cause interference with TCR. Our structural investigations of over 200 TCR-peptide MHC complexes, however, reveal that SCT MHC's linkers are positioned apart from crucial TCR, peptide, and MHC interfaces (Figure 1). Furthermore, we use mammalian cells to express our SCT MHC rather than the common way of producing it from E. coli expression and then refolding it from the inclusion body. Thus, our SCT MHCs offer improved stability and sensitivity compared to conventional MHC complexes with separate chains, making the SCT design a promising advancement for immune research. Form 2: Chimeric MHCs for Reduced Off-Targets Humoral immune responses to MHC-I seldom induce T-cell receptor mimic (TCRm) antibodies. Furthermore, many antibodies are thought to detect the α3 domain of MHC-I and the β2 microglobulin (β2m), which are not directly involved in delivering the target peptide. Substituting the MHC-I α3 and β2m domains with their murine counterparts preserved the MHC's antigen specificity for T-cells and supplied an acceptable antigen for TCRm antibody recognition. Here, we engineered our own chimeric human-murine MHCs designed to be expressed in mammalian systems to retain their innate conformation and activity (Figure 2). This should increase the likelihood of generating peptide-specific antibodies while decreasing the frequency of non- specific antibodies and making screening less

labor-intensive. Form 3: prMHC (Peptide-Ready MHC) for Rapid Neoantigen Loading Jurkat cell Jurkat cell with 1G4 TCR Vendor M: PE-labeled MHC tetramer (HLA*01:01) pre-loaded with NY-ESO-1 peptide Human APC-equivalent prMHC tetramer (HLA- A*02:01), His Tag mixed with NY-ESO-1 peptide The Peptide-Ready MHCs (prMHC) offer unparalleled ease and convenience for scientists. By providing MHC molecules without pre-loaded peptides, scientists gain the flexibility to select and load any peptide of their choice, tailored precisely to their experimental needs. This ready-to-be-load peptide feature not only saves valuable time spent on peptide-MHC complex preparation (Figure 3), but also offers the highest level of flexibility and experimental control.

834. Nonclinical Development of T-Plex Component TSC-204-A0101: A Natural TCR-T Cell Therapy for the Treatment of MAGE-A1- and HLA-A*01:01-Positive Cancers

Shazad A. Khokhar, Shubhangi Kamalia, Daniel C. Pollacksmith, Jenny Tadros, Sveta Padmanabhan, Drashti Shah, Amanda Kordosky, Maytal Bowman, Tary Traore, Victor Ospina, Alok Das Mahapatra, Sanket Revadkar, Kimberly M. Cirelli, Vivin Karthik, Kenneth L. Jahan Jahan, Nicolas Gaspar, Livio Dukaj, Jin He, Ryan E. Kritzer, Alexander Cristafaro, Chandan K. Pavuluri, Elisaveta Todorova, Tyler M. Sinacola, Savannah G. Szemethy, Kyra N. Sur, Vandana Keskar, Carolyn Hardy, Hsin-Ho Huang, Zhonghua Zhu, Sarah C. Potter, Kenneth J. Olivier, Nancy Nabilsi, Antoine J. Boudot, Sonal Jangalwe, Ribhu Nayar

Tscan Therapeutics, Inc., Waltham, MA

Background: T-Plex is an autologous TCR-T cell therapy product for the potential treatment of solid tumors comprising customized combinations of two to three TCR-T cell components that recognize different tumor antigens presented on specific HLA class I molecules. T-Plex cell therapy products are engineered using a transposon-based vector encoding the therapeutic TCR, CD8α and CD8β co-receptors, a CD34 epitope tag, a dominant-negative TGFβRII (DN-TGFβRII), and a mutated form of dihydrofolate reductase (DHFRdm). TSC-204-A0101 is intended for the treatment of MAGE-A1- and HLA-A*01:01-positive cancers. The TCR-T cells recognize the cancer-testis antigen MAGE-A1, which is highly expressed in solid tumors but is virtually absent in healthy tissues except testis. **Methods:** The TSC-204-A0101 TCR is a naturally occurring TCR discovered using TScan's proprietary ReceptorScan platform. TSC-204-A0101 TCR-T cells engineered using a process representative workflow for the planned manufacturing process were used to investigate the *in vitro* pharmacology and toxicology of TSC-204-A0101. TSC-204-A0101 was evaluated for avidity and target-dependent cytotoxicity, proliferation, and cytokine secretion *in vitro* as well as for anti-tumor efficacy *in vivo*. The contribution of DN-TGFβRII was assessed by measuring resistance to TGFβ-mediated induction of pSMAD2 of TCR-T cells. Further, TSC-204-A0101 was assessed for risk of alloreactivity and off-target recognition using TScan's SafetyScan screen. Furthermore, to assess the risk of off-target/off-tumor activity, TSC-204-A0101 TCR-T cells were tested for their reactivity to an extensive panel of 53 healthy human primary cells, including those expressing high levels of the putative off-targets of TSC-204-A0101. Finally, to demonstrate the benefit of combining individual TCR-T cell components as part of the T-Plex product for treatment of heterogenous solid tumors, target cancer cell populations were produced to simulate target and/or HLA loss. **Results:** TSC-204-A0101 displayed high avidity for the cognate peptide and target-dependent secretion of proinflammatory cytokines, cytotoxicity, and proliferation of both engineered CD4+ and CD8+ T cells and successfully controlled the growth of HLA-A*01:01+ MAGE-A1+ tumors in mice. TGFβ-mediated pSMAD2 induction was strongly decreased in TSC-204-A0101 TCR-T cells demonstrating the resistance of TCR-T cells to immune-suppressive effects of TGFβ. Further, TSC-204-A0101 displayed no alloreactivity to the 110 most common class I HLAs in the US population. Although putative off-targets were identified in the SafetyScan screen, TSC-204-A0101 showed no reactivity to normal primary cells. Lastly, when facing a target cell population simulating heterogeneous target expression, TSC-204-A0101 combined with a second TCR-T cell component in a defined T-Plex product led to the efficient killing of both cell types. **Conclusions:** TSC-204-A0101 exhibits high specificity and potency against MAGE-A1- and HLA-A*01:01-positive tumor cells with no projected allo- or off-tumor reactivity. Based on these results, an IND for the clinical development of TSC-204-A0101 has been filed with plans in place to incorporate TSC-204-A0101 in TScan's ongoing T-Plex clinical trial master protocol.

835. Non-Clinical Development of T-Plex Component TSC-201-B0702: A TCR-T Cell Therapy Directed to a Novel HLA-B*07:02 Restricted MAGE-C2 Epitope for the Treatment of Solid Tumors

Hannah L. Bader, Amanda Kordosky, Sonal Jangalwe, Alok D. Mohapatra, Dalena Nguyen, Debanjan Goswamy, Maytal Bowman, Nivya Sharma, Victor Ospina, Shazad A. Khokhar, Shubhangi Kamalia, Sveta Padmanabhan, Drasthi Shah, Daniel Pollacksmith, Sanket Revadkar, Kimberly M. Cirelli, Kenneth Jahan, Livio Dukaj, Jin He, Ryan E. Kritzer, Alexander Cristofaro, Chandan Pavuluri, Zhonghua Zhu, Carolyn Hardy, Hsin-Ho Huang, Olivia Pryor, Elisaveta Todorova, Tyler M. Sinacola, Savannah G. Szemethy, Kyra N. Sur, Vandana Keskar, Andrew Ferretti, Ribhu Nayar, Antoine J. Boudot, Cagan Gurer

TScan Therapeutics, Waltham, MA

Introduction: T-Plex is an autologous TCR-T cell therapy product comprised of two to three TCR-T cell components for the potential treatment of solid tumors from TScan's ImmunoBank, a repository of therapeutic TCRs each recognizing a different antigen presented by an HLA class I molecule. By combining components from the ImmunoBank, a multiplexed product is customized to match the target and HLA expression pattern of a patient's tumor. Each component of T-Plex is engineered using a transposon-based vector encoding the therapeutic TCR, CD8α and CD8β co-receptors, a CD34 epitope tag, a dominant-negative TGFβRII (DN-TGFβRII), and a mutated form of dihydrofolate reductase (DHFRdm). TSC-

201-B0702 is a new component of the ImmunoBank designed to recognize an HLA-B*07:02 epitope derived from the cancer-testis antigen MAGE-C2, which is frequently overexpressed in solid tumors but is absent in healthy tissues except testis. **Methods:** A novel HLA-B*07:02-restricted T cell epitope of MAGE-C2 was discovered with TScan's proprietary TargetScan platform and TScan's ReceptorScan platform was used to identify a potent naturally occurring TCR recognizing this epitope. The MAGE-C2 specific TCR was then used to build TSC-201-B0702. TSC-201-B0702 TCR-T cells were engineered using a full-scale representative workflow for the planned clinical manufacturing process and were used to investigate the *in vitro* pharmacology and safety of TSC-201-B0702. The specificity of TSC-201-B0702 for the HLA-B*07:02 restricted MAGE-C2 epitope was tested, and target-dependent cytotoxicity, proliferation and cytokine secretion was evaluated *in vitro* and *in vivo*. Resistance to TGFβ—conferred by expression of DN-TGFβRII—was evaluated by assessing TGFβ-mediated induction of phospho-SMAD2, and resistance to suppression of IFN-γ secretion. Further, TScan's SafetyScan was used to investigate allo- and off-target reactivity. In addition, the reactivity of TSC-201-B0702 TCR-T cells to a panel of 54 healthy HLA-B*07:02-positive human primary and iPSC-derived cells isolated from tissues that are traditionally assessed in toxicology studies was evaluated. **Results:** TSC-201-B0702 TCR-T cells recognized their cognate MAGE-C2 peptide in a dose dependent manner and displayed potent target-dependent secretion of inflammatory cytokines, cytotoxicity, and proliferation of both engineered CD4+ and CD8+ T cells. Moreover, TSC-201-B0702 TCR-T cells displayed anti-tumor activity against A101D xenografts in mice. Target-dependent IFN-γ production was maintained in the presence of physiological levels of TGFβ, and TGFβ-mediated phospho-SMAD2 induction was strongly reduced. Further, TSC-201-B0702 displayed no alloreactivity to the 110 most common class I HLAs in the US population. Although two putative off-targets were identified in the SafetyScan screen, TSC-201-B0702 showed no off-tumor reactivity to normal primary or iPSC-derived cells. **Conclusions:** TSC-201-B0702 exhibits high specificity and potency against MAGE-C2-positive, HLA-B*07:02 tumor cells with no projected allo- or off-tumor reactivity. Based on these results, an IND for the clinical development of TSC-201-B0702 has been filed with the aim of incorporating TSC-201-B0702 in the ongoing T-Plex Phase 1 clinical trial master protocol.

836. THRIVE™ Non-Viral Targeted Transgene Knock-In Platform Generates Specific and Potent TCR T Cell Products for the Treatment of Solid Cancers

Santosh Narayan[1], Nathaniel Swanson[1], Allison Drain[1], Ken Gareau[1], Nicholas Rouillard[1], Jinsheng Liang[1], Hui-Wen Liu[1], Lauren Brown[1], Rebecca Lamothe[2], Philip D. Greenberg[3], Christopher A. Klebanoff[4], Ankit Gupta[1], Damien Hallet[1], Gary Shapiro[1], Kim Nguyen[1], Loic Vincent[1]

[1]Affini-T Therapeutics, Watertown, MA,[2]Metagenomi Inc., Emeryville, CA,[3]Program in Immunology and Translational Science and Therapeutics Division, Fred Hutchinson Cancer Research Center, Seattle, WA,[4]Memorial Sloan Kettering Cancer Center, New York, NY

Introduction: Selective and homogenous expression of oncogenic driver mutations such as $KRAS^{G12D}$ and $p53^{R175H}$ make them ideal targets for cancer therapies. Adoptive T cell therapies with engineered T cell receptors (TCRs) recognizing mutated driver proteins have led to durable responses in patients with solid tumors. Lentiviral vector (LVV)-based T cell manufacturing suffers from limited cargo size, heterogeneous transgene expression, high manufacturing cost and complexity, and the potential for oncogenesis due to random genomic insertions. To overcome these challenges, we have developed THRIVE™, a non-viral, gene-editing-based T cell manufacturing platform that achieves high rates of targeted transgene insertion and robust cell expansion, and yields potent TCR T cell therapies. Methods and Results: An efficient and specific novel type V CRISPR-Cas system was used for targeted transgene knock-in (KI) at the *TRAC* locus via homology directed repair (HDR) in primary human $CD4^+$ and $CD8^+$ T cells. The optimized HDR template and engineering process achieved efficient KI and expression of multi-cistronic constructs up to 7 kb in size that included: 1) a TCR specific for an oncogenic driver mutation, 2) a CD8αβ coreceptor allowing for effective $CD4^+$ T cell target recognition and stimulation, and 3) T cell function-enhancing synthetic fusion proteins. Unlike LVV-mediated random insertions, genotypic analysis of THRIVE-produced T cells showed targeted transgene insertion at the *TRAC* locus that also disrupted the endogenous TCRα chain, resulting in increased surface expression of the transgenic oncogene-specific TCR and enhanced antigen sensitivity. Thus, THRIVE gene-edited T cells demonstrated improved efficacy as compared to LVV-engineered T cells both *in vitro* during repeat tumor cell challenges and *in vivo* after single administration in mice harboring tumor xenografts. Gene-editing safety evaluation demonstrated specificity with no off-target activity in high sensitivity (~0.1%) NGS-based analyses or any gene-editing-related chromosomal rearrangements. When evaluated at expected clinical scale, the THRIVE platform yielded up to 50% transgene-positive T cells that showed a favorable T stem cell memory/ T central memory phenotype and >50-fold expansion achieving anticipated dose levels for clinical trials. Conclusion: The non-viral targeted knock-in platform, THRIVE, enables safe and cost-effective T cell engineering with high efficiency. THRIVE-engineered oncogene driver-specific TCR T cells demonstrated superior cytotoxicity against tumor cells relative to LVV-engineered cells both i*n vitro* and *in vivo*. These data support the clinical readiness and advancement of the THRIVE platform for engineering TCR T cell therapy for the treatment of advanced solid malignancies.

837. Generation of Allogeneic CAR T-Cells Using an Easy, Safe, and Effective Non-Gene Editing Technology

Matteo Rossi, Fanny Huberty, Jérôme Marijsse, Thuy Nguyen, Céline Jaques-Hespel, Eytan Breman

Celyad Oncology, Mont-Saint-Guibert, Belgium

Chimeric antigen receptor (CAR) T-cells have revolutionized the treatment of hematological malignancies. However, to date only approximately 10% of eligible patients can receive this treatment, due to the several drawbacks of autologous CAR T-cell production (difficulty to obtain high quality starting material from patients, limited manufacturing capacity, complex logistics, long vein-to-vein time, high costs). Allogeneic, "off-the-shelf" CAR T-cells could help

circumvent these issues, but their successful clinical application relies on the ability to manage graft-versus-host disease (GvHD) and host-versus-graft (HvG) reaction. To address these issues, a variety of gene-editing strategies have been attempted to ablate the expression of the molecules primarily involved in GvHD (T-cell receptor, TCR) and HvG (Human Leukocyte Antigen, HLA, class-I and class-II) on the CAR T-cell surface. However, these strategies are burdened by the technical complexity of effectively targeting multiple genes simultaneously and by the potential risk for introducing harmful genomic aberrations. As an alternative, we successfully applied our proprietary, clinically validated, non-gene editing technology to the generation of allogeneic CAR T-cells, by targeting genes essential in GvHD, HvG and T-cell apoptosis. Our approach is based on a microRNA (miRNA)-derived short hairpin RNA (shRNA) platform, which does not require the direct alteration of the genome, thus ensuring a high safety profile. Moreover, our small miRNA expression cassette, able to target up to four different genes simultaneously, can be deployed in a single construct with the other components required for CAR T-cell generation, easing the technical aspects of allogeneic CAR T-cell manufacturing. Most importantly, our technology allows for the fine-tuned modulation of gene expression. This is especially relevant for targets such as HLA class-I, whose complete ablation triggers recognition by NK cells, calling for further interventions (e.g., HLA-E overexpression). Thanks to the tunability of gene expression achievable with our platform, we obtained functionally relevant downregulation of HLA class-I to avoid recognition by the recipient's T-cells, ensuring at the same time sufficient expression to let CAR T-cells escape NK recognition. Allogeneic CAR T-cells generated through our miRNA-based shRNA technology displayed protection against GvHD, HvG and apoptosis in specifically designed *in vitro* assays, leading to significantly improved CAR T-cell survival while maintaining comparable efficiency to their non-engineered counterparts. Overall, we provide *in vitro* proof-of-concept that our miRNA-based shRNA technology can improve CAR T-cell viability by allowing evasion from GvHD and HvG and target-cell induced apoptosis. This in turn demonstrates the feasibility of the generation of allogeneic CAR T-cells via a non-gene editing approach, making our technology stand out as an easy, safe, and effective strategy for the generation of allogeneic CAR T-cells.

838. Two Distinct Activation Methods Yield Clinical-Scale Expansion of Peripheral Blood Derived γδT Cells

Chengkang Zhang, Amy Burkall, Elizabeth Kagan, Karen Roderick, Erin Brooks, Kenneth Low, Alexis Bossie

Lonza Walkersville Inc, Rockville, MD

Gamma delta (γδ) T cells have the inherent ability to infiltrate solid tumors and can directly recognize and kill transformed cells independently of HLA-antigen presentation. Moreover, γδ T cells do not cause graft-versus-host disease (GVHD) and hence, provide a promising platform for the development of cell therapies targeting solid tumors. However, due to the low prevalence of γδ T cells in peripheral blood, it remains a challenge to generate sufficient numbers of γδ T cells *ex vivo* to produce a clinical dose. In this study, we demonstrate that billions of γδ T cells can be generated from peripheral blood in the 2L rocking motion platform with two distinct activation methods, by using TheraPEAK® T-VIVO® Medium - a chemically defined, non-animal origin, serum-free medium. In the first method which uses 6μM zoledronic acid as the activating agent, more than 1e9 Vδ2 T cells are produced within 14 days starting from 500e6 peripheral blood mononuclear cells (PBMCs). Furthermore, using a process that specifically depletes αβ T cells from PBMCs prior to anti-CD3 and anti-CD28 co-stimulation, billions of γδ T cells - including both Vδ1 and Vδ2 T cell subsets - are produced from the same PBMCs source. The γδ T cells expanded using either activation method exhibit innate cytotoxicity towards the K562 cell line and produce cytokines including IFN-γ and TNF-α *in vitro*. Both activation methods and expansion protocols may be utilized to generate sufficient numbers of γδ T cells to support clinical applications.

839. Single-Cell, Pooled CRISPR Screening Reveals T Cell Intrinsic Regulators of Exhaustion in the Context of Chronic Antigen Stimulation

Glenn G. Wozniak[1], Sahil Joshi[1], Kristina Vucci[1], Angela Boroughs[1], Andrew Cardozo[1], Ashley Cass[1], Bob Chen[2], Julie Chow[1], David DeTomaso[1], Celine Eidenschenk[2], Jake Freimer[2], Brendan Galvin[1], Katie Geiger-Schuller[2], Levi Gray-Rupp[1], Jan-Christian Huetter[2], Jacob Levine[2], Allyson Merrell[1], Catherine Oh[1], Soyoung Oh[2], Sascha Rutz[2], Jill Schartner[2], Mandi Simon[1], Pratiksha Thakore[2], Li Wang[1], Emily Wheeler[2], Grace Zheng[1], Orit Rozenblatt-Rosen[2], W. Nicholas Haining[1], Ira Mellman[2]

[1]Arsenal Biosciences Inc., South San Francisco, CA,[2]Genentech Inc., South San Francisco, CA

T cell therapies for solid tumors are limited by T cell exhaustion resulting from chronic antigen stimulation, and by an immunosuppressive tumor microenvironment. Engineering chimeric antigen receptor (CAR) T cells that are able to resist exhaustion represents a promising strategy in the development of potent immunotherapies. To this end, we sought to identify T cell intrinsic genetic perturbations that can prevent exhaustion, a state not only characterized by altered cell function but also by distinct transcriptional and epigenetic alterations. In order to screen many perturbations at once for their effect on exhaustion, we established a pooled, in vitro, CRISPR/Cas9-based screening approach paired with multimodal, single cell readouts in the context of chronic antigen stimulation. To achieve this we developed a lentiviral delivery method to generate pools of genetically engineered CAR T cells, wherein each cell carries a CRISPRko, CRISPRi or CRISPRa perturbation to achieve up- or downregulation of a single target gene. Subjecting these T cell pools to chronic antigen stimulation using a cell-based repetitive stimulation assay (RSA) recapitulated key hallmarks of T cell exhaustion, including loss of proliferation and effector function. Multimodal, single cell characterization of engineered T cells throughout the assay revealed not only acquisition of transcriptional and epigenetic signatures of exhaustion, but also a diverse landscape of perturbation-dependent T cell states. Moreover, we observed clustering of perturbed genes with similar biological functions based on similar gene expression changes allowing for prioritization of genetic perturbations to be tested in next-generation CAR T cells.

These results demonstrate the utility of pooled CRISPR screening to measure complex phenotypes and to direct the development of advanced CAR T cell therapies.

840. Sugar-Conjugated Poly(Beta-Amino Ester) Nanoparticles for Targeted mRNA Delivery to Immune Cells *In Vivo*

Tiarra R. Warren, Stephany Yi Tzeng, Jordan J. Green
Johns Hopkins University, Baltimore, MD

Introduction. Intracellular mRNA delivery systems have been remarkable in the context of vaccination, spurring the exploration and development of a wide array of carriers. There is an opportunity for these carriers to be designed to exhibit unique tropisms tailored for various therapeutic targets. Poly(beta-amino ester)s (PBAE) are cationic, hydrolytically degradable polymers that can deliver various nucleic acid cargos and reach several organs and tissues. However, there is a need for further enhancement of delivery efficiency and specificity following systemic injection. This study investigates the utilization of sugar-targeting ligands to enhance the precision of mRNA/PBAE nanoparticles towards immune cells. *Methods*. PBAEs were synthesized using Michael addition reactions, with the structure optimized for mRNA delivery. Nanoparticles (NPs) were formed by mixing polymer, mRNA, and sugar conjugates in buffer and allowing self-assembly to occur. For *in vivo* transfection studies, Cre mRNA NPs were injected into Ai9 mice intravenously (loxP-flanked STOP cassette removal led to tdTomato expression), sacrificed 3 days post-injection and organs were collected. For uptake studies, Cy3-labeled mRNA NPs were injected into C57BL/6 mice intravenously and sacrificed 2 hours post-injection. The tissues were processed into single cell suspensions and prepared for flow cytometry. Staining included markers for neutrophils, dendritic cells, Kupffer cells, M1 monocytes and macrophages. Fluorescence of Cy3 indicated particle uptake and expression of tdTomato indicated successful particle transfection via flow cytometry. *Results*. Within liver samples, NPs without sugar targeting displayed high cellular internalization into M1 monocytes and macrophages (20%) and Kupffer cells (40%), a liver-specific macrophage population (Fig. 1A). These populations also had positive transfection of ~10% and ~5%, respectively. Sugar-targeted nanoparticles (Fig. 1B), dramatically reduced total NP uptake in the liver, including especially by Kupffer cells to a measured value of Cy3 uptake of ~2%. Positive transfection of neutrophils, Kupffer cells, monocytes and macrophages was observed in the range of 3-7%. Interestingly, the results indicate that the efficiency of uptake leading to successful transfection (instead of NP degradation) improved with the sugar-mediated uptake. The results also indicate that immune cells that only internalized a small amount of Cy3-labeled NPs (such that the fluorescence signal did not shift significantly by FACS), nonetheless were able to lead to successful Cre-mediated recombination (which has a stronger fluorescence signal). Cellular uptake and transfection of other organs with these particles (and analogs) is also currently being investigated and will be further discussed. *Conclusion*. The inclusion of sugar-conjugated to PBAE/mRNA nanoparticles reduces uptake by Kupffer cells in the liver and increases the efficiency of transfection in immune cells post cellular uptake, including in monocyte and macrophage populations in the liver. These sugar-conjugated PBAE/mRNA particles hold promise as a targeted platform for effective gene delivery, specifically designed to target monocytes and macrophages.

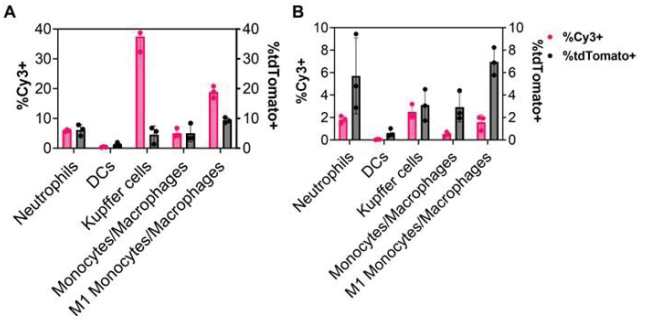


Figure. 1 *In vivo* biodistribution and transfection in the liver by PBAE mRNA nanoparticles following intravenous administration. (A) NPs without sugar conjugation. (B) NPs with sugar conjugation.

Wednesday Posters: Other Cellular and Regenerative Therapies

841. The Role of ZEB1 in Neural Stem Cells; Powerful Strategy for Stem Cell Therapy in Multiple Sclerosis

Elham Poonaki[1,2], Ulf Dietrich Kahlert[3], Ali Gorji[2], Sven G. Meuth[1]
[1]Department of Neurology, Heinrich Heine University, Düsseldorf, Germany,[2]Department of Neurosurgery, University of Münster, Münster, Germany,[3]Molecular and Experimental Surgery, Faculty of Medicine, Magdeburg, Germany

Stem cell therapy is one of the main remedies for curing some chronic neurological disorders such as multiple sclerosis (MS). However, how to overcome the failure of neural cell transplantation following treatment remains controversial. The key role of Zinc Finger E-Box Binding Homeobox 1 (ZEB1) has a well-established role in both inflammatory signaling and epithelial-to-mesenchymal transition (EMT) regulation. Since neuroinflammation is one of the main hypotheses for the failure of stem cell transplantation in MS, this work studied the inflammatory response of neural stem cells (NSCs) by down-regulating the ZEB1 gene. First, we looked at ZEB1 expression in neural stem cells (NSCs) isolated from the subventricular zone (SVZ) of rat brains and differentiated into mature myelinating oligodendrocytes (OLs) using our standard methodology. The ZEB1 gene was then downregulated in neuronal stem cells, and its effect on migration and neuroinflammatory cytokines like IL6, IL1β, TNFα, and IL17 was studied using immunocytochemistry (ICC), qPCR, wound healing, ROS, and colony formation in a simulated inflammatory condition with LPS soup derived from brain microglia. The results demonstrated that silencing the *ZEB1* gene significantly affected the expression of neuroinflammatory cytokines, colony formation, and migration in neuronal stem cells. In conclusion, more research is needed to fully understand the role of ZEB1, even though targeting it may be useful for localizing neuronal stem cells.

842. Preclinical Characterization and Emerging Clinical Trial Results for NRTX-1001 - A Human Pluripotent Stem Cell-Derived Inhibitory Interneuron Cell Therapy for Drug-Resistant Chronic Focal Epilepsy

Marina Bershteyn, Yves Maury, Luis Fuenteabla, Robin Zhou, Steven Havlicek Havlicek, Sonja Kriks, Seonok Lee, Geetha Subramanyam, Meliz Sezan, Mansi Parekh, Eric Sevilla, Alexandra Vogel, Philip Hampel, Brianna Feld, Rose Larios, Ji-Hye Jung, Tia Kowal, Shrei Madrid, Alessandro Bulfone, John Hixson, Gautam Banik, Catherine Priest, David Blum, Cory R. Nicholas

Neurona Therapeutics, South San Francisco, CA

Epilepsy is one of the most common central nervous system disorders, characterized by hyperactive neuronal networks that precipitate and propagate seizures. Pallial inhibitory GABAergic interneurons regulate excitatory neural activity and, when lost or dysfunctional, can lead to epilepsy. Anti-seizure drugs (ASDs) largely dampen overexcitation and decrease seizures, but can cause adverse effects due to systemic administration. Moreover, one-third of individuals with epilepsy have drug-resistant seizures that are not ameliorated with ASDs. For some people with focal epilepsy, surgical resection or laser ablation of a seizure focus can be an effective option; however, these procedures are tissue-destructive, risk permanent cognitive deficits, and are not indicated for all. Cell therapy represents an alternative therapeutic strategy to administer inhibitory interneurons that secrete GABA to the seizure focus to potentially suppress chronic seizures. NRTX-1001 is an investigational cellular therapeutic candidate comprising GABAergic, post-mitotic interneurons of a specific pallial lineage derived from human pluripotent stem cells. Clinically compliant processes were developed to reliably manufacture and cryopreserve NRTX1001, with an assessment of cell identity, composition, neuronal migration, GABA secretion, sterility, and viability. Following transplantation into the rodent brain, human graft composition and neuronal subtype fate were investigated for up to 18 months using single nuclei RNA sequencing, demonstrating long-term persistence and stable composition of GABAergic pallial interneuron subtypes. A single intrahippocampal administration of NRTX-1001 into mice with chronic drug-resistant temporal lobe epilepsy (TLE) resulted in pronounced and dose-dependent suppression of focal seizures and reduction of hippocampal pathology. Approximately two-thirds of epileptic mice were stably seizure-free after receiving NRTX-1001. A phase I/II clinical trial (NCT05135091) is underway to evaluate NRTX-1001 safety and efficacy in people with drug-resistant TLE. The initial stage of the trial includes an open-label dose escalation in up to 10 subjects, and we will discuss the preliminary clinical data. The first two subjects are now past one year after receiving NRTX-1001, and have reported >95% seizure reduction from baseline, improved performance on select visuospatial neurocognitive memory tests, and an absence of any serious or severe adverse events to date. In summary, these preclinical and clinical data highlight the promise of human inhibitory interneuron cell therapy for the potential future treatment of chronic focal epilepsy.

843. The Preclinical and First-in-Human Study of iPSC-Derived Human Forebrain Neural Progenitor Cell Injection-hNPC01 in Treating Chronic Ischemic Stroke

Jing Fan[1,2], Anxin Wang[1,2], Fang Ren[1,2], Yinggang Yan[2], Shuning Zhang[1,2], Jasmine Wang[1]

[1]Hopstem Biotechnology, Houston, TX,[2]Hopstem Bioengineering Ltd. Co., Hangzhou, China

The hNPC01 injection, is an injection containing Human Forebrain Neural Progenitor Cells (hFNPCs) derived from induced Pluripotent Stem Cell (iPSC) and manufactured under GMP condition. This allogenic cell therapy product contains more than 95% hFNPCs and its progeny cells, and was able to differentiate further into functional cortical neurons and glia cells both *in vitro* and *in vivo*. In previous mechanism and preclinical studies, hNPC01 showed potential in promoting functional recovery of ischemic stroke rat and cynomolgus monkey models and forming neural circuitry *in vivo*. The preclinical safety study programs including tumorigenicity study in NOG mice up to 52 weeks indicated a good safety profile of hNPC01. A phase I open-lable dose-escalation clinical study of hNPC01 injection treating chronic ischemic stroke by intracerebral injection is approved by NMPA and currently undergoing. The current observation of first dose cohorts showed good safety and tolerance of hNPC01, as well as sign of motor function improvement in volunteer subjects with ischemic stroke onset 17 to 40 months prior to the enrollment. It is expected that the 12 month safety observation and exploration of evaluation tools will provide more valuable information for the future development of iPSC cell therapy for stroke or traumatic brain injury caused brain injury patients with chronic symptoms.

844. Transplantation of Gene-Edited Hematopoietic Stem Cells Rescues Neuronal and Cardiac Complications in a Mouse Model of Friedreich's Ataxia

Anusha Sivakumar[1], Alexis N. Corl[1], Angelyn B. Solis[1], Lilas Rony El-Hachem[1], Rishika Murthy[1], Rafael Andres Badell-Grau[2], Rita Wan[1], Eric Thai[1], Veenita Khare[3], Stephanie Cherqui[4]

[1]Department of Pediatrics, Division of Genetics, University of California, San Diego, La Jolla, CA,[2]UCSD, La Jolla, CA,[3]UCSD, San Diego, CA,[4]University of California, San Diego, La Jolla, CA

Friedreich's ataxia (FRDA) is a multi-systemic, autosomal recessive, neurodegenerative disorder caused by homozygous GAA repeat expansion mutation in intron 1 of frataxin gene (*FXN*). FRDA is characterized by ataxia, areflexia, sensory loss, muscle weakness, and cardiomyopathy. Symptoms typically begin between 5 to 15 years of age and patients will be in wheelchair within 10-15 years of onset. This intronic expansion reduces FXN expression, a mitochondrial protein required for its homeostasis. Currently, there is no effective treatment for FRDA. We previously showed complete rescue of the neurologic, muscular, and cardiac complications in the YG8R mouse model of FRDA with a single, systemic infusion of wild-type hematopoietic stem and progenitor cells (HSPCs). We showed that the rescue was mediated by FXN transfer from the engrafted HSPC-derived microglia/

macrophages to neurons/myocytes. We next developed an ex *vivo* CRISPR/Cas9 gene editing approach that excises GAA expansion in FRDA patients' $CD34^+$ HSPCs as a potential autologous transplantation therapy. We are now investigating the *in vivo* safety and efficacy of our CRISPR/Cas9 gene editing approach in YG8s$(GAA)_{>800}$ mice (YG8JR) carrying the human *FXN* (h*FXN*) with >800 GAA repeats. Two-month-old myeloablated YG8J mice were transplanted with either *ex vivo* gene edited $Sca1^+$ HSPCs (GAAed) or mock-edited HSPCs (Mock) and analyzed 6-7 months later. Despite limited gene editing efficiency in the cell product (mean 15.7%), we observed substantial amounts of gene edited HSPC-derived cells in the hematopoietic tissues: ~ 5.25% in bone marrow cells, ~ 8.3% editing in blood, 19% in spleen and 16.51% in thymus, and in the primary organs affected in FRDA such as heart (2.4%), spinal cord (1.1%), cerebellum (0.69%), and skeletal muscle (4.4%). Significantly increased FXN expression was observed in the affected organs that led to significant tissue preservation. In the brain, the density of cerebellar granular cell layer neurons, essential for motor coordination, were comparable to wildtype mice whereas the non-treated and mock transplanted YG8JR exhibited significant neuronal loss. Furthermore, non-treated and mock YG8JR mice exhibited significant cardiac defects such as hypertrophic cardiomyocytes, infiltration and proliferation of non-myocytic cells, ultrastructural myofibrillar and mitochondrial abnormalities. In contrast, these defects were mostly absent in the YG8JR mice treated with GAAed HSPCs. Taken together, these promising findings demonstrate that single infusion of *FXN* gene corrected HSPCs engraft in the bone marrow niche to become a reservoir of healthy cells that can engraft into all injured organs for their long-term preservation. This work will support the clinical translation of this approach for FRDA.

845. Hematopoietic Stem Cell Gene Therapy for Mucopolysaccharidosis Type IIIC

Rafael A. Badell-Grau, Kasra Pakravesh, Rola Chen, Eric Thai, Frankie Son, Kalvin Duong, Pauline Losay, Rushil Pithia, Anusha Sivakumar, Veenita Khare, Alexis N. Corl, Christine Tran, Jefferey Esko, Stephanie Cherqui

UCSD, La Jolla, CA

Mucopolysaccharidosis type IIIC (MPSIIIC) or Sanfilippo Syndrome type C is a severe neurodegenerative lysosomal storage disease (LSD) caused by loss-of-function of the lysosomal transmembrane protein Heparan-α-glucosamine N-acetyltransferase (HGSNAT). MPSIIIC has no available treatment and is characterised by accumulation of glycosaminoglycans (GAGs). This disease presents with developmental delay and neuronal cell loss leading to death from severe neurodegeneration. Our group has shown that transplantation of wildtype hematopoietic stem cells (HSPCs) rescue cystinosis, another LSD due to mutations in a transmembrane lysosomal protein. One of the mechanisms of rescue involved lysosomal cross-correction from HSPC-derived macrophages to the disease cells via tunnelling nanotubes. We believe that the same principles could be used to treat MPSIIIC.We generated a novel MPSIIIC mouse model by knocking-out exon 2 in *Hgsnat* and confirmed disease phenotypes such as GAG accumulation, enlarged liver, lysosomal expansion, neurological defects, increased inflammation, and presence of disease specific non-reducing end carbohydrates. We transplanted MPSIIIC mice with HSPCs isolated from WT GFP-transgenic mice or $Hgsnat^{-/-}$ mice as controls. WT-HSPC transplant improved neurological and gait defects, hepatomegaly, urine retention, as well as decreased inflammatory markers in the treated MPSIIIC mice. Nonetheless, whilst we see engraftment of HSPC-derived cells, tissue expression of *Hgsnat* was low and no significant reduction of total GAG storage was observed. These data suggest that endogenous *Hgsnat* is not well expressed in macrophages/microglia explaining the limited impact on GAG accumulation.Limited efficacy of WT-HPSCs has also been reported in MPSIIIA, which was overcome by using gene-modified HSPCs to overexpress *SGSH*. Therefore, we are developing an autologous transplantation of ex-vivo gene modified HSPCs. We have generated self-inactivated (SIN)-lentivirus vector containing human *HGSNAT* cDNA (NM_152419) and performed *in-vitro* testing. Following transduction of mice and patient cells lines, we observed increased *HGSNAT* expression, lysosomal localization, recovery of HGSNAT enzyme activity, decreased GAG storage, and decreased lysosomal accumulation. We also transplanted gene-modified HSPCs in MPSIIIC mice, and have confirmed engraftment of our treated mice at 2 months post-transplantation. This study represent the first step towards the clinical translation of an ex vivo gene therapy approach for MPSIIIC.

846. Intrauterine Cellular Therapy Using a Non-Human Primate (NHP) Model

Julie Yeo[1], Nuryanti Johana[1], Yi Wan Tan[1], Yiping Fan[1], Citra N. Mattar[2,3], Fritz Lai[4], Qingfeng Chen[4], Arijit Biswas[2,3], Mahesh Choolani[2,3], Simon Waddington[5], William Peranteau[6], Alan W. Flake[7], Els Verhoeyen[8], Jerry Chan[9]

[1]KKH Women's and Children's Hospital, Singapore, Singapore,[2]National University of Singapore, Singapore, Singapore,[3]National University Health System, Singapore, Singapore,[4]Institute of Molecular and Cell Biology, Singapore, Singapore,[5]University College London, London, United Kingdom,[6]The Children's Hospital of Philadelphia, Philadelphia, PA,[7]Childrens Hospital of Philadelphia, Philadelphia, PA,[8]INSERM/CIRI/EVIR, Lyon, France,[9]KK Women's and Children's Hospital / Duke NUS Medical School, Singapore, Singapore

In utero haematopoietic stem cell transplantation (IUHSCT) for the treatment of congenital hematological disorders has been proposed to be an ideal form of therapy. This is due to the immature immune system of the early gestation fetus that allows the development of donor-specific tolerance. Hopefully within the fetal environment, the haematopoietic stem cells (HSC) would be able to survive and proliferate. In this work, we have used a macaque model to transplant GFP-labelled maternal cells into the fetus in order to study the rate of engraftment and fate of the maternal cells. Maternal HSC were harvested from bone marrow aspirates, magnetically enriched for CD34+ cells, and labeled with eGFP using a simian immnodeficiency virus (20-70% GFP+). Transduced CD34+ cells were CD26 inhibited prior to intravenous injections at ~ $1x\ 10^8$ cells/ estimated fetal weight (kg) with a final 1-3% CD3+ in the inoculum into 0.4-0.5 gestation fetal macaques. Following transplant, fetal subjects (n=6) were delivered via cesarean section and hand-reared. Fetal tissue harvested at 12 weeks post-IUHSCT showed chimerism levels ranged from 0.05% to 3.31%. Whereas cord blood collected at birth was evaluated for presence of GFP cells by both qPCR and FACs, demonstrating levels to be very low

if not absent (<0.05%). Mixed lymphocyte reaction between treated offspring and same-donor (maternal) CD45 cells showed evidence of tolerance towards maternal cells. Postnatal same donor boosting of offspring with GFP labelled cells was performed after busulfan treatment. Chimerism in the blood and bone marrow was still low (<1%) after 20weeks post boost. NSG (Nod-Scid Gamma) mouse model was used to evaluate how long the GFP labelled cells were able to proliferate and continue to express GFP *in vivo.* GFP-labelled HSC were injected into irradiated newly born mice. Engraftment was assessed through regular blood sampling and labeling of NHP and mice blood cells by FACs analysis. Results show that NHP chimerism in the NSG blood was low <1% even after 14 weeks. Cells expressing GFP in blood circulation decreased at 14 weeks in some animals. NHP chimerism levels were higher in the bone marrow and spleen than peripheral blood at 14 weeks, although less than 1%. These tissues also have GFP expressing NHP cells in the tissues indicating that the GFP can still be expressed at 14 weeks post injection. Prenatal maternal donor IUHSCT with postnatal boosting has been shown to be efficacious in murine and canine models. However, we were unable to demonstrate that maternal donor cells were able to engraft in a fetal macaque model.

847. Rational Inclusion of Antimetabolites to ADC-Based Non-Genotoxic Conditioning Strategies for the Transplantation of Engineered Hematopoietic Stem Cells

Jennifer Okalova, Jordan S. Alexander, Gianna M. Branella, W. Hunter Baldwin, Chengyu Prince, Seema R. Patel, Andrew Fedanov, Shanmuganathan Chandrakasan, Christopher B. Doering, H. Trent Spencer

Department of Pediatrics, Aflac Cancer and Blood Disorders Center, Emory University, Atlanta, GA

Hematopoietic stem cell (HSC)-directed lentiviral (LV) gene therapy is a promising curative strategy for monogenic diseases. However, therapeutic application of genetically engineered HSCs is limited by potential infertility and genotoxicity associated with pre-transplant non-targeted conditioning, such as total body irradiation (TBI) or alkylating chemotherapies. Since conditioning is needed to create space in the bone marrow (BM) niche for transplanted HSCs, the development of non-genotoxic conditioning regimens is a high priority translational objective. Indeed, we pioneered the use of LV-engineered HSCs for the treatment of hemophilia A (HA) by incorporating a bioengineered high expression fVIII variant, termed ET3, which is currently under clinical investigation [ClinicalTrials.gov identifiers: NCT04418414 and NCT05265767]. To achieve targeted HSC killing, we and others tested an antibody-drug-conjugate (ADC) using anti-CD117 (c-kit) and saporin (SAP), which selectively depletes CD117-expressing HSCs and progenitor cells by ribosome inactivation. Even though ADC-based conditioning shows preclinical promise, robust engraftment of genetically engineered HSCs continues to be a clinical limitation. This study investigates the hypothesis that current non-genotoxic conditioning regimens with ADCs like CD117-SAP are not effective as single agents due to a lack of deep HSC/progenitor cell depletion. Herein, we found administration of CD117-SAP in HA mice induces residual HSC exit from a quiescence state and entry into a proliferative state, as determined by flow cytometric analysis of isolated murine HSCs using defined HSC markers coupled with Ki-67 and DAPI staining. Thus, to achieve sufficient HSC/progenitor clearance, we tested intermittent IV injections of the antimetabolites 5-fluorouracil (5-FU) and fludarabine (Flu) post-ADC treatment and confirmed augmented clearance by flow cytometry and colony-forming unit assays of BM harvested from conditioned HA mice. Although 200 mg/kg of either antimetabolite alone is well tolerated, addition of CD117-SAP did not affect the maximum tolerated dose (MTD) of Flu but lowered the MTD of 5-FU to 50 mg/kg. This suggests antimetabolites can display different degrees of synergy with CD117-SAP and, in turn, distinct toxicity profiles. Subsequent HSCTs with unmodified Sca-1+ HSCs isolated from either GFP or CD45.1 donor mice resulted in >80% engraftment in HA (CD45.2+) recipient mice pre-conditioned with both CD117-SAP and the antimetabolite. However, in the context of 5-FU, deeper depletion must be coupled with additional T-cell immunosuppression, e.g. anti-thymocyte globulin (ATG), to permit unmodified Sca-1+ HSC engraftment. Inconsistent outcomes with mouse-specific ATG reagents in the setting of our murine HSCTs with CD117-SAP led us to incorporate the immunoglobulin G-degrading enzyme of *Streptococcus pyogenes* (IdeS) to ensure cleavage of residual circulating ATG antibodies at the time of transplantation, which resulted in robust engraftment (>80%). Together, these studies show CD117-SAP and antimetabolites display synergistic clearance of proliferating progenitors, resulting in long-term engraftment of transplanted HSCs comparable to TBI. Ongoing studies explore transgene tolerance, durability, and immunogenicity of engrafted ET3-modified cells after HSC-directed LV gene therapy for HA. Therefore, antimetabolites such as 5-FU and Flu provide proof of concept for the repurposing of existing small molecule drugs in non-genotoxic conditioning platforms for HSCTs.

848. Human Donor Islets and iPSCs, Encapsulated in Immuno-Privileged Next-Generation Hydrogels, Are Viable and Functional *In Vitro* and *In Vivo*

Harald Stover

Allarta Life Science, Dundas, ON, Canada

Introduction & Objective: Encapsulation carries the promise to physically immuno-protect cell clusters, to provide a safer and more durable vehicle for cell therapies. This expands the potential for patients to access these cell therapies without the use of immunosuppressive regimens. Our proprietary synthetic hydrogels can support different cell types and cell densities, and here, we report the assessment of *in vitro* and *in vivo* functionality. **Methods:** Next-Gen hydrogels were implanted in mice, rats and pigs to assess tissue response. Human donor islets and human iPSCs were encapsulated in next generation shape-agnostic hydrogels at different cell densities and in vitro viability and function was assessed, then they were implanted in healthy or streptozotocin (STZ)-induced diabetic immunocompetent rodents and pigs to assess their *in vivo* functionality. **Results:** Our hydrogels successfully supported cell function *in vitro* and had no-to-minimal tissue response with strong robustness in the various animal species. Encapsulated human iPSCs have good *in vitro* viability and proliferation and maintain good viability signals 3 months post-explant. Human donor islets, encapsulated at different cell densities, had good

in vitro function in response to glucose stimulation, and showed dose-dependent *in vivo* response in rodents. The cells also demonstrate good function in pigs with reduced blood glucose and increased C-peptide levels. **Conclusion:** Our proprietary hydrogels enable delivery of both primary islets and iPSCs, and demostrated robustness without eliciting particularly strong host reponses in a variety of animal models. Encapsulated cells remain viable and functional *in vitro* and *in vivo,* without any immunosuppression. We are currently scaling to therapeutic doses in large animals, in pursuit of a curative therapy based on an optimal balance of cell protection and functionality.

849. Immune-Stealthy Hepatocytes Engraft and Expand in the Allogeneic Mouse Liver

Anne Vonada, Leslie Wakefield, Markus Grompe, Amita Tiyaboonchai

Oregon Health and Science University, Portland, OR

Hepatocyte transplantation is a cell therapy approach with applicability to a variety of liver disorders, including both inherited and acquired conditions. Unlike gene therapy, for which the genetic cargo must be customized for each condition, hepatocyte transplantation allows wild type hepatocytes to be used as a therapeutic for a wide variety of disease indications. Many attempts at hepatocyte transplantation in humans have achieved partial efficacy, but the benefits are consistently lost in long-term follow up due to rejection of transplanted cells. Standard immunosuppression regimens, similar to those given after whole organ transplantation, are insufficient to prevent allorejection. The beneficial effects of hepatocyte transplant are typically lost by 1 year post-transplant. Ex vivo engineering of the patients' own cells to correct the disease-causing mutation (i.e., use of an autologous cell source) is a possible solution to this problem, although this approach is invasive and low-throughput. An optimized approach would involve creation of immune-stealthy hepatocytes to allow production of an off-the-shelf cell therapy product. Approaches to engineering "universal donor" cells have been reported for T-cells and induced pluripotent stem cells (iPSCs) by genetic manipulation of major histocompatibility complex (MHC) presentation. These modifications include knockout of the beta-2-microglobulin (*B2m*) gene to prevent MHC class I presentation, class II major histocompatibility complex transactivator (*Ciita*) knockout to prevent MHC class II presentation, and transgenic expression of immune-inhibitory ligands to prevent NK cell responses. Similar methods are expected to be applicable to hepatocytes, but these have never been tested in vivo. To assess whether a similar approach is applicable to hepatocyte transplantation, primary hepatocytes from a transgenic tdTomato reporter mouse on the C57BL/6 background were triple edited in vitro. This included knocking out *B2m* and *Ciita* with CRISPR ribonucleoprotein (RNP) complexes, along with lentiviral overexpression of Cd47. An additional single-edited cell group included only RNP-mediated knockout of B2m. Edited donor cells were transplanted into recipient mice on an allo-mismatched 129S4 background as well as congenic C57BL/6 controls. All recipient mice were deficient in fumarylacetoacetate hydrolase (Fah), which leads to a strong selective effect for wild type hepatocytes. Transplantation of triple-edited donor cells produced stable engraftment in 6 out of 9 recipients for at least 2 months. Interestingly, transplantation of allogeneic donor cells deficient in only B2m also led to engraftment and clonal expansion in 8 out of 13 transplanted animals. Control animals transplanted with B2m-proficient hepatocytes in the same allotransplantation paradigm never showed any engraftment (0/8) and congenic controls always displayed engraftment. In some recipients, transplantation of B2m-deficient allogeneic hepatocytes resulted in complete liver repopulation, subsequent weight stabilization and long-term survival of the recipient animals. These data indicate that knockout of *B2M* may be the only genetic modification needed for creation of immune-stealthy donor hepatocytes. B2M-deficient donor hepatocytes may represent an off-the-shelf cell therapy product enabling hepatocyte transplantation without immunosuppression.

850. Engineered T Cells as a Platform to Treat Enzymopathies

Evan W. Kleinbohl[1], Kanut Laoharawee[1], Jacob D. Jensen[1], Joseph J. Peterson[1], Nicholas J. Slipek[2], Bryce J. Wick[1], Matthew J. Johnson[2], Beau R. Webber[2], Branden S. Moriarity[2]

[1]University of Minnesota - Moriarity Lab, Minneapolis, MN,[2]University of Minnesota, Minneapolis, MN

Inherited metabolic disorders account for nearly seventy percent of lysosomal storage diseases. While treatments with enzyme replacement therapy (ERT) regiment are possible for several of these diseases, enzyme replacement halflife/turnover is too short to be a lifelong solution for patients with most diseases. To build a longer-term therapeutic modality, we aimed to harness T cells to deliver therapeutically relevant levels of replacement proteins for lysosomal storage diseases, specifically mucopolysaccharidosis type I (MPS I). T cells are capable of migrating throughout all of the body's tissues, can persist for many years, and have highly validated gene engineering protocols, making them one of the most attractive platforms for cell-based systemic enzyme replacement therapy. This study shows IDUA delivery via engineered T cells can sustainably correct enzyme deficiencies systemically. Within a preclinical immunodeficient MPS I mouse model, a single dose of engineered memory CD4+ T cells resulted in detectable IDUA activity levels surpassing heterozygous levels in the blood and organ tissues, leading to reduced glycosaminoglycans (GAGs) waste product accumulation and alleviation of MPS I indications. Furthermore, 10 weeks post T cell delivery, no significant difference in organ GAG accumulation was observed in MPS I treated and IDUA heterozygous mice. This therapeutic effect was observed through the course of our 22 week study. Immunohistochemistry of liver and brain samples showed a >2 fold decrease in lysosomal associated membrane protein 1 (LAMP1), a hallmark of lysosomal storage disease manifestations, further indicating alleviation of MPS I. This therapeutic strategy is being further improved with newly developed methods for in vivo cell activation and enhancing the ability of IDUA to cross the blood brain barrier using protein fusions. These promising findings underscore the potential of our cell-based therapy platform for enzyme replacement, not only in MPS I, but also in addressing a spectrum of enzymopathies and protein deficiencies.

851. Optimization of Lysosome Extraction for Future Downstream Applications in Cell Therapy

Ilana Chefetz Menaker
Biomedical Sciences, Mercer University, Macon, GA

The lysosomes are a membrane-enclosed recycling bin/suicidal bag organelles that digest a variety of biomolecules such as proteins, carbohydrates, lipids, and nucleic acids. The digestion process is performed by lysosomal enzymes that require an acidic pH for optimal activity. Dysfunction of lysosomal proteins causes Lysosomal Storage Diseases and currently enzyme replacement therapy or gene therapy are being clinically developed. Interestingly, recent studies confirmed the lysosome transfer between cells, suggesting use of healthy lysosomes as cell therapy. After lysosome substrates are broken down, their components can be recycled and reused. Besides their role in feeding cells from the inside, lysosomes have a central role in metabolic growth signaling. Thus, targeting lysosomes, could be a logical approach in metabolically deranged conditions such as cancer. Additionally, lysosomes are detrimental for completion of autophagy, a process that is often reduces efficiency of cancer therapeutics, triggering pro-survival of cancer cells. Thus, it is important to be able to isolate lysosomes without other organelle contamination using a validated protocol that will ensure purity and intactness for downstream applications and reproducibility. While several methods have been developed, including immunoprecipitation of the exogeneous lysosomal gene TMEM192-3-HA following its overexpression using magnetic beads, we decided to asses a commercially available the lysosome extraction kit (Thermo Fisher) that utilizes gradient and ultracentrifugation that results in appearance of three phases with the upper phases containing lysosomes. Following series of optimizations of sonication frequency, we were able to confirm enriched expression of the lysosome protein LAMP1 in the upper phase that also contained a significant contamination of mitochondrial proteins as assessed by COXIV expression. We further optimized this procedure, by modifying the gradient conditions of Opti Prep cell separation media and subdividing the upper phase into 4 different phases. Based on the purity of fractions (Lamp1/COXIV) we choose 2 gradient conditions and collected two upper phases (out of 4) with highest LAMP1 expression to validate our optimization in comparison to the intermediate phase that includes mitochondria. The fraction purity was further confirmed by the ratio of the lysosome protein LAMP2 and the mitochondria protein SDHA. All collected fractions were sent to proteomic analysis to establish optimal conditions for the lysosome enrichment signature. For label free quantification LC-MS/MS, peptides would be reconstituted in 5% Acetonitrile (ACN) and 5% formic acid (FA) and analyzed using a nano-UHPLC-Dionex Ultimate 3000 system connected to an Orbitrap Fusion Lumos mass spectrometer. In summary, we developed a method that allows extraction of intact pure lysosome fractions to allow downstream cell therapy applications and analyzed proteomic content to compare purity and enrichment across different experimental conditions.

852. Genetically Engineered Macrophage Cell Therapy Reverses Liver and Lung Fibrosis in Preclinical Models

David Christopher Sloas, Yumi Ohtani, Angela Mosebarger, Ben Johnson, Nancy Carson, Julia Smith, MacKenzie Villano, Joanna Schmidt, Sascha Abramson, Thomas Condamine, Michael Klichinsky, Bradley Zinker
Carisma Therapeutics Inc., Philadelphia, PA

Introduction: Fibrosis of the liver and lung can lead to organ failure and the need for transplantation. Currently available therapies are unable to reverse established fibrosis. The development of fibrosis is an interplay of inflammatory and pro-fibrotic pathways leading to collagen deposition and disease progression. Macrophages play a central role in the development and resolution of fibrosis, and pre-clinical/Phase I studies have previously shown the feasibility, safety, and potential efficacy of non-genetically modified macrophage cell therapy in liver cirrhosis. We genetically engineered macrophages targeting relaxin, IL-10 or TGFbR2 signaling pathways and assessed the efficacy of engineered macrophage cell therapy in preclinical models of liver and lung fibrosis. **Methods:** Primary macrophages were efficiently transduced to express anti-inflammatory or anti-fibrotic payloads, such as IL-10, relaxin, and dominant negative (DN) TGFbR2. Engineered primary murine and human macrophages were highly viable and durably expressed functional payloads. Liver fibrosis efficacy was investigated in vivo in the hallmark CCl_4 mouse model of established fibrosis. Mice were challenged with CCl_4 2X/week for 6 weeks. At week 4, mice were intravenously dosed once with vehicle control, untransduced macrophages (UTD-M), or engineered macrophages then sacrificed 2 weeks later (n=9/group). A non-CCl_4 group was included as a non-disease control and efficacy was examined histologically. Separately, we investigated lung fibrosis efficacy utilizing the hallmark bleomycin mouse model of pulmonary fibrosis. Following bleomycin exposure, all mice were dosed once with either vehicle control, UTD-M, or engineered macrophages (n=10-15/group). A non-bleomycin group (non-disease control) was included and efficacy was determined via hydroxyproline biochemical analysis. **Results:** Genetically engineered macrophages were able to traffic to fibrotic tissues in two independent models and durably expressed their payloads. Picrosirius red and Masson's trichrome staining, independent collagen biomarkers, were significantly elevated in livers following CCl_4 exposure and significantly reduced upon UTD-M (55% reduction relative to control) and relaxin-IL10 (116% reduction relative to control) macrophage treatment. Furthermore, relaxin-IL10 macrophage treatment significantly reduced fibrosis compared to UTD-M, demonstrating an enhanced benefit with genetic macrophage modification. In the lung fibrosis model, a significant reduction in lung hydroxyproline levels in the relaxin-IL-10 and TGFbR2 DN (90% reduction relative to control) engineered macrophage treated groups vs bleomycin control was observed. Macrophage cell therapy was well tolerated in both fibrosis models with no weight loss observed. **Conclusion:** Macrophages engineered to co-express relaxin and IL10 reversed established liver fibrosis, and macrophages engineered to express DN TGFbR2 ameliorated pulmonary fibrosis. These results demonstrate the pre-clinical feasibility, safety, and efficacy of genetically modified macrophage cell therapy for the treatment of fibrosis.

853. Impact of Origin and Delivery Route on Efficacy of Mesenchymal Stromal Cell-Based Therapy in Acute Lung Injury

Jayita Barua[1], Loredana Asarian[1], Keara L. McElroy-Yaggy[2], Daniel Weiss[1]

[1]Dept. of Medicine, Larner College of Medicine, University of Vermont, Burlington, VT,[2]Dept. of Medicine, Stem Cell Core, University of Vermont, Burlington, VT

In-vivo models of acute lung injuries (ALI) have been widely used to evaluate efficacy of mesenchymal stromal cell-based (MSCs) therapies and investigating the underlying mechanisms. LPS, a gram-negative bacterial endotoxin delivered intra-tracheally has been used in mouse models to mimic the hallmarks of acute respiratory distress syndrome (ARDS), a form of ALI. However, the methodologies to treat LPS-treated mice with MSCs lack consensus. This makes it difficult to compare the studies for their outcomes to determine efficacy. Unsurprisingly, contrasting outcomes from non-COVID ARDS clinical trials also reflect similar issues. This led us to examine experimental variables in our mouse model, such as species matching between donor and recipient, different routes to deliver MSCs in LPS-treated mice. 2.5 ug of O55:B5 LPS (or vehicle) is administered intra-tracheally to C57BL/6J (B6) mice (8-12 weeks old, male) to induce lung injury. 4 hours post-LPS, human bone marrow derived MSCs (hBM-MSCs) or mouse BM-MSCs are delivered either intratracheally or subcutaneously. Mice are harvested at day 3(D3) and disease outcomes, such as total protein content of bronchoalveolar lavage fluid (BALF) and percentage of infiltrated neutrophils in alveoli are measured as indicators of breach of alveolar-capillary barrier and inflammation respectively. Lungs are gravity-fixed with 4% formalin, sectioned and stained with Eosin-Hematoxylin (H&E) to determine cellular infiltration, thickening of the alveolar septa and presence of protein rich edema fluid. Intra-tracheally delivered $1x10^6$ mouse MSCs were shown to lower the neutrophil infiltration significantly (**;p=0.0097, n=3),but failed to lower the protein concentration in BALF of LPS-treated mice. H&E-stained lung sections also corroborated that data. In the following pilot experiment, we tested the efficacy with subcutaneous delivery of mMSCs dose-dependently. At a higher dose ($1x10^7$) mMSCs, delivered subcutaneously were shown to lower the neutrophil infiltration significantly (*;p=0.04, n=4 in LPS, n=2 in LPS-MSC groups). However, protein concentration in BALF was not lowered at D3. Currently, we are in the process of repeating the experiments with higher sample number and a later timepoint for harvest to measure the outcomes. In conclusion, our studies showed that syngeneic, but not xenogeneic MSCs partly mitigate LPS-induced lung injury. In addition, subcutaneous delivery of mMSCs may prove to have better therapeutic potential in our disease model than intra-tracheal delivery. The presence of proteins in alveoli can be attributed to the early examination (D3) of BALF and remain to be tested at day 7. It can also be evaluated for the presence of other proteins such as cytokines (TNF-α, IL-10, IL-6, IFN-γ etc.), chemokines (CXCL1/2 , SDF-1α, CXCL5 etc.) that could add to the BALF protein repertoire. Once established, our model can provide the platform to investigate the pertinent questions about the underlying mechanisms of MSC-therapy, not only in lung disease, but also in other acute inflammatory diseases.

854. *In Vitro* Production of Somite-Derived Muscle Stem Cells from Human Pluripotent Stem Cells for Cell Replacement Therapy in Duchenne Muscular Dystrophy

Yannis Djeffal[1,2,3], Lu Yan[1,2,4], Yuchuan Miao[1,2], Alejandra Rodriguez-de-la-Rosa[1,2], Yori Endo[5], Indranil Sinha[5], Frédéric Relaix[3], Emanuela Gussoni[6], Allon Klein[4,7], Olivier Pourquié[1,2,4]

[1]Department of Pathology, Brigham and Women's Hospital, Boston, MA,[2]Department of Genetics/Harvard Stem Cell Institute, Harvard Medical School, Boston, MA,[3]Univ Paris Est Créteil, INSERM, IMRB, Créteil, France,[4]Somite Therapeutics, Boston, MA,[5]Division of Plastic Surgery, Brigham and Women's Hospital, Boston, MA,[6]Harvard Medical School/ Children's Hospital, Boston, MA,[7]Department of Systems Biology, Blavatnik Institute, Harvard Medical School, Boston, MA

Cell replacement therapy is an innovative approach to treat Duchenne muscular dystrophy by transplanting functional muscle stem cells directly to muscle. We have developed a differentiation process that can yield somite cells from human pluripotent stem cells (PSCs). Somites are transient structures in developing embryos which give rise to diverse cell types including skeletal muscle, dermis, bone, cartilage, tendons, and brown adipose. We have developed a stepwise myogenic induction protocol that recapitulates the developmental trajectories of skeletal muscle, resulting in the efficient production of myofibers and satellite cells (muscle stem cells) in vitro. To meet the requirements for the cell therapy applications, we optimized the protocol using a newly developed bioinformatics pipeline. Compared to our original protocol, this optimization allowed us to double the proportion of satellite cells in human iPS-derived myogenic cultures. Additionally, adjusting the differentiation duration led to the identification of a satellite cell population with enhanced maturity and increased cell fusion ability. In proof-of-concept experiments, these in vitro-generated cells were transplanted into mice to assess their in vivo functionality. When transplanted in irradiated pre-injured immunocompromised mice in which leg muscles are atrophied, in vitro-produced human muscle stem cells could generate up to 80% of the grafted Tibialis Anterior muscle cells, demonstrating a restoration of force production. This preclinical success underscores the potential of in vitro-produced somite-derived cells as a promising source for cell replacement therapy in muscle-related diseases and injuries. The regenerative capacity of these cells, characterized by self-renewal and differentiation into mature functional muscle fibers, positions them as top-tier cell sources for long-term patient benefits. In conclusion, our established protocols serve as a significant advancement in the production of somite-derived muscle cells, providing a proof of concept for their promising use in cell replacement therapy. This successful strategy also lays the groundwork for exploring other somite-derived cell lineages.

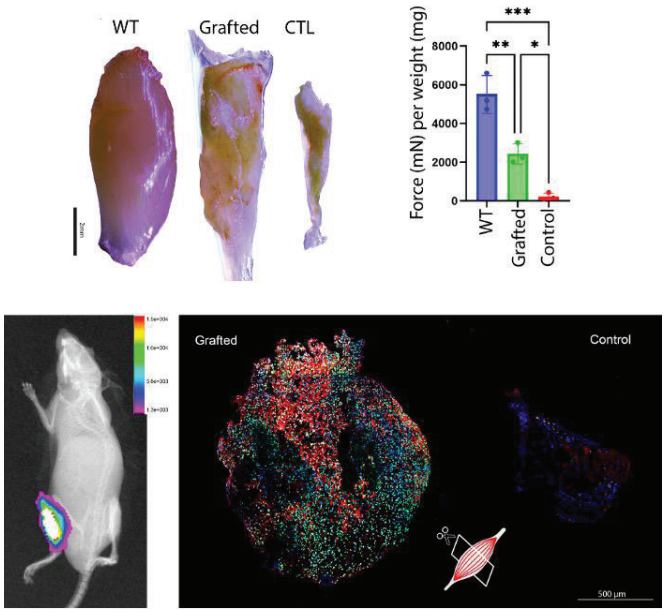


Figure: Top. (Left). Comparison between wild type (WT), grafted, and control (CTL) dissected Tibialis Anterior muscles one month after irradiation and cardiotoxin injection with (grafted) and without (CTL) injection of human iPSC-derived satellite cells. (right), restoration of force production in grafted muscles. Bottom. (Left) detection of the luciferase signal in the grafted human satellite cells after one month. (Right) comparison between sections of grafted and control TA muscles after one month showing the expression of human dystrophin (red) and human laminB2 (nuclei, green).

855. Cell-Based Treatment of Skeletal Muscle Atrophy Induced by Amyotrophic Lateral Sclerosis

Raphael Severino Bonadio[1], Silvia Scalabrin[1], Mattia Polato[1], Alessandro Bertoli[2], Maria Lina Massimino[3], Stefano Cagnin[1]

[1]Department of Biology, University of Padova, Padova, Italy,[2]Department of Biomedical Science, University of Padova, Padova, Italy,[3]CNR, Padova, Italy

Introduction. Amyotrophic Lateral Sclerosis (ALS) is the most common adult motor neuron (MN) disease whose causes remain largely unknown and whose treatment options are ineffective in the long term. The hallmark of ALS is selective and progressive degeneration of lower and upper MNs, leading to spasticity, weakness, and muscle atrophy. Several studies suggest that non-coding RNAs are central to the onset and progression of ALS. Most ALS studies are neurocentric and assume that MN dysfunction is the main origin of the disease and that muscle changes are the consequence. Growing evidence, however, suggests that molecular alterations in muscle fibers in the presymptomatic phase may trigger ALS pathology. Methods. We analyzed noncoding and coding genes differentially expressed in skeletal muscle of SOD1*G93A mice, an established model for studying ALS. We identified as altered a signature involved in the maintenance of muscle mass. We produced cells stably expressing miRNAs that modulate this signature and tested their ability to affect non-engineered cells in a paracrine manner. Conclusions. We have shown that cells overexpressing a specific miRNA sequence (patenting process) produce thicker myotubes and secrete the same miRNA to affect other non-engineered muscle cells. Furthermore, the thickness of myotubes from ALS mice was improved not only by transfecting them with the DNA construct we developed, but also just by using the RNA secreted by the transfected cells. This is a promising approach to delineate or rescue muscle atrophy that occurs in various pathological conditions not only of skeletal muscle but also of the nervous system.

856. Development of Novel Immunological-Directed Synthetic Biology-Based Microbiome Therapeutics

Christian Furlan Freguia[1], Govindaraj Anumanthan[2], Ananta Prasad Arukha[2], Bikash Sahay[2], Gary Fanger[1]

[1]Rise Therapeutics, Rockville, MD,[2]University of Florida, Gainsville, FL

As cell therapies offer a promising prospect for the treatment of several diseases, engineered probiotics represent the new frontier of non-viral cell-based drug-delivery platforms, produced by leveraging synthetic biology to modify natural probiotics through gene editing. Using probiotics as non-viral chassis to deliver therapeutics offers many advantages over other viral-based gene delivery approaches, such as reduced pathogenicity, low cost, and ease of manufacturing. In addition, oral engineered probiotics can directly interact with the gut microbiome, which has emerged as a vital regulator of the host immunity by the induction, functional modulation, or suppression of local and systemic immune responses. Therefore, a promising novel strategy to manage human diseases is to leverage the body's own natural microbiome-associated immune regulatory mechanisms with oral engineered probiotic therapy. Rise Therapeutics' oral platform consists of GRAS probiotics, engineered to express novel microbial-derived immunomodulatory proteins. Our lead clinical program, R-3750, consists of the food-grade *Lactococcus lactis* engineered to delivered Surface layer protein A (SlpA) from a genome integrated operon. R-3750 has been tested in several murine models of colitis. In the T cell-induced colitis model, oral administration of R-3750 at dose of 1E8 CFU was sufficient to significantly diminish intestinal pro-inflammatory cytokines (IL-1β, TNF-α, IL-6, and IL-17), and modulate the immune response by reducing inflammatory T cells ($CD3^+CD4^+IL\text{-}17^+$ and $CD3^+CD4^+Ror\gamma T^+$) and by upregulating regulatory T cells ($CD3^+CD4^+IL\text{-}10^+$ and $CD3^+CD4^+FoxP3^+$). Oral administration of R-3750 also restored gut epithelium barrier integrity as shown by upregulation of tight junction protein genes (*tight junction protein 2* and *3*) and mucin production genes (*mucine 2* and *mucine 3*). When the gut microbiome was analyzed, mice that received R-3750 showed higher relative abundance of species. Notably, R-3750 mechanism of action was corroborated using *in vitro* models where co-incubation of R-3750 with human or murine-derived dendritic cells similarly resulted in expression of IL-10. Single and repeat dose murine toxicology studies were performed with R-3750. In those studies, oral administration of R-3750 at doses 100-fold higher than the clinical dose, showed no toxicity as measured by hematology and clinical chemistry testing, and histopathology. Importantly, there was no R-3750 exposure detected in the blood of any animals using a validated qPCR assay, while fecal levels of R-3750 were detected in the treated animals, demonstrating that R-3750 remains confined in the

gut. R-3750 is currently in a Phase 1 clinical study (NCT05666960) in patients with mild to moderate ulcerative colitis. This study consists of a dose escalation stage, in which three doses of R-3750 are tested; and an expansion stage, where additional patients are enrolled at the highest tolerated dose. The study is currently enrolling. Overall, non-viral engineered probiotic-based therapies, like R-3750, represent a new strategy to tackle hard-to-treat diseases by allowing to treat patients sooner, improve compliance, and, for a drug like R-3750 which remains localized to the oral-gut cavity and is not systemically delivered, minimizes potential adverse reactions.

857. Building Synthetic Biosensors Using Red Blood Cell Proteins

Taylor F. Gunnels[1], Taylor B. Dolberg[2], Te Ling[3], Kelly A. Sarnese[2], John D. Crispino[3], Joshua N. Leonard[2]

[1]Department of Biomedical Engineering, Northwestern University, Evanston, IL,[2]Department of Chemical and Biological Engineering, Northwestern University, Evanston, IL,[3]Department of Hematology, St. Jude Children's Research Hospital, Memphis, TN

The success of engineered immune cell therapies for cancer treatment has opened the door for building cell-based devices for other applications and using alternative cell types. An exciting such direction is developing engineered red blood cells (RBCs) as *in situ* biosensors for long-term physiological monitoring and diagnostics. In this context, RBCs are an attractive platform because of their exposure to important biomarkers in blood and their long circulation time. A key biological hurdle to generating RBC biosensors is that red blood cells undergo dramatic cellular changes during their maturation process, including expelling their nucleus and changing the protein composition of the plasma membrane. As a result, traditional genetic engineering approaches are of limited use directly, and we largely lack tools to convert RBCs into cell-based biosensors. We set out to address this need towards the goal of building RBC biosensors for physiological monitoring. In this study, we demonstrate proof-of-concept for a technology that may ultimately enable the engineering of RBCs for *in situ* diagnostic purposes. Specifically, we built protein-based biosensors that respond to an extracellular cue via reconstitution of a split fluorescent protein or luminescent enzyme. We chose to use membrane proteins found natively in mature RBCs as biosensor scaffolds to overcome traditional limitations associated with erythropoiesis such as protein turnover and membrane remodeling. Prototyping designs via transient transfection of biosensor-encoding plasmids in a model cell line generated useful insights into how design considerations such as scaffold choice, linker length, and output protein modulate the ligand-induced signaling of this biosensor platform. Promising designs were stably implemented genetically into an erythroid precursor cell line and more deeply characterized for *in vitro* biosensor performance and kinetics. Finally, erythroid precursor cells containing the biosensor were injected into mice and successfully detected and reported on the presence of a model ligand. We anticipate this technology will be of interest in the cell therapy and diagnostic fields towards the goal of *in situ*, real-time physiological monitoring.

858. Enhanced Human CD34+ Cell Engraftment and Differentiation Depend on Pre-Conditioning Regimen in NSG and NBSGW Mouse Models

Bala Sai Sundarasetty, Maj-Kristin Holz, Fabian Musch, Johanna Mueller, Celeste Rizzello, Jens Dorna, Eileen Rauch, Claudia Pertab, Anke Beutel, Timo Kraushaar, Joachim Roeder, Maximilian Bielohuby

CSL Behring Innovation GmbH, Marburg, Germany

Immunodeficient mice are invaluable tools to study modeling of the human hemato-lymphoid system post hematopoietic stem cell (HSC) transplantation and has paved way in understanding of hematopoiesis and safety of the novel cellular therapeutics, in particular gene modified HSCs. One important consideration here, is to make the hematopoietic niche available to the transplanted HSCs by reducing the cellularity in mouse bone marrow, either by sublethal irradiation (X-ray or ^{137}Cs) or by the myeloablative drug busulfan. Here we compared the effects of irradiation vs busulfan pre-conditioning in two different, frequently used immunodeficient mouse strains: NOD.SCID.IL2rγ$^{-/-}$ (NSG) and NOD.Cg-Kit^{W-41J} Tyr^{+} Prkdc^{scid} Il2rg^{tm1Wjl}/ThomJ (NBSGW) mice and assessed the HSC engraftment and the HSC ability to give rise to various immune cell lineages post transplantation. NSG mice were pre-conditioned with irradiation (X-ray; 270cGy) or Busulfan (clinical grade; 25 mg/Kg or 35 mg/Kg) whereas NBSGW mice were either not pre-conditioned or pre-conditioned with a low dose of Busulfan dose (12.5 mg/Kg). All mice received CD34^{+} cells (mobilized peripheral blood derived CD34^{+} cells; 2x10^{6} cells/mouse) after pre-conditioning and the human cell engraftment was assessed by flow cytometry up to 16 weeks post transplantation. NBSGW mice without preconditioning supported the engraftment of human HSCs (week 10: 25% hCD45^{+} and 20% at termination). In comparison, mild pre-conditioning of NBSGW mice with Busulfan has improved the human cell engraftment (week 10: 53%) and the engraftment levels were sustained till the end of the study. At week 10 post transplantation, NSG mice pre-conditioned with a high dose of busulfan supported the engraftment of human cells at a higher rate compared to the low dose of busulfan (51% and 30% hCD45^{+} for 35 and 25 mg/kg, respectively). Notably, NSG mice conditioned with 35 mg/kg showed a constant increase of human cells in the periphery compared to the lower dose of busulfan (at termination: 70% vs 21% respectively). However, the high dose of busulfan conditioning led to higher mortality (50% survival) compared to low dose busulfan conditioning (90% survival). NSG mice irradiated did support the human cell engraftment albeit with a lot of variability (cohort 1: >40% vs cohort 2: <20%). Moreover, irradiation led to other side effects, such as tooth loss and reduced food intake. In conclusion, busulfan conditioning showed a better and less variable pre-conditioning in NSG mice compared to irradiation. Mild pre-conditioning in NBSGW mice improved the human cell engraftment compared to the non-conditioned NBSGW mice but appears to increase anemia. Overall, different options to improve the engraftment rate of HSCs in mice exist, however, the higher engraftment rate may be connected to higher dropouts in mice and therefore needs to be carefully considered, depending on the specific study requirements.

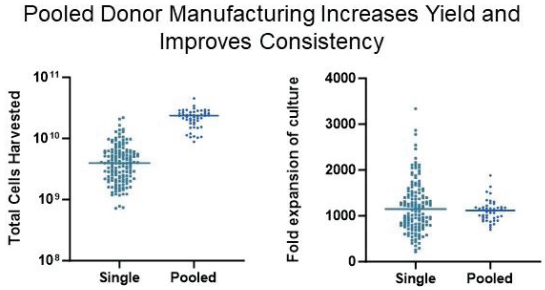
Wednesday Posters: Cell Therapy Product Engineering, Development, and Manufacturing

859. Overcoming Current Challenges in Allogeneic Cell Therapy Manufacturing by Using Pooled Donor Cord Blood CD34+ Cells as Starting Material

Joe Blake, Carrie Stoltzman, Håkan P. Svensson, Colleen Delaney

Deverra Therapeutics, Seattle, WA

Background & Aim: Allogeneic cellular immunotherapies hold the promise of improved clinical access as compared to autologous approaches, but many biological and regulatory challenges remain including optimal starting material, donor variability, scalability, cost of goods, in vivo persistence, and ability to cryopreserve formulated product. Most allogeneic cell therapies start with single-donor source material (e.g. cord blood, peripheral blood) with resulting high lot-to-lot variability, limited scalability, and high costs. To overcome these common challenges, we developed a manufacturing process to expand enriched CD34+ cells from pooled cord blood units (CBU) and then differentiate them to immune effector cells. This allows scalability and improves lot-to-lot consistency. Pooled donor products from this platform are used without HLA-matching and have now been used in four clinical trials to date, two with an expanded stem/progenitor cell product and two with an unmodified NK cell therapy derived from CB CD34+ cells. **Methods & Results:** For our expanded stem/progenitor cell product, CBU from FDA-licensed banks were thawed, pooled, and CD34-enriched. CD34+ cells were plated in vessels coated with a proprietary Notch ligand and cultured for 14-16 days before harvesting. Final products were cryopreserved in fixed doses, tested and released for clinical use. Using the same process to generate our expanded stem/progenitor cell product, data were collected for 55 pooled-donor (8 CBU: n=53, 6 CBU: n=2) and 155 single-donor manufacturing runs. Pooling CBU increased the consistency and output of the CD34+ enrichment process. Total cell fold expansion after culture was comparable with less variation observed in the pooled donor runs, while total cell output was increased by pooling CBU (Figure 1). Total number of CD34+ cells generated after culture was also increased, and purity of CD34+ was higher at harvest. Single and pooled donor products were cryopreserved and used in clinical trials as off-the-shelf universal donor products, including 184 infusions of pooled donor product; to date there have been no product-related SAEs.



Discussion: Pooling CBUs streamlined manufacturing and led to a large increase in cell product output and consistency without compromising growth or quality. Generating large batches of product reduces manufacturing cost and increases lot-to-lot consistency versus multiple small single donor manufacturing runs. Further, without requirement for HLA matching, our clinical products are manufactured, cryopreserved and released ahead of patient need and are available on-demand. Data described here reflect manufacturing using pools of 6-8 CBU, but the process is scalable to pool larger numbers of CBU. We now leverage this pooled donor expansion platform for generation of large numbers of progenitor cells which are then differentiated into immune effector cells, including myeloid cells (in preclinical development) and NK cells that have been safely used in 2 trials to date.

860. Optimising Lentiviral Transduction Using Microfluidics

Lindsey A. Clarke, Nicholas Akosa, Maria Kalli, Antonio de Grazia, James Kusena, Césaré M. Cejas, Antoine Espinet

MicrofluidX, Stevenage, United Kingdom

Background & Aim: Transduction via lentiviral methods in conventional bioreactors are susceptible to efficiency limitations, particularly often requiring high multiplicity of infection (MOI) to achieve acceptable efficiency levels. High MOIs typically translate to high experimental costs and sometimes even risk, with some studies reporting toxicity. In this study, we demonstrate that even at lower MOIs, we optimise the contact probability of the virus reaching the cell by leveraging the advantages of microfluidics. Methods: We fabricated a bespoke microfluidics-based bioreactor that takes advantage of high surface-to-volume ratios. This results in a reduced fluid height and elongated form factor. Using an exemplar CAR-T transduction process with Primary T-cells with CD19 lentiviral vector, we compare transduction efficiency in these bioreactors with conventional transduction methods. Results: Using a design of experiment methodology, we find that these novel microfluidics-based bioreactors benefit from high cell densities, and seeding by cell density (cells/cm2) similar to adherent cell culture, as opposed to cell concentration (cells/mL). This approach increases contact with the vector; thereby fostering a conducive environment that maintains paracrine signalling. We also show that despite the low fluid height environments, we still maintain high cell viability under optimal seeding conditions. Overall, we achieved a three-fold increase in transduction efficiency in these microfluidics-based bioreactors compared to conventional methodology. Conclusion: Here we demonstrate that even at low MOIs, a higher transduction efficiency can be achieved in comparison to conventional methodology. Ongoing work explores the impact of this low volume environment on transduction enhancers as well as the effect of agitation to further increase probability of contact between lentiviral particles and target cells. We continue to investigate new methods & protocols to collectively improve the transduction efficiency while reducing viral vector usage, ultimately contributing to reducing overall costs of CAR-T processes.

861. Non-Viral Cell Engineering of Peripheral Blood Natural Killer Cells Using a GMP-Compliant, Scalable Electroporation Platform

Lauren E. Unsworth, Ashley B. Strickland, Cornell Allen, James Brady
MaxCyte, Inc., Rockville, MD

Natural killer (NK) cells are an integral part of the innate immune system as they recognize an abundance of surface receptors on target cells without the need for antigen specificity and recruit and activate other immune cells by secreting a myriad of cytokines. The hallmark of NK cells is their cytotoxic function which enables them to recognize and attack tumor or infected cells by releasing granules that induce apoptosis of target cells. Electroporation (EP) is a non-viral method for cellular engineering that has been used with virtually all cell types and has been specifically shown to be an effective cell engineering method for peripheral blood NK cells. Advantages of electroporation include highly efficient transfection with a variety of molecules (e.g., DNA, RNA, and ribonucleoprotein (RNP) complexes), regulatory acceptance, and cGMP compatibility. In this study, we optimized conditions for electroporating primary NK cells with the GMP-compliant MaxCyte® ExPERT GTx™ electroporation platform, with specific focus on maximizing cell viability, cell recovery, transfection efficiency, cell expansion and functionality. Previous studies showed that expansion of NK cells before electroporation was necessary for effective transfection, and therefore larger scale platforms were required, leading to the use of more costly reagents. Here, we showed that the MaxCyte ExPERT GTx could genetically modify naïve or expanded primary NK cells using mRNA, RNP complexes or plasmid DNA at both a small ($1x10^6$ cells in 25µl) and larger scale ($1x10^8$ cells in 5ml). We observed over 90% gene expression and viability following mRNA electroporation and over 60% expression and viability after minicircle DNA (mcDNA) loading at both small and larger scales. We also evaluated the effect of commercially available DNA sensing pathway inhibitors to determine if transfection efficiency and cell viability could be increased when loading small and large plasmid DNA. Furthermore, we knocked-out the NKG2A inhibitory receptor at different timepoints during expansion by loading a sgRNA-Cas9 RNP complex and looked at post-electroporation effects. With our optimized transfection conditions specific for NK cells, we determined that electroporation had minimal impact on cell viability, cell expansion, surface marker expression, and killing capacity.

862. Potency Assurance Testing of a CAR-T Cell Therapy Product: Evaluating T Cell Function Using Multiple Quantitative Bioassays

Katrina Adlerz, Ellie Jafari, Morgan Trapp, Jon Pileggi, Chantale Bernatchez, Priya Balasubramanian
CTMC (Cell Therapy Manufacturing Center), Houston, TX

Background & Aim: Cell therapy is a promising treatment for cancer patients, with six CAR-T therapies approved by the FDA. One of the challenges on the path to approval, however, is establishing a strategy to ensure product potency. In fact, the newly issued FDA draft guidance "Potency Assurance for Cellular and Gene Therapy Products" acknowledges the specific challenges that product developers of complex cell therapies face. This draft guidance outlines recommendations for a Potency Assurance Strategy including a potency assay or assays that measure at least one potency-related Critical Quality Attribute (CQA) related to the product's bioactivity while avoiding redundant assays measuring various biological activities that occur in a stepwise chain. Here, we describe results of measuring the T cell Function of a CAR-T Product across multiple bioassays. We also compare critical aspects of the assays including precision, specificity, range, and robustness towards selection of a potency assay for evaluating T cell function of an early-stage investigational new drug. **Methods, Results, & Conclusion:** Autologous CAR-T cell product was manufactured from CD4/CD8-selected T Cells from three healthy donor apheresis. The T cells were activated, transduced with a CAR lentiviral vector, expanded, and cryopreserved. The cryopreserved final drug product was then tested for T Cell function in four different assays: 1) IFNγ secretion after TCR-activation 2) IFNγ secretion after antigen-specific stimulation, and two cytotoxicity assays with readouts by 3) bioluminescence and 4) cell impedance using the Agilent xCELLigence Analyzer. A concordance in trends was observed between the different assays with donor-to-donor biological variability a significant driver of T Cell function. Assays were also evaluated for suitability for use as product release in accordance with the FDA draft guidance and ICH recommendations, including precision, specificity, range and select robustness parameters. All assays showed high specificity with low IFNγ secretion or killing in non-stimulated or non-transduced samples. Precision varied among the assays with ELISA-based readouts having better precision with lower coefficient of variation and a broader range. Finally, CAR-T cells from the same apheresis but manufactured with two different processes were assessed. Significant differences were observed between the manufacturing processes. The results described here are impactful in understanding how to assess CAR T Cell function and towards defining the Potency Assurance Strategy for an early-stage investigational new drug.

863. Expansion of Stem-Like T Cells by Protein Nanoscaffold-Based NanoSpark™ STEM-T Activator

Zachary I. Imam, Donna Marsh, Luke Haines, Curtis D. Hodge
Nanotein Technologies, Berkeley, CA

Cellular therapies are the cancer treatment of the future. CAR-T cells are the most clinically and commercially advanced of anti-cancer cellular therapies. Despite their effectiveness, CAR-T cell therapy has encountered some obstacles including manufacturing failures, long manufacturing times, and short-lived or poor treatment outcomes. One solution to these problems is optimizing the activation and expansion step of the manufacturing process. Here, we used a novel protein-based T cell activation technology called NanoSpark STEM-T, a soluble reagent that promotes the growth of stem-like, less differentiated T cells with a bias towards clinically relevant CD8+ stem-like central memory T cells (Tscm) during 7 to 10-day expansions of donor T cells. We used NanoSpark to expand T cells from multiple donors, using several cell culture medias, different cytokine concentrations and combinations, and comparing expansion in conventional 6-well plates and G-Rex6M bioreactors over the course of a 10-day manufacturing process. NanoSpark STEM-T formulation achieved great expansion of total live T cells, CD8+ cells, and CD8+ Tscm cells during the 10-day

expansions. We quantified the fold-change of total live cells from day 0, quantifying Tnaive, Tscm, Tcm, Tem, and Temra cell phenotypes, and created the stemness score metric to quantitatively determine the stemness of the expanded T cell population on day 10 of expansion. Our results indicated that NanoSpark STEM-T readily expanded CD8+ T cells and preserved their stemness. These results were conserved across donors, different cell culture media, cytokine combinations and concentrations, and in different cell growth vessels.

864. Decentralized Manufacturing of Cell and Gene Therapies: A Novel Approach

Emily Heffernan[1], Forrest Brown[2]

[1]Arcadis, Cary, NC,[2]Germfree, Ormond Beach, FL

Since the approval of the first cell and gene therapies in 2017, demand and approvals for ATMPs continues to accelerate. While these therapies are life altering for the patients they serve, they have also been associated with such concerns as high price tags and critical supply chain shortages. This has raised concerns with the ability to provide access to patients with serious indications– particularly in developing nations. Historically, centralized manufacturing has for many entities led to economies of scale and thus the lowest cost model for manufacturing. However, due to the personalized nature of cell and gene therapies (CGT), the advantages to the economies of scale that favor centralized manufacturing are less prominent. Global regulators are concerned with issues arising from CGT cold-chain logistics. For example, the EU guideline on *Good Clinical Practice specific to Advanced Therapy Medicinal Products* notes: "Storage, transport and handling conditions have the potential to negatively impact the quality of ATMPs." For such reasons, decentralized manufacturing should be considered as an alternative to enable greater patient access. We will review specific benefits and disadvantages, as well as alternative contemporary models, of decentralized CGT manufacturing. Pharma 4.0 initiatives including process automation support manufacturing consistency in decentralized manufacturing approaches. A case study exploring the use of stand-alone cleanroom modules for decentralized manufacturing will also be reviewed and discussed.

865. Rapid and Precise Process Monitoring with Label-Free Laser Force Cytology™ Driving Improvements in CGT Manufacturing Quality and Consistency

Sean J. Hart

LumaCyte, Charlottesville, VA

The development and production of life saving advanced therapies can be challenging and time-consuming due to the complex nature of the cellular starting materials, manufacturing processes, and challenges around final product yields. Many current analytical tools face challenges in terms of speed, precision, reproducibility, and the high resource requirements needed to operate them. LumaCyte will present applications of Laser Force Cytology as a rapid process analytical technology (PAT) for biologics production monitoring which enables rapid characterization of cellular starting materials, the ability to precisely monitor and quantify biological processes affording developers and manufacturers a deeper understanding of cellular behavior across important bioprocess and production steps. Laser Force Cytology delivers novel label-free quantitative data in minutes to support rapid process optimization and scale up. The ability to minimize variability and batch failures during the manufacturing process with a real-time PAT can enable significant improvements in production quality and consistency. Process development and manufacturing efforts will benefit from a powerful yet simple to use analytics platform, enabling ease of deployment across multiple sites and the entire manufacturing process helping to minimize challenges around comparability.

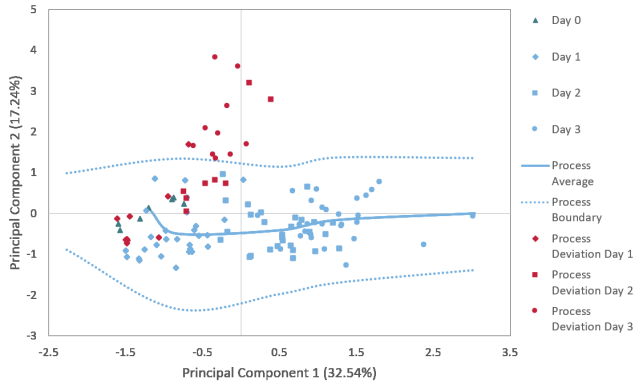


Figure: T Cell activation multivariate process road map showing successful process data for three patients (blue), and a process deviation of cells treated with doxorubicin (red). Data collected across five independent experiments using Radiance Laser Force Cytology instrument.

866. A Multiplexed and Scalable Continuous-Flow Electroporation Paltform for Efficient Cell Transfection

Harold Craighead, Jacob VanderBurgh, Stephen Levy, Grant Corso

CyteQuest, Ithaca, NY

Electroporation has emerged as an alternative to viral vectors for cellular reprogramming for CAR-T cell therapies. However, widespread adoption requires addressing difficulties with traditional electroporation such as process variability, arduous empirical process optimization, and limited throughput for cellular manufacturing. We addressed these issues with an electroporation platform that provides flexible, 8-channel multiplexed and efficient optimization capabilities, combined with ease of scaling from low-volume research/optimization to high-volume cell manufacturing. Flexibility and ease of scaling are enabled by a continuous-flow, planar microfluidic flow chip (Fig. 1A-B). The flow chip ensures cells experience reproducible electric fields and provides seamless scalability of experimental throughput by scaling the channel width. Up to 8 of these flow chips are incorporated into a continuous-flow microfluidic system that uses automated liquid handling to simultaneously test up to 8 chemical conditions combined with a range of flexible electrical conditions for rapid optimization of transfection. As a demonstration of the optimization capabilities of the system we transfected Jurkat cells with plasmid DNA encoding GFP within the multiplexed optimization system. We measured GFP expression and viability 24 hours post-transfection using flow cytometry, and a unifying metric, efficiency was calculated as the

product of GFP expression and viability. We simultaneously screened 8 different plasmid concentrations and 9 different electrical waveforms, depositing electroporated cells into a 96-well plate. We found that in this case, the optimal plasmid concentration was 37.5 μg/mL (Fig. 1C). Next, using this plasmid concentration, we rapidly screened hundreds of different voltage waveforms of varying shapes, durations, and amplitudes using varied parameters using ~20 μL of cells per condition. As a demonstration of the scaling capabilities of the system, we used the parameters optimized in the low-volume flow chip with 2 mm channel width to perform electroporation in a higher throughput flow chip with a 10 mm channel width. By keeping the channel height constant and proportionally increasing the flow channel width (from 2 mm to 10 mm) with the volumetric flow rate, cells experience identical mechanical, electrical, and chemical environments resulting in essentially identical transfection performance without re-optimization. Notably, we found that GFP expression and viability values were less than 5% different from Jurkat cells transfected in the 2 mm and 10 mm channel widths, demonstrating the ability to seamlessly scale processed cell volumes. (Fig. 1D). These results demonstrate the possibilities with a flexible electroporation platform for rapid, multiplexed optimization that is easily scaled to high-volume cell manufacturing without re-optimization. These capabilities bypass limitations of traditional electroporation equipment, creating a promising platform for non-viral cellular reprogramming for cell therapies.

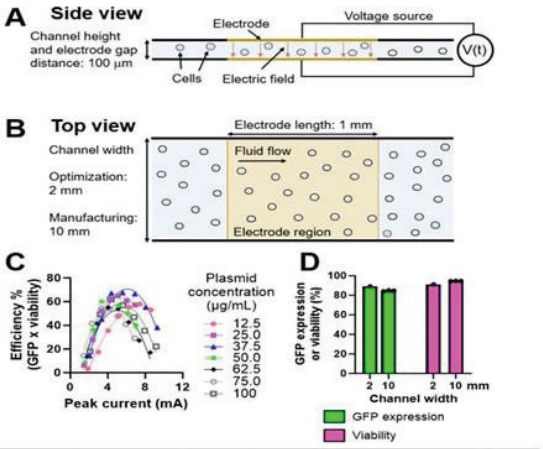


Figure 1: (A) Side and (B) Top view schematics of microfluidic flow chip. (C) Efficiency (GFP expression x viability) plotted as a function of plasmid concentration or peak current experienced by the cells. (D) GFP expression and viability data from Jurkat cells transfected with plasmid DNA encoding GFP in a flow chip with either a 2- or 10-mm channel width.

867. Closed Automation Enables Quick Fill-Finish of Large-Scale T Cell Therapy While Maintaining Consistency and Quality of the Final Product

Minsung Park[1], Meredith Safford[1], Jade Scheers[2], Lora Hammill[2], Despina Pleitez[1], Sarah Campion[2], Gibb L. Stuart[3], Mindy M. Miller[3], Smith Trevor[3], Alex Sargent[1]

[1]Process Development, Charles River Laboratory, Hanover, MD,[2]Analytical Development, Charles River Laboratory, Hanover, MD,[3]Terumo Blood and Cell Technologies, Lakewood, CO

Scaling up the manufacture of cell therapies can be complex and challenging. Maintaining critical quality attributes of the cell product during its final formulation and fill-finish into greater quantities of containers can be difficult and laborious. Here, we tested the automated FINIA platform to efficiently scale up the formulation and fill-finish of a T-cell product, and then assessed cell quality and product consistency across different sub-lots filled during this expanded process. We found that this automated system could be effectively scaled to four times its singular capacity in a two-hour time interval, and reduced operator hands-on time by 60%. The variation in cell product volume across all containers was less than 5%. Analysis of the different sub-lots of the final product revealed high cell viability and consistent T-cell phenotype, with a high proportion of effector and central memory T-cells and low expression of T-cell senescence or exhaustion markers. The functionality of the T-cell product was compared by measuring cytokine response after restimulation, with secreted levels of effector cytokines like IFN-γ and TNF-α being similar across the different sub-lots. Collectively, these results show that automation with the FINIA platform can scale up the formulation and fill-finish of a cell manufacturing process while maintaining the phenotype and functionality of the cell product. Demonstrating this automated, scalable approach to fill-finish that can maintain product uniformity and quality is important for the future development of cell therapies.

868. Efficient T-cell Activation and Expansion for CAR-T Therapy is Dependent on a Complex Interplay of the Starting Donor Population, Activation Antibodies and Expansion Platform

Matt Heidtman, Brandon Wagner, Christine Goetz, Nidhi Nath

Antibody Development, Bio-Techne, Minneapolis, MN

Chimeric antigen receptor (CAR) modified T cell therapies have revolutionized the area of cancer treatment, showing long term remission and minimal toxicity in many refractory and relapsed patients that have few available alternative treatments. Even with these promising results, the field continues to make improvements in targeting new cancer types, engineering new and more potent CAR-T cells, and improving manufacturability to reduce cost and time to therapy. Critical early steps in CAR-T manufacturing are isolation and enrichment of T cells from PBMCs followed by T cell activation and expansion using CD3 and CD28 antibodies. Commonly used activation methods involve anti-CD3/anti-CD28 coated culture dishes or bags, antibody coated-magnetic beads, direct antibody addition as soluble factors, and conjugated-polymeric matrices, among others. In

this study we explore two interconnected factors that may impact the T cell activation profile and phenotype. In the first study, we analyzed the effect of 3 different antibody-based activation methods (soluble, plate-bound, and bead-bound) on T cell activation/expansion by using chimeric (human and mouse) CD3 and CD28 antibodies. We observed similar levels of T cell expansion over 14 days across the different activation methods, using cells obtained from 4 donors. Interestingly, we found that bead-based expansion of T cells resulted in a lower percentage of cells expressing a subset of activation markers (CD25 and CD38) and a higher proportion CD45RO+ memory T cells relative to plate-bound and soluble antibody platforms. Furthermore, T cells expanded by stimulation via bead-bound antibodies displayed higher levels of the exhaustion marker, PD-1, but lower levels of TIM-3 and LAG-3 compared to stimulation via soluble and plate-based antibodies. These phenotypic differences may have important consequences for efficacy in a clinical setting. In a subsequent study we tested whether altering the ratios of CD3/CD28 antibodies resulted in differences in T cell phenotype after expansion. Surprisingly, we found that increasing the amount of the CD28 antibody relative to constant CD3 antibody did not impact T cell expansion or phenotype over a 14-day process. These results suggest that addition of other agonist antibodies (e.g., anti-CD2, anti-CD137) or altering the amount of CD28 more extensively, may be necessary to generate a desired phenotypic outcome. Taken together, our results highlight the importance of CD3/CD28 antibody presentation when used to stimulate T cell proliferation, particularly when developing expansion protocols for routine, reproducible manufacture of cell therapies. Among various modes of cell activation, we further noted significant differences in the resulting activated T-cell population, underscoring the importance of monitoring cell phenotype during the production of CAR-T therapies. This work further confirms the need for larger studies to analyze the T cell expansion and phenotypic profiles from a wide cohort of individuals of different sex and ages. This will build a knowledge base to meet the need for truly personalized T cell activation profiles to deliver highly effective T cell therapies.

869. Aptamer-Based, Traceless Selection of CD62L$^+$ CD8$^+$ T Cells for Improved Adoptive T Cell Manufacturing

Abe Y. Wu[1], Emmeline L. Cheng[1], Nataly Kacherovsky[1], Stephen J. Salipante[2], Michael C. Jensen[3], Suzie H. Pun[1]

[1]Department of Bioengineering, University of Washington, Seattle, WA,[2]Department of Laboratory Medicine, University of Washington, Seattle, WA,[3]Seattle Children's Research Institute, Seattle, WA

INTRODUCTION: CAR T cells manufactured from more defined, less differentiated T cell subsets have shown greater persistence and potency than those derived from more differentiated effector cells. However, current selection relies on antibody-based magnetic activated cell sorting (MACS) technology, which is costly and largely precludes multiple positive selections in series due to the surface retention of magnetic beads. Consequently, selection of T cell subsets requires a combination of positive and negative selection strategies that can result in low product purity and yield, presenting a significant challenge for clinical translation. Previously, we discovered a CD8 aptamer (CD8Apt) and developed an aptamer-based method to tracelessly isolate CD8$^+$ cells for CAR T cell therapy[1]. In this work, we discovered a high-affinity CD62L aptamer (CD62LApt) and applied CD62LApt and CD8Apt to tracelessly and sequentially select less differentiated CD62L$^+$CD8$^+$ T cells with high yield and purity. **RESULTS:** We conducted cell-SELEX for 8 rounds to identify T cell-binding aptamers and discovered an aptamer that binds to CD62L (CD62LApt). CD62LApt binds to primary human T cells with K_D=0.31nM and to soluble CD62L with K_D=5.30nM. Using CD62LApt and our previously discovered CD8Apt, we designed a method to tracelessly and sequentially select CD62L$^+$CD8$^+$ T cells from human PBMCs. In each selection step, we show >95% capture of all CD8$^+$ and CD62L$^+$ cells (Fig. 1A). After selection, we show that CD3$^+$CD8$^+$CD62L$^+$ T cells are highly enriched from 5.18 ± 2.16% in the PBMCs to 69.7 ± 8.16% in the post-selection product (antidote elution), comparable to antibody-selected cells (Fig. 1B and C). However, the aptamer-based, serial positive selection approach delivers an overall CD8$^+$CD62L$^+$ yield of 59.14 ± 2.55% that is significantly higher than that from antibody-based selection (Fig. 1D). **CONCLUSIONS**: These results suggest that the aptamer-based approach enables the precise selection of T cell subsets with high yield for manufacturing CAR T cells from less differentiated cell subsets in a mostly synthetic system. We are currently evaluating the performance of CAR T cells manufactured from our aptamer-selected cells in in vivo tumor models.

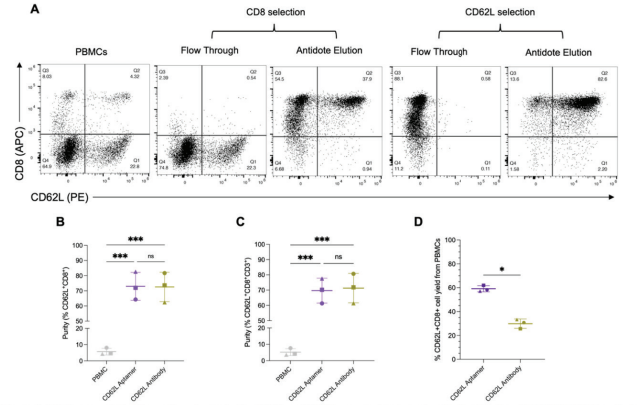


Figure 1. (A) Representative flow cytometry analysis of CD8 and CD62L expression in each selection fraction. (B) CD62L$^+$CD8$^+$ purity in the final selected fraction. (C) CD62L$^+$CD8$^+$CD3$^+$ purity in the final selected fraction. (D) CD62L$^+$CJ8$^+$ yield from PBMCs.

1 Kacherovsky, N. *et al.* Traceless aptamer-mediated isolation of CD8(+) T cells for chimeric antigen receptor T-cell therapy. *Nat Biomed Eng* **3**, 783-795, doi:10.1038/s41551-019-0411-6 (2019).

870. Impact of Media Exchange Strategy on T-Cell Expansion

Lindsey A. Clarke, Noman Naeem, Richard Baxter, Nicholas Akosa, Maria Kalli, James Kusena, Césaré Cejas, Antoine Espinet

MFX, Stevenage, United Kingdom

Background & Aim: Media supplementation (replacement/addition/perfusion) of Primary T-cell culture plays a crucial part in maintaining robust expansion ex vivo in a variety of systems and is key to achieving high cell densities. Scaled-down bioreactors are advantageous for understanding the impact that media exchange strategies can have as smaller environments help users to better comprehend the underlying cell biology by allowing rapid generation of data in a cost effective manner. Existing automated systems for manufacturing and process development have high price-points that curtail accessibility.

Therefore, users rely on current research tools and systems - largely labour-intensive - where manual feeds limit the opportunity to closely monitor the expansion process and control cell growth densities. Methods:We designed a machine platform that automates media replacement via continuous perfusion. This system uses a bespoke scaled-down microfluidics-based bioreactor, designed specifically for automation integration while still offering optimal conditions for cell growth. Using Primary T-cells from different donors (both fresh & frozen) as well as a cell line (Jurkats), we demonstrate the performance of this machine platform in delivering consistent media continuously by investigating its impact on cell expansion numbers, viability, phenotype and morphology. We also compare results from automation with that of a manual batch-fed media exchange strategy on the same bioreactor. In addition, we contrast the performance of our bioreactor vs. conventional industry standard tools. Results:We demonstrate via automation that controlled frequent media replenishment regularly help minimise lactate build-up and maintains more conducive microenvironments for cell growth than more standard manually-operated protocols. Through automation, we can push cell densities even higher and by multiplexing multiple bioreactors can perform parallel multivariant experiments, thereby permitting more efficient process optimisation at scaled-down level. Some of the challenges of automated continuous perfusion strategy are cell loss and cell shear. However, with this bespoke microfluidics-based bioreactor, we demonstrate that we are able to maintain high cell retention efficiencies with low shear and consistent viable growth.. Conclusion:Our study highlights the benefits of automation to improve T-cell expansion for more efficient process development using a lower-cost machine platform with custom-designed microfluidics-based bioreactors. Future works aims to explore the feasibility of bringing other unit operations such as transduction and washing into the same microfluidics bioreactor.

871. Considerations in Raw Materials when Manufacturing Cell Therapies and Other Biologics

Rebecca Warfield[1], Spencer Chiang[2], Yanke Liu[2], Jane Liu[2]

[1]ACROBiosystems, Newark, DE,[2]ACROBiosystems, Beijing, China

Growth factors are a cornerstone in the intricate landscape of cell therapy manufacturing, playing multifaceted roles essential to produce biologics and other advanced therapeutic medicines. These biomolecules are intricately utilized throughout various manufacturing phases. As growth factors, they stimulate cell reproduction for desired therapeutic compounds as well as differentiate stem cells into cells of interest. After the initial adoption of several cell therapies, focus has become on ensuring manufacturing consistency, quality, and reliability in biopharmaceutical manufacturing. As such, quality control of cell therapies requires a multi-faceted approach for QC of not only the final therapeutic, but also raw materials that go into manufacturing cell therapies. Herein, we explore the different key methods and new assay validation procedures behind GMP product development to ensure a safe raw material for use in therapeutic manufacturing. We also highlight key facets of growth factor 'grades' that are associated with different stages of cell therapy manufacturing. As the biopharmaceutical industry continues to evolve and innovate, cell therapy manufacturers need to further emphasize the need for suppliers to ensure these tools are high-quality and provided at the required structure to continue to provide the foundational elements in pursuing improved global healthcare outcomes.

872. Closed Modular Clinical-Scale Manufacturing of CAR T Cells for Cancer Immunotherapy Using Activated and Non-Activated T Cells

Akshaya Chandrasekaran[1], Ranganatha Somasagara[1], Monique LaCourse[1], Anna Liu[1], Arseny Smirnov[1], Gregory Bonello[1], Namritha Ravinder[2]

[1]Thermo Fisher Scientific, Carlsbad, CA,[2]ThermoFisher Scientific, Carlsbad, CA

Introduction: Rapid developments in the field of chimeric antigen receptor (CAR) T-based immunotherapies and the need for generating efficient and cost-effective cell therapies have increased the demand for manufacturing workflow improvements to improve patient access while maintaining quality control and performance. Conventional CAR T cell manufacturing workflows involve T cell activation, followed by payload delivery, and *ex vivo* expansion. However, the activation and expansion of CAR T cells leads to their progressive differentiation, T cell exhaustion, and ultimately results in resistance to CAR T therapies. Accumulating evidence in mice and humans suggests that T cell differentiation negatively correlates with long-term anti-tumor activity, with early memory T cells allowing for greater and persistent anti-tumor effects. More recently, it has been established that a modified cell manufacturing process implementing activation after Cas9 delivery minimizes occurrence and impact of chromosome loss in the manufactured product. Non-activated cells are also desirable as a starting material to achieve shorter workflow times critical to point-of-care and decentralized therapy manufacturing and reduced costs. Here we demonstrate a closed and modular system for the generation of CAR T cell products starting from non-activated/resting and activated T cell populations. Methods: We use an automated counterflow centrifugation method where PBMCs are isolated from fresh leukopaks using the Rotea™ system and then downstream T cell enrichment is performed. Electroporation (EP) reactions for payload delivery are set up and optimized at small scale using Neon™ NxT™ platform and then scaled up using CTS™ Xenon™ Electroporation System. Transfection efficiency and cell viability are measured by flow cytometry and resting T cell phenotype is monitored pre- and post-EP to monitor maintenance of stemness in comparison to the starting population. Electroporations are performed using the CTS™ Xenon™ Lower Conductivity Buffer that enables higher energy setting on the Xenon system for hard-to-transfect cell types thereby providing a wider design space for process development and commercial manufacturing. Cells are activated post-electroporation and expanded for downstream functional assays. For the activated T cell protocol, PBMCs are isolated from frozen leukopaks using the Rotea™ system and activated using CD3/CD28 CTS™ Dynabeads™. The activated T cells are then resuspended in CTS™ Xenon™ Genome Editing Buffer and electroporated to deliver the Cas9 RNP complex and donor DNA expressing anti-CD19 CAR. Post-expansion, cells are washed and concentrated using Rotea™ and analyzed for transgene expression and

cryopreserved for downstream functional studies. Targeted Amplicon-seq validation assay (TAV-seq) confirmed no detectable off-targets in CAR T cells and functional cancer cell cytotoxic assays against CD19 presenting-Nalm-6 cells showed effective killing by anti CD19 CAR-T cells. Conclusions: In summary, we established a closed modular workflow for CAR T cell generation using automated instrumentation for cell processing and gene delivery thereby minimizing human intervention in the cell therapy manufacturing process. The transfection reagents and gene editing payloads in combination with the closed automated workflows described here will help reduce labor-intensive open system hurdles and improve the engineering of hard-to-transfect primary cells used for the development of efficient adoptive immunotherapies.

Wednesday Posters: Pharmacology/Toxicology Studies and Analytics/Assay Development

873. Validation of AntiAdeno Associated Virus Serotype rh10 (AAVrh.10) Total and Neutralizing Antibody Immunogenicity Assays

Elizabeth Butala-Flores, Thien Nguyen, Luke Armstrong, Amanda Hays
BioAgilytix, Durham, NC

Purpose/Background: Immunogenicity assessments used for inclusion/exclusion for safety and efficacy are critical for the development of Adeno-Associated Virus (AAV)-based gene therapies. There are different assay formats that can be used and that are being used for this context and yet it is not clear which of the assays should be implemented at what stage of drug development. AAVrh.10 has gained interest for use in gene therapies given its superior transduction efficiency, however there is limited information in the literature on anti-AAVrh.10 antibodies and their clinical impact. Herein, we highlight the validation of a total antibody assay (TAb) and a neutralizing antibody (NAb) assay for detection of anti-AAVrh.10 antibodies to be used in enrollment of patients into an AAVrh.10 gene therapy clinical study. Both assays were compared in terms of sensitivity, drug tolerance, and precision, along with a concordance analysis to determine if both assays produce similar results if used in enrollment. Methods/Materials: For the TAb assay, a sequential bridging ECLIA assay was developed. Briefly, MSD standard binding plates were coated with unlabeled AAVrh.10 for capture, followed by blocking then incubation with human serum and detection with a ruthenium labeled AAVrh.10-Luc and MSD Read Buffer T. A signal within the well correlates with the presence of TAb in the sample. For the NAb assay, an AAVrh.10 vector containing firefly luciferase reporter gene was applied to a transduction permissive cell line, 2V6.11. Cells were seeded in 96-well Poly-D-Lysine coated plates and incubated overnight at 37°C/5% CO_2. The next day, AAVrh.10-Luc vector was mixed with human serum samples, added to the cell plate and placed in an incubator for 24 hours. One-Glo reagent was added to the plate before reading luminescence on a Biotek Cytation 3. The presence of NAb in human serum samples inhibits the luminescence-generating activity of the virus, thereby reducing signal in the assay. Both assays were validated including determination of screening cut-points in accordance with the 2019 FDA immunogenicity guidance. Results: Both assay formats (TAb and NAb) were validated to determine sensitivity, cut-point determinations, precision, drug tolerance, and selectivity. Both assays were compared in terms of sensitivity and other performance parameters. In addition, a concordance analysis was performed to understand the agreement in response between samples that were evaluated in both the TAb and NAb assays. Of the 140 screened samples, 34% (48 samples) screened negative in both the TAb and NAb assays (TAb-/NAb-), whereas 55% (77 samples) screened positive in both assays (TAb+/NAb+). Five samples (3.6%) that screened positive in the TAb assay did not demonstrate positivity for neutralizing antibodies to AAVrh.10 (TAb+/NAb-) and 10 samples screened negative in the TAb and positive in the NAb (TAb-/NAb+). Further evaluations were performed to understand the antibody titer response for samples that screened positive in one assay and negative in the other. Conclusions: The information we present herein suggests a concordance between TAb and NAb assays that demonstrates the suitability of either to be used as a screening assay for patient enrollment into AAVrh.10 gene therapy clinical studies. This information contributes to the acceleration of drug development and provides the industry with information on assays that can be used for patient enrollment and as a potential companion diagnostic for AAVrh.10 gene therapies.

Correlation of TAb and NAb assay			
		Anti-AAVrh.10 NAb	Anti-AAVrh.10 NAb
		Negative	Positive
Anti-AAVrh.10 TAb	Positive	5	77
Anti-AAVrh.10 TAb	Negative	48	10

874. Qualification of a Universal IFN-gamma ELISpot Assay That Can Monitor Cellular Immune Responses in Gene Therapy, Cell Therapy, and Vaccine Development

Lu Zhang, George Zhu, Hongrong Hu, Tongyuan Li, Dongbei Li, Xiao-Yan Cai
Accurant Biotech Inc., Cranbury, NJ

Gene therapy, cell therapy, and clinical vaccine development all require cellular immunity testing performed in a regulated environment to ensure that they are safe and effective. Enzyme-Linked Immunospot (ELISpot) assays are currently the most commonly used cellular immunoassays that can measure cytokine-secreting cells at the single cell level in a semi-quantitative manner. In our current study, we developed and qualified a universal and sensitive interferon-gamma (IFN-γ)-based ELISpot method that can monitor T-cell immune responses during pre- and post-dose periods in clinical trials. This method explored the following parameters: Lower limit of quantification (LLOQ), upper limit of quantification (ULOQ), precision tested at two different levels of CEF (Cytomegalovirus, Epstein-Barr virus, and Influenza virus epitopes)-stimulated spots, linearity, robustness ranges, and plate and sample storage stability. We

tested this method using PBMCs collected and isolated in different ways, and designed each of these platform parameters so that they could be utilized by a wide variety of future assays. For each individual therapy development, the confirmatory cut point (CCP) of test article-specific antigens, limit of detection (LOD), and minimum immune response (MIR) have been established at the 95th percentile, using 28 PBMC lots to achieve reasonable statistical power. This qualified IFN-γ ELISpot platform has been utilized in several gene therapy clinical studies (AAV2, AAV9 etc.), demonstrating the value of this universal ELISpot methodology to reliably measure cellular immune responses against AAV gene therapy.

875. Comparison of a Microfluidic Generic Total Antibody (TAb) Assay with Independent Neutralizing Antibody (NAb) and TAb Assays for the Detection of Pre-Existing Antibodies Against AAV Vectors

Eric Feeley[1], Frida Löthberg[2], Kira Hedström[2], Jordi Rodo[3], Lone Frier Bovin[3], Selvi Celik[3], Cecilia Klint[3], Maria Gianneli[2]

[1]Gyros Protein Technologies, Warren, NJ,[2]Gyros Protein Technologies, Uppsala, Sweden,[3]Svar Life Science AB, Malmo, Sweden

PURPOSE: In clinical trials with adeno-associated virus (AAV) vectors, screening for participants lacking pre-existing neutralizing antibodies is crucial for effective gene therapies. Various methods, such as Neutralizing (NAb) assays and Total Antibody (TAb) assays, are used to screen for anti-AAV antibodies. NAb assays assess how NAbs and other non-antibody factors hinder AAV transduction as measured by the expression of a reporter gene mediated by the AAV therapy. TAb assays, with their simpler design and operational ease, typically require serotype-specific assays or AAV capsid labeling (when bridging format is used), introducing time and variability. We developed a generic anti-AAV TAb immunoassay on the automated Gyrolab platform that enables the detection of anti-AAV antibodies in human samples, eliminating the need for serotype-specific assays or AAV capsid labeling. The assay's robustness was confirmed through comparison with independent NAb and TAb assays. METHODS: Anti-AAV TAb analysis in an automated, CD-based format was performed on the Gyrolab microfluidic platform with commercially available Gyrolab Generic Anti-AAV kit reagents on human serum samples. NAb assays were completed using SVAR Life Science iLite® AAV Neutralizing Platform for 2 different serotypes (AAV2 and AAV8), whereas SVAR Life Science ELISA TAb assay was performed for serotype AAV2. RESULTS: The NAb results for 12 human samples are given in % neutralization (transduction inhibition). For this comparison, a cut point was set at 35% for AAV2, with samples reading above qualifying as positive and samples reading below qualifying as negative. For AAV2, 8/12 NAb-samples correlated well with the Gyrolab microfluidic assay. Samples S58, S72 and S74 came in as negative in the NAb assay and positive in the Gyrolab assay while S69 qualified as positive in the NAb and negative in the Gyrolab assay. For AAV8, with a cut point of 38%, the NAb and Gyrolab assays correlated completely. In the comparison of the AAV2 ELISA TAb assay to the Gyrolab assay, samples quantifying above 1 ug/mL in a 1/30 dilution in the TAb are considered positive. There is a clear correlation between samples qualifying as positive in both assays. The TAb data for samples S58, S69, S72 and S74 is consistent with the Gyrolab assay. This speaks to the conclusion that the differences in qualification for these samples are due to the assay formats (NAb vs TAb) rather than an inaccurate read. CONCLUSIONS: We have developed a Gyrolab generic assay that allows for the qualitative assessment/screening of binding antibodies against AVV vectors for different serotypes. The results correlate strongly with independent NAb and TAb assays despite a small sample size. This highlights the potential of our Gyrolab assay as a stand-alone or complementary tool for the assessment and screening of pre-existing anti-AAV antibodies in patients.

876. The Role of Thermal Stability in AAV Titration of Engineered Variants

Emilia Araujo Zin[1], Melissa Desrosiers[1], Guillaume Labernede[1], Camille Robert[1], Deniz Dalkara[2]

[1]Institut de la Vision, Paris, France,[2]Institut de La Vision / Sorbonne Universites, Paris, France

Determining accurate and reproducible titers for recombinant adeno-associated virus (rAAV) productions is essential to their development and downstream therapeutic applications. However, reproducible and reliable titering is a bottleneck in the field of AAV-mediated gene therapy. Moreover, the fast growing number of engineered AAV capsids further complicates the generalization of titering schemes, sometimes resulting in low and unreliable viral titers, questioning their adequacy for clinical development and use. Traditionally, AAV titers are expressed as genome copies or viral genomes per milliliter. Before amplifying viral genomes through qPCR or ddPCR, the free-floating DNA requires enzymatic digestion. In this study we propose for the first time that the DNase inactivation step, at 65°C or above, is a critical bottleneck for variants who have a melting temperature within the DNase inactivation temperature range. Here we measure melting temperatures for engineered AAV variants and demonstrate a correlation between lower thermal stability and titer variability emphasizing the need for titering methods that do not require heating of the sample. This finding is especially important for work involving engineered AAV variants with lower thermostability.

877. Evaluating Transduction Efficiencies of AAV Vectors in Human iPSC-Derived Cell Types

Ravi Vaidyanathan[1], Rebecca Fiene[1], Morten Seirup[2], Trish Hoang[2], Jeff Nelson[2], Brett Johnson[3], Belen Torres[3], Sreenath Palle[3], Emigdio Reyes[3], Margrethe Boyd[4], Ana Wang[4], Anthony Essex[4], Simon Hilcove[1], Coby Carlson[1]

[1]FUJIFILM Cellular Dynamics Inc. - Madison WI, Madison, WI,[2]Promega Corporation, Fitchburg, WI,[3]FUJIFILM Diosynth Biotechnologies, College Station, TX,[4]PhenoVista Biosciences, San Diego, CA

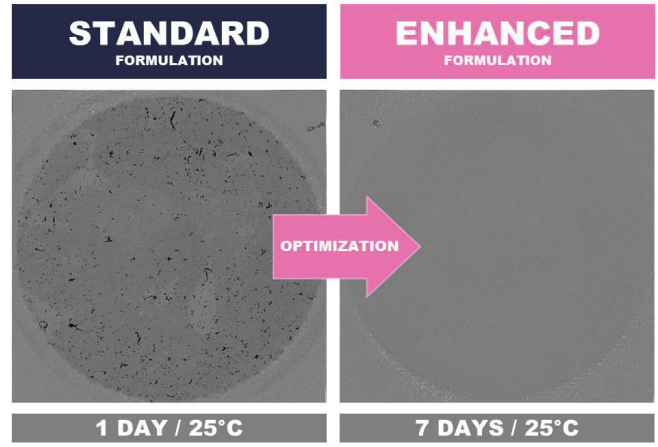
INTRODUCTION: The differentiation of induced pluripotent stem cells (iPSC) into specialized cell types of the human body represents a major advancement for the development of biologically relevant in vitro models. These cellular systems enable "disease-in-a-dish" studies and are compatible with various gene targeting approaches, including

adeno-associated viral (AAV) vectors, to directly correct the genetic mutation. However, the permissibility of AAV transduction across different iPSC-derived cell types needs to be defined. This requires the implementation of a sensitive and versatile technology to monitor transduction efficiencies and quantify the impact of various factors, including viral vector serotype, multiplicity of infection (MOI), choice of promoter, transduction media, and/or timing of transduction. **METHODS:** Commercially available cryopreserved human iPSC-derived "iCell" products from FUJIFILM Cellular Dynamics, Inc. (FCDI) were used according to manufacturer's recommendations. Viral vectors were engineered with the AAV NanoLuc®-HaloTag® Dual Reporter System and viruses were generated by Promega. A subset of parallel experiments utilized AAV reagents from commercial vendors driving the expression of green fluorescent protein (GFP), comparing CAG and CMV promoters were also used in this study. These panels of AAV reagents was used to transduce several different iCell® products at MOI ranging from 10 to 10^6. Transduction efficiency was monitored either kinetically for 48 hours or via endpoint assay measuring luminescence on a plate reader or fluorescence by flow cytometry or imaging. **RESULTS:** iCell products (including astrocytes, cardiomyocytes, hepatocytes, neurons, and retinal pigmented epithelial (RPE) cells) were transduced with different serotypes (including AAV1, 2, 4, 5, 6, 8, and 9) containing various promoters (CAG, CMV, or Synapsin) in serum-free media. Broadly speaking, AAV6 showed the highest transduction efficiency across iPSC-derived cell types. Moreover, Synapsin drove higher expression in neuronal cells, whereas CMV was better in other cells. CAG was also very effective in cardiomyocytes. Neuronal cell types required longer transduction times (i.e., 96 h exposure to AAV) than did cardiomyocytes (24 h to peak signal, for example). In general, transductions were more efficient at earlier timepoints in culture (i.e., Day 7 vs. Day 10 for cardiomyocytes and Day 14 vs. Day 21 for RPE) with some exceptions. Importantly, the fluorescent aspect of the reporter enabled imaging of the cells to verify no impact to cell health or morphology post-transduction, in addition to efficient expression of the transgene.**CONCLUSION:** iCell products make a great model to evaluate transduction efficiency of different AAV serotypes preclinically. The systematic exploration of the numerous variables that impact the efficiency of AAV transduction for any given cell type is required. Cryopreserved cells from commercial sources that are manufactured reproducibly at scale are an ideal reagent for use in these studies because of their "thaw-and-go" consistency. Proper handling and storage of AAV material is critically important as it can affect transduction efficiency. Finally, the combination of iPSC-derived cells and AAV technology to modulate in vitro cellular disease models for pre-clinical studies and potentially even potency release assays is a powerful system that is now available as part of a drug manufacturing process for material release.

878. Combination of BMI, DLS and Visual Inspection to Resolve Particle Formation Issues in Formulations Commonly Used in Gene Therapy

Astrid Stockinger, Philipp Beck, Sonya Schermann
Ascend, Planegg, Germany

The development of gene therapies necessitates a rigorous approach to ensure product safety and efficacy. Historically, consideration of subvisible particles as a parameter in formulation development was deferred to later stages due to resource-intensive methods like light obscuration (LO) and micro flow imaging (MFI). The **scarcity of material** in **early-stage gene therapy** requires the exploration of **low-volume, high-throughput methods** for early detection of both **subvisible** and **visible particles**. This study highlights the effectiveness of **backgrounded membrane imaging (BMI)** as a low-volume method to identify optimal buffer conditions that mitigate subvisible and visible particle formation **early in formulation development**. Starting from a standard PBS AAV formulation, we **screened a range of excipient conditions at room temperature over the course of 7 days** to discover the optimal formulation for prevention of particle formation. The low volume requirement of BMI enabled us to perform this study with only **150 µL of sample per condition**. Complemented by dynamic light scattering (DLS) and visual inspection (VI), our findings showcase the successful resolution of particle aggregation by introducing a common surfactant to the formulation buffer and determining the optimal concentration. The use of BMI allows particles in both the **subvisible & visible range (2 µm - 5 mm)** to be characterized at low volumes and **complements DLS data**, which captures smaller aggregates (less than 1 µm) and **visual inspection**, which primarily identifies larger particles (> 150 µm). Nonetheless, a potential blind spot exists for particles between 1 µm and 2 µm, posing a challenge to achieving thorough characterization of the entire particle size range. However, the **combination of all three methods represents a comprehensive and synergistic approach** to mitigate the challenges associated with particle formation in formulation development for gene therapies.



879. *In Vitro* Assessment for Innate Immune Activation by AAV

Hideyoshi Kawasaki[1], Daisuke Sasaki[1], Shogo Matsumura[1], Geoffrey de Couto[2], Takeshi Sato[2], Ko Omura[2], Katsuyuki Kazusa[1]
[1]Exploratory Pathophysiology, Non-Clinical Biomedical Science, Applied Research & Operations, Astellas Pharma Inc., Tsukuba, Japan,[2]Safety Assessment, Integrative Biology, Astellas Gene Therapies, South San Francisco, CA

Adeno-associated virus (AAV) vectors are widely used in gene therapy, but immune response against AAV is known as one of the concerns. It

was reported that unmethylated CpG dinucleotides contained in the AAV genomes can induce activation of innate immune response via Toll-like receptor 9 (TLR9). It has also been reported that the production of type I interferon via TLR9 activation in plasmacytoid dendritic cells (pDCs) contributes to the activation of CD8+ T cell responses to AAV capsid. Because these activated CD8+ T cells have been reported to lower transgene expression via cytotoxicity to transduced cells, it is considered that to minimize the innate immune activation by AAV is valuable from the perspective of maintaining transgene expression and reduction of safety risks. Moreover, because it is known that there are species differences in immune responses, estimating the potential risk in humans at the non-clinical stage will lead to lower safety risks in the clinical setting and increase the probability of development success. Therefore, the purpose of this study is to establish an *in vitro* assay using human-derived samples to predict the innate immune response to AAV in humans. In this study, healthy volunteer-derived whole blood or frozen primary pDCs were used as human-derived samples. We analyzed secreted type I interferon levels in response to AAV as an indicator of innate immune activation. In contrast to AAV alone, addition of anti-AAV antibody led to robust type I interferon secretion. We analyzed the variability between donor lots, reactivity of multiple AAV serotypes, and the impact of TLR9 inhibition. To assess the implementation of this assay with higher throughput, we investigated innate immune signal response to AAV using THP-1 reporter cells. The results of these experiments will be shared in this presentation. In this study, it was concluded that innate immune response against AAV can be evaluated with *in vitro* assay using human-derived samples. This assay may improve gene therapy product development by selecting AAV genomic sequences with lower innate immune activation, lower safety risks, and greater pharmacological efficacy. Funding Statement: This research was funded by Astellas.

880. IdeS *In Vitro* Specificity Testing and GLP-Compliant Toxicology Study Yielded No Off-Target Substrate Cleavage or Test Article Related Toxicity

Scott N. Ashley, Sarah T. C. Elliott, Kelvin See, Saghana Muraleetharan, Rhyan Paul, Stephanie Kutza, Sayali Saykhedkar, Andria Culbert, Timothy S. Luongo, Jeffrey M. Alexander, Christopher R. Riling

Spark Therapeutics, Philadelphia, PA

AAV-based gene therapy has demonstrated promise in its ability to treat genetic disease. Pre-existing humoral immunity to the AAV vector may present a barrier to access for AAV-based gene therapy because a significant proportion of the population has naturally occurring antibodies that neutralize AAV vectors and inhibit transduction when delivered systemically. Overcoming this obstacle is critical for broad access to AAV-based gene therapy as a therapeutic. IgG-degrading enzyme (IdeS) is a cysteine endopeptidase derived from *Streptococcus pyogenes* that progressively cleaves the lower hinge region of IgG, generating F(ab')2 and Fc fragments and reducing IgG-mediated neutralization. Utilizing IdeS as a pre-treatment to eliminate anti-AAV IgG may enable treatment of seropositive patients with preexisting immunity to AAV. To evaluate the potential safety risks of using IdeS as a pre-treatment for AAV-based gene therapy, we investigated the specificity of IdeS through an *in vitro* characterization of potential substrates and evaluated the *in vivo* tolerability of IdeS in the rabbit model. To determine the specificity of IdeS, we digested a panel of human proteins selected on a variety of characteristics including predicted binding to IdeS and prevalence in human serum. IdeS demonstrated singular specificity as cleavage was only observed in the immunoglobulin IgG isotype. To investigate the safety of IdeS, we conducted a GLP-compliant toxicology study in New Zealand white rabbits, an animal model in which IdeS has been shown to be pharmacologically active. Both female and male rabbits were randomly assigned to one of four dosing groups given a single intravenous dose of IdeS at 0 mg/kg, 2.5 mg/kg, 7.75 mg/kg, or 20 mg/kg with half of each group taken down on study day 3 and remaining half taken down on study day 29, resulting in no significant pathology or mortality when monitored over 3 or 29 days. IdeS treatment demonstrated a range of 99.2% to 99.7% mean IgG cleavage despite a relatively short range of mean serum IdeS half-life, 4.63-8.15 hours. There were no test article findings in clinical observations, macro and microscopic histopathology, hematology, clinical chemistry, and coagulation. These findings provide evidence for specificity and tolerability of IdeS.

881. Optimal Prescreening Number of Non-Human Primates (NHPs) for Adeno-Associated Virus (AAV) Neutralizing Antibodies (NAb) in Gene Therapy Studies

Rajeev K. Dhawan, Connor John Balzer, Sarah Bernard, Lachelle Campbell, Brittany Egan, Donald Nicholson

Charles River Laboratories, Wilmington, MA

Adeno-associated virus (AAV) is the preferred vector for gene therapy studies. However, presence of neutralizing antibodies (NAb), from prior exposure to the virus, can limit its use in patients or research animals. Prior to studies, it's a common practice to prescreen the animals to detect the preexisting NAb using cell based assays. This may need screening of 3X to as high as 10X the number of NHPs enrolled in studies to find enough seronegative animals for the AAV vector serotype being utilized. Given today's NHP supply shortages, it's becoming difficult to find enough animals to accommodate testing of large numbers leading to increased costs and delays in studies. Using the data collected by our lab over the past 2 years, we sought to identify variables that can better predict the likelihood of AAV seropositivity in NHPs to minimize the number of animals required for prescreening to achieve the desired total of seronegative NHPs. To this end, data for four different AAV serotypes including AAV2, AAV8, AAV9 and AAVrh74 from nearly 2500 cynomolgus macaque sera was collated and analyzed. Results showed that seroprevalence ranged between 50% and 80% for all serotypes with AAV2 being the most prevalent and AAV9 the least. Both sex and age (2-3 years old) did not influence the percentage of AAV NAb positive NHPs for any of the tested serotypes. Small variations in seroprevalence were found in NHPs from different countries of origin and even different colonies from the same country. For example, between two Asian countries, one showed ~15% higher prevalence of AAV9 NAbs but ~10% lower prevalence of AAV2 NAbs. Variations in AAV seroprevalence was observed between different serotypes for NHPs sourced from different countries and vendors. However, no big change was seen between males vs. females. Additionally, in some cases, seroconversion was observed between

prescreening and the study start date, so it is advisable to screen NHPs as close to the start of a study as possible. To summarize, when screening NHPs to select subjects for study, it's important to consult the NHP supplier for suggestions about optimal screening timing and numbers based on specific seroprevalence observed in their colonies.

882. Comprehensive Immunogenicity Analysis of AAV Therapies: Correlating Anti-AAV Antibodies (NAbs/TAbs) with Complement Activation Dynamics

Jordi Rodo[1], Klaudia Kulak[2], Viktoria Kozma[2], Cecilia Klint[2], Michael Tovey[3], Michael Schwenkert[2], Lone Frier Bovin[2], Therese Segerstein[2]

[1]Svar Life Science, Cambridge, MA,[2]Svar Life Science, Malmö, Sweden,[3]Svar Life Science, Paris, Sweden

The extensive study of adeno-associated virus (AAV) vectors as carriers in gene therapy has progressed the field from a promising concept to a tangible medical reality. Despite these advancements, significant bottlenecks persist in the development of gene therapy products, particularly in meeting stringent regulatory requirements. A key challenge is the influence of antibodies against AAV on the efficacy of in vivo AAV-mediated gene therapy. This includes issues like capsid neutralization and triggering of immune reactions, notably the activation of the complement system. Currently, it is recommended by different industry leaders to analyze both the presence of total binding antibodies (TAbs) as well as neutralizing antibodies (NAbs). Additionally, assessing patient sensitivity to complement activation due to AAV adds a critical dimension to immunogenicity evaluation. Here we present the evaluation on 100+ samples of Svar's AAV immunogenicity solutions, which includes the assessment of anti-AAV antibodies (both NAbs and TAbs) and each sample's sensitivity to AAV-mediated complement activation. Our approach first employs a novel cell-based iLite® AAV platform for accurate NAb assessment to detect NAb-mediated reduction of AAV transduction. Secondly, TAbs are assessed with a standardized immunoassay with a high sensitivity, allowing the evaluation of the presence of anti-AAV antibodies Finally, the potential for AAV-mediated complement activation is evaluated in each sample, a crucial step in identifying susceptibilities that may lead to adverse effects. This evaluation is adaptable for both pre- and post-AAV administration scenarios. These three assays combine the advantages of both immuno- and cell-based assays. By offering reliable and customizable solutions for assessment and differentiation of humoral antibody responses against AAV vectors we respond to the expert demand for such assays. Svar's AAV immunogenicity solutions represent a unique toolbox designed to accelerate and support the clinical development of todays and tomorrow's AAV-mediated gene therapies.

883. Anti-Drug Antibody Assay for an Antisense Oligonucleotide

Mary Wojcinski, Leslie Kuiper, Jessica St. Charles

Charles River Laboratories, Mattawan, MI

Antisense oligonucleotide (ASO) drugs have been emerging as a gene therapy for protein misfunction as opposed to protein replacement. ASOs bind RNA to produce non-disease state proteins or inhibit the production of disease-causing proteins. To evaluate safety, the anti-drug antibody (ADA) response needs to be recorded. Due to the small size of an ASO, the development of an ADA assay was challenging; however, the resulting assay was similar to an ADA assay for an antibody. We developed a regulated ADA assay to an ASO to support a nonclinical cynomolgus monkey study. The assay had a 5'-Biotion-ASO as capture. The positive control was produced by immunizing rabbits with the ASO and isolating the ADA from the serum using affinity chromatography. ADA presence was detected by commercially available anti-ADA conjugated antibodies. Because the PC was produced in rabbits and the samples were being collected in monkeys, two detection antibodies were used to detect both PC and samples simultaneously. Development began with testing feasibility using the MSD. Detection concentrations were determined by coating the PC directly to the plate and testing different concentrations of anti-species-sulfotag. The concentration at which both detections overlapped was the optimal detection condition. The challenging aspect of MSD was sensitivity. These curves did not have workable S:N values. Therefore, different MRDs and buffers were tested. As no option improved S:N, the assay was switched to absorbance-based on the spectrophotometer using anti-species-HRP as detection. The detection assessment was performed again to determine the most optimal concentrations. Once determined, matrix testing was performed, and a theoretical screening cut point factor and sensitivity were calculated. The sensitivity curve had better S:N values when compared to MSD. There was also decent variability between the lots with a theoretical S:N value of 1.412. During this assessment, the plate was inadvertently shaken at the coating step. This run had higher than previously seen %CVs. Therefore, it was determined that the assay plate should not be shaken at any step. Drug tolerance was assessed next to determine the amount of drug tolerated by the assay as well as the concentration at which to test confirmatory sensitivity. Drug tolerance was sufficient based on PK data available. Confirmatory sensitivity performed well and a concentration of the ASO to do confirmatory at was determined. The assay was validated with a workable screening cut point factor (1.48) and confirmatory cut point (26.8%) that resulted in a highly sensitive assay (85.2 ng/mL). Even with the unique properties of an ASO, we saw values that are optimal for an ADA to an antibody-based drug. The assay also tolerated drug beyond the Cmax seen with the PK. Sample analysis is currently on-going. Of the 200 samples, 35 screened positive and 12 confirmed positive.

884. Can AI Improve Compliance, Consistency, and Efficiency for CGTP Data Analysis?

Stephanie Pasas-Farmer[1], Carrie Vyhlidal[2]

[1]BioData Solutions, LLC, Lawrence, KS,[2]KCAS Bioanalytical & Biomarker Services, Olathe, KS

Introduction: With the promise of treating cancer, Parkinson's, and other rare diseases, cell and gene therapy products (CGTPs) are gaining traction in the biopharma industry. As of 2023, the FDA has approved 34 CGTPs and over 1500 clinical trials are currently ongoing globally. Despite the increasing number of CGTP drug candidates, the bioanalysis supporting these programs is still fraught with many hurdles. These challenges include limited or no well-defined guidance; lack of harmonization of validation approaches and acceptance criteria; and limited software tools to facilitate the management of the high

volume of bioanalytical data being generated. Artificial intelligence (AI) offers a unique opportunity to alleviate these data analysis and throughput challenges by automating key processes that reduce time and increase efficiencies while maintaining data security, integrity, and compliance, such as 21 CFR part 11. More importantly, AI-enabled auditing data tools can facilitate consistency in scientific approaches and standardization of acceptance criteria. Challenges: While molecular assays are not new to the world of drug development, their application in CGTPs is more recent. Consequently, the regulations are either not specific or have yet to be established. Validation approaches and acceptance criteria lack harmonization. This results in inconsistencies in data analysis across and within studies. Additionally, a lack of tools for tabulating the high volumes of raw data used for FDA submissions often leads to transcription errors, data analysis errors, and overburdened quality control staff. Hypothesis and Solution Workflow: To overcome data analysis challenges facing molecular assays in highly regulated environments, an AI-enabled automation prototype was developed for evaluation. The tool would replace the common manual, repetitive, error-prone tasks and increase the efficiency of the laboratory scientists while improving the quality and compliance of the results and requiring minimal training. Firstly, a scientist would upload text/csv data file(s) to a validated and secure system with some minimal input fields. Due to the lack of harmonization around acceptance criteria, the system would permit study-specific customizable acceptance criteria to accommodate discovery data. Next, automated data analysis would substitute human processes of flagging of data outside of acceptance criteria are performed per regulatory guidance and industry best practices; tabulating raw data to reportable tables; and interim quality control (QC) and quality assurance (QA) checks for data transcription. Finally, data output would be generated. Results: The process of performing data calculation, creating reportable excel tables, and performing interim checks was completed in less than 1 minute. To make it more robust, R code simultaneously validates all calculations for additional validity and data integrity. It eliminated the need for interim QC and QA checks for transcription errors and data calculations as the data from the instrument is uploaded directly to the tool and converted to reportable output. Based on data gathered from end users, the automation tool prototype yielded time savings of 96.4%, going from 5.8 FTE to 0.2 FTE (46.4 work hours to 1.6 work hours) for a project with 15 plates. The reported times include extracting reports from instruments, uploading files, performing calculations, completing interim QC and QA checks, and populating the tables. Additionally, cost savings from the time saved were 97.3% and despite customization costs, an ROI of 600% was observed. Some unmeasurable benefits of automated data analysis and tabulation include the time given back to the scientists to focus on more important tasks that require human discretion and decision-making capabilities.

Wednesday Posters: Molecular and Cellular Methods

885. Ex-Vivo Platforms for Assessing Cell and Gene Therapy Agents in the Human Cornea and Retina

Khoa D. Tran, Megan M. W. Straiko, Katie Breitinger, Mark S. Ellison

VisionGift, Portland, OR

Gene therapy holds great promise for treating ocular disorders, particularly those affecting the corneal endothelium and the retina. To facilitate the preclinical assessment of gene therapy agents targeting these tissues, we established two tissue culture platforms for evaluating the efficacy of therapeutic interventions using human cornea and retina tissues from post-mortem donors. The first platform utilizes whole corneas, providing a native and intact tissue where the corneal endothelium can be treated with AAV, LNPs, small molecules, and other agents designed for genetic or cellular modifications. After treating the endothelium (for up to 4 hours), the cornea is cultured in organ culture media for up to one month at 31°C. Following the culture period, the cornea can be processed using methods compatible with different techniques for downstream analysis. These methods may include fixation for histological analysis, placement in preservation solutions, and flash freezing with LN_2. The endothelium can also be isolated by removing Descemet's membrane and corneal endothelial cells from the whole corneal structure, enabling cell dissociation, sorting, and molecular analysis of an enriched cell population of corneal endothelium. AAV-mediated transduction of GFP reporter genes resulted in detectable expression of GFP in corneal endothelial cells at 2 weeks post-transduction and was maintained for up to 1 month. The second platform employs retina explant cultures (retina only, as well as chorioretinal tissues). Explants are cultured on polycarbonate membranes in transwell culture plates at 37°C with 5% CO_2. Explants can be treated with test articles as described above, and the explants can be cultured for up to 1 month. Tissue preservation at experimental endpoints is similar to the methods described above. Previous success includes AAV-mediated gene editing and promoter-driven reporter gene expression at 2 weeks and 1-month post-transduction. Our results indicate that both platforms successfully replicate key aspects of the human cornea and retina, allowing for the assessment of gene therapy agents in a more native environment. The platforms can be used to examine vector transduction efficiency and biological responses, making them ideal for pre-clinical studies to support the optimization of treatment strategies for corneal and retinal disorders in a more clinically relevant context.

886. Broadly Expressed Synthetic NRF1-Array Mini-Promoters for Application to Gene Therapy and Disease Modeling Vectors

Robert D. Nicholls[1], Ting Zhang[2], Wan Zhu[3], Emma J. Hundertmark[4], Jia Qi Cheng Zhang[1], Marie A. Johnson[1], Haley L. Crable[1], Erik A. Koppes[1], Dwi U. Kemaladewi[1], George K. Gittes[2]

[1]Pediatrics, UPMC Children's Hospital of Pittsburgh, Pittsburgh, PA,[2]Surgery, UPMC Children's Hospital of Pittsburgh, Pittsburgh, PA,[3]Human Genetics, University of Pittsburgh School of Public Health, Pittsburgh, PA,[4]Bioengineering, University of Pittsburgh, Pittsburgh, PA

Introduction: Adeno-associated virus (AAV) has strict size limitations for packing into a viral particle, which previously limited the size of encoded therapeutic proteins or genome editing applications. To overcome this size limitation of AAV vectors and to provide a modular component for gene regulation in any vector type, rather than the commonly used CMV promoter-enhancer (613-nt) or many cell-type specific promoters for gene expression, we rationally designed mini-promoters of length 34-nt to 210-nt. **Results:** These mini-promoters are tandem arrays of 2-10 units in length of motifs (consensus is 5′-YGCGCANGCGCR-3′) for a transcription factor, NRF1, with 10-11 nucleotides spacing equal to one-turn of the DNA α-helix between binding sites to allow each NRF1-dimer to interact for potential cooperative binding. An additional set of NRF1-array mini-promoters includes a TATA-box motif. Indeed, use of NRF1-arrays of 1, 2, 4, 6, 8 and 10 units displayed cooperative increases in luciferase expression within transfected SK-N-SH neuroblastoma cells. Subsequent characterization has focused on the 4xNRF1 and 10xNRF1 promoters, with high levels of mNeonGreen or luc-EGFP expression detected from mammalian expression plasmids in rat INS-1 and mouse MIN6 insulin-secreting β-cells, mouse αTC1-6 glucagon-secreting α-cells, mouse C2C12 myoblasts, mouse mHippoE-2 hippocampal neurons, as well as human HEI-193 schwannoma (peripheral nerve) and HEK293T (adrenal) cell lines. Next, robust expression was also demonstrated by fluorescence microscopy and luciferase assays using each of these two promoters in an AAV6-luc-EGFP vector following viral transduction of rodent β- and α-cell lines, primary human α-cells, and by tail vein or pancreatic duct infusion of each virus in wildtype mice with *in vivo* imaging of bioluminescence, clear expression was observed in the liver and pancreas, respectively, and confirmed by tissue dissection. Critically, the 4xNRF1 mini-promoter was also utilized in a miniaturized CRISPR-activation (CRISPRa) AAV9 vector for therapeutic *Lama1* gene upregulation in a *Lama2*-deficient muscular dystrophy mouse model, resulting in reduced hindlimb paralysis, improved mobility, body weight, and survival. **Conclusions:** Previously generated synthetic promoters have typically been based on random DNA motifs or utilized endogenous promoter fragments, but few have the broad expression profile or minimal size as for our panel of NRF1-array mini-promoters. Miniaturization of promoter and encoded functions will aid *in vitro* and *in vivo* disease modeling and gene therapy applications, not only for AAV but also for non-viral approaches such as lipid nanoparticle (LNP)-mediated nucleic acids delivery.

887. A Novel Next-Gen Sequencing Library Preparation Method to Sequence Small, Long, and Low-Quality (Fragmented/ Degraded) with Low (*Pico*-Grams) Quantity of DNA/RNA Templates for Better Diagnostics, Biomarker Discovery, and Research

Praveensingh B. Hajeri[1], Masato Yamamoto[2], Adam Herman[1], Nuri Alpey Temiz[2]

[1]Department of Surgery, University of Minnesota, Minneapolis, MN,[2]University of Minnesota, Minneapolis, MN

Background and limitations of current technologies: Next-generation sequencing (NGS) of RNA and DNA is a method of high-throughput sequencing of biological samples. There are several well-established methods in published literature and also commercially available in the form of products/kits dedicated to sequencing total RNA, DNA, exome, small RNAs, and selected panels of gene/ loci to address specific issues. However, it has been a great challenge to sequence highly fragmented and degraded DNA/RNA due to the limitations of library preparation methods. This affects the sequencing of clinical samples, biological research materials, liquid biopsy, FFPE tissue, exosomes, forensics, cell-free RNA/DNA, and archeology, among several other types of materials. A large fraction of archived and even fresh samples do not generate high-quality data, which cripples basic research, biomarker discovery, and diagnostics. Currently, it is also impossible to sequence small RNAs alone or small and long RNAs together with pico-gram quantities of total RNA template. ***Methods and results:*** We have addressed these limitations by developing a novel method to prepare NGS libraries from pico-gram quantities of total RNA templates. Our methods could efficiently capture RNA of all sizes (20bp and above, including small and long RNAs) simultaneously and detect 2-10 times more unique transcripts at a similar sequencing depth than the current state-of-the-art methods. We could capture 20,000 to 100,000 more transcripts than an established method in a proof-of-concept study. We could capture small (as small as 20 bases and above) and long RNAs in the same library using very low (pico-grams) quantities of total RNA. ***Applications and broader impacts:*** Our method is applicable to highly fragmented and degraded samples like biological samples, clinical samples, FFPE tissue, cell-free RNA/DNA, liquid biopsies, archeological samples, and also normal samples. The variations and errors observed in NGS data due to differential degradation between samples are negligible in our method. This helps generate consistent and high-quality sequencing data. We could generate 2-10 times more high-quality data with as little as 500pg of total RNA, which is close to the total RNA obtained by a single cell and hence applies to single-cell sequencing. The method is compatible with automation as well. Due to its high efficiency and sensitivity, we think our method will enable better diagnostics, biomarker discovery, and research.

888. Harnessing the Power of Multiomics from Fresh and Fixed Patient Sample Characterization

Andrea O'Hara, Yang Han, Yongjun Fan, David Corney, Chris Mozdzierz, Haythem Latif, Ginger Zhou
Azenta Life Sciences, Burlington, MA

The omics era has greatly expanded the repertoire of approaches available for researchers and clinicians to unravel the complexity underpinning human health: Next Generation Sequencing (NGS) approaches can characterize genomes, epigenomes, transcriptomes and proteomes. The analyses are critical to assess in individuals both pre- and post-treatment during therapeutic development and early-stage clinical trials. Peripheral blood mononuclear cells (PBMCs) offer a non-invasive approach that, when combined with omics tools, can provide a near holistic view of immune processes across patient cohorts. Meanwhile, Formalin Fixed Paraffin Embedded (FFPE) tissues are a staple in clinical diagnostics and an ideal means to store archival tissue but can be difficult to work with in traditional NGS assays. Here we detail workflows using both fresh and fixed patient samples to rapidly produce a diverse set of multiomics results including genomics, epigenomics, transcriptomics and proteomics. For fresh blood draws, this starts with automated sample handling and processing to ensure high viability and yield of PBMCs, along with simultaneous plasma separation and collection. Samples are then aliquoted and simultaneously processed for whole exome sequencing, single cell RNA sequencing, epigenetic characterization and Olink biomarker analysis. For fixed tissues, FFPE blocks were serially sliced into various FFPE slides, with a single slide H&E stained. Individual slides were then utilized for genome, epigenome, single cell RNA-seq, and digital spatial profiling. Genome information was captured using hybrid capture based approaches followed by deep NGS on an Illumina platform and analyzed for a variety of variants and tumor mutational burden. DNA methylation was detailed using target capture probes targeting DNA methylation sites. Single cell approaches were applied to explore the transcriptome using 10X Genomics scRNAseq kit, and Digital Spatial Profiling (DSP) was done using the NanoString GeoMx Whole Transcriptome Atlas and immunostaining. With these robust workflows, all these datatypes can be produced within days of fresh or fixed sample receipt using minimal sample amounts. High throughput integrative omics workflows, as described here, drive greater insights in human health, allowing for a rapid combined approach to address the biological questions at hand.

889. Validated Solution for High Throughput Analyses with Flexible Mods for Custom Assay Design

Hubert F. Arokium[1], Howard Rosoff[2], Francis Kim[2], Sean Hsu[2], Rebekkah Hesson[1], Patrick Trainor[3], Victoria M. Velazquez[1], Karim Khoury[1], Sam Pan[2], Sapna Varia[1]
[1]AmplifyBio, West Jefferson, OH,[2]AmplifyBio, South San Francisco, CA,[3]BioinfoScientist, Louisville, KY

Introduction: Fast expanding parameters and criteria for determining safety, efficacy, and biodistribution analyses of Cell and Gene Therapies require sizeable datasets looking at complex overlapping variables. Interpretation requires high throughput biostatistical analyses for achieving the desired results in individual preclinical studies. Performing intricate data analysis requires considerable effort and precise execution, which can lead to a depletion of resources, risk data integrity, and a turnaround time that is often months when relying solely on rudimentary IT tools like spreadsheets. The goal of this study was to design, develop, and validate a more efficient tailor-made solution using modern data automation practices and ETL (extract, transform, load) techniques to reduce the time it takes to perform repetitive analyses and use the best analysis strategy for critical preclinical safety and biodistribution data sets. *Approach:* Using a well-qualified Digital PCR (dPCR and RT-dPCR) biodistribution method as a test case, the data pipeline was designed to use single and multi-plex dPCR and RT-dPCR output results from a QIAGEN QIAcuity dPCR system. We defined the system's capabilities to include statistical analyses, reportable values, statistical results with output files, the ability to generate various summary plots and not alter any raw data points. *Results:* The use of the validated data pipeline system successfully demonstrated the technical performance of dPCR and RT-dPCR data through the application of method validation precision standards and established pass/fail benchmarks. The Data pipeline performed as designed and met all the validation requirements for i) data handling/ user defined access ii) performing technical quality control of the dPCR runs by flagging the results that fall outside of the acceptance criteria and distinguishing valid data from flagged data and iii) exporting data to a centralized data for pickup by biostatistical analysis modules. It featured user-friendly components, such as an exported Excel file with a "valid results" tab, showcasing data meeting the set criteria, and a "review" tab. This "review" tab displayed all relevant precision calculations (like %CV, mean, standard deviation), along with any technical indicators, for straightforward examination by a subject matter expert. Pipeline operation included separate Development and Production environments, the use of source code from an industry standard library, access and functionality controls, and a strict requirement that the pipeline itself did not affect the raw data. The data pipeline was able to accurately facilitate the comparative analysis of target gene copies or gene expression as defined by method validation precision standards and plate pass/fail criteria. In summary, the use of the validated data pipeline in a GxP setting ensures adherence to high standards of technical quality, data integrity, and traceability. Significantly, the ability to analyze samples within minutes or hours, as opposed to days or weeks, opens up possibilities for study sponsors to conduct extra analyses, thereby enhancing the depth of understanding derived from multi-faceted interpretation of study outcomes.

890. A Comprehensive ddPCR Portfolio for In-Depth DNA Impurity Profiling

Sonya M. Schermann, Sabine Geiger, Renée Kober, Felicia Thoennissen, Marina Magerl, Sophie Beer, Markus Hoerer
Ascend, Planegg, Germany

DNA impurities present in recombinant adeno-associated virus (rAAV) products pose a potential risk to patients and therefore need to be thoroughly understood. Methods are required for quality control (QC) of final product and also for extended product characterization. Here we discuss some of the ways in which multiplex digital droplet polymerase chain reaction (ddPCR) can be used in these two

applications. For QC release, multiplexing can be used to monitor multiple DNA impurities in a single assay, reducing the number of assays required for release of product and thereby minimizing the time and cost of batch release. Here we present a duplex method for monitoring residual DNA impurities of different origin and discuss aspects of method development and qualification/validation of such assays. For extended product characterization, we show how multiplexing can be used in a variety of applications, for example in understanding the length profile of encapsidated plasmid or host cell DNA, which is an important attribute when considering potential risk to patients of such encapsidated sequences. Long read NGS sequencing established in-house is used as an orthogonal method to validate the results obtained. We also demonstrate applications in which multiplex ddPCR can be used to interrogate whether two different sequences are present in the same capsid, for example residual rep and cap DNA, an application which can be used to predict the potential for rAAV batches to contain replication competent AAV (rcAAV). Taken together, such multiplex ddPCR methods represent powerful tools to determine not only the presence, but the amount, size and identity of encapsidated residual DNA and as such can be used both in QC and in extended characterization of rAAV products.

891. Development and Qualification of a Quality Control Panel for the Characterization of Recombinant Adeno Associated Viral Vectors

Francesca Rossetti[1], Veronica Meraviglia[2], Anna Longatti[2], Giulia Maria Nava[2], Matteo Paolillo[2], Marco Rausa[2], Chiara Portugalli[2], Chiara Boroni[2], Grazia Frizzale[2], Claudia Piovan[2], Margherita Neri[3], Giuliana Vallanti[2]

[1]AGC Biologics - Milan, Bresso (Milano), Italy,[2]AGC Biologics, Milano, Italy,[3]AGC Biologics, Milan, Italy

AGC Biologics is a leading global Contract Development and Manufacturing Organization (CDMO), providing world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA, viral vectors and genetically engineered cells. Recombinant Adeno Associated Viral Vectors (rAAVs) produced with transient transfection in HEK293 are extensively used for genetic modification in gene therapy treatments. Companies that decide to go down the road of AAV production face two major challenges. The first challenge is the availability of an efficient and scalable off-the-shelf vector production processes. AGC Biologics is developing different systems for AAV production at large scale, in adhesion and suspension, flexible to be adapted to different GOI and serotypes. The second challenge is to develop a solid analytical panel for vector characterization. A full quality control plan has been designed for the release of clinical lots, in accordance with GMP rules, EU and US Guidelines, CBER Points and ICH. The panel designed foresees all the assays to assess the identity, potency, safety and purity of the vector. The tests to determine vector particles, vector genome and infectious particles (potency test) were developed and qualified. Both assays for vector genome and infectious titer have been designed in order to be quickly applied to all different serotypes and GOIs and to be easily switched to product specific assays, according to the different applications. Assays for the determination of full/empty ratio, vector aggregation and determination of AAV viral capsid proteins have settled scouting different technologies. Even if the gold standard for F/E is Analytical Ultracentrifugation (AUC), other techniques are now acquiring visibility, due to their easier set up, time-saving and lower cost. Anion-Exchange Chromatography (AEX) performed by UHPLC instrument has been chosen as good strategy for the separation of full and empty capsids. Data obtained have been compared with the ratio calculated from viral genome titer and vector particles titer, and with orthogonal procedure AUC in outsourcing for AAV6 and AAV8 samples. Percentage difference among the different procedures is ≤ 36%. Introduction of Dynamic Light Scattering instrument for the determination of vector aggregation helps to nicely verify aggregates removal throughout the purification process, with percentage of aggregates that span from 10% to 40% in the post affinity sample to below 1% in the final purified vector analysing six different serotypes. AGC biologics is then putting a lot of effort for the assays for residuals characterization to have from one site more sensible assays to meet guidelines request for in vivo applications and form the other to have precise, accurate and high throughput assays, that can be coupled with automatic station. Assay for the determination of DNA size, total DNA, E1A DNA, HCP, benzonase are some of the assays ready to use for vector release. In the context of a comprehensive CDMO offer, a higher value is added considering that more than 90% of the assays is performed in house, optimizing volume and number of samples dedicated to QC analysis.

892. Short- and Long-Read Sequencing for Integration Site Analysis (ISA) of Viral Vectors for Gene Therapy

Ben Niu, Andrea O'Hara, Candy Mao, Brian Sereni, Brad Scherer, Haythem Latif, Ginger Zhou

Azenta Life Sciences, Burlington, MA

While lentivirus has historically been the viral vector of choice for gene therapy, recombinant adeno-associated viral (rAAV) vectors have seen widespread application as a gene therapy delivery platform in preclinical and clinical studies. Unlike lentiviral vectors, which are fundamentally integrated into the host genome, recombinant AAV largely remains episomal after transduction into the nucleus of the host cells; however, data has shown AAV may integrate into the host genome in a random, non-homologous manner. The frequency of AAV integration has been estimated at 0.1% to 10% in hepatocytes. In consideration of the theoretical risk of tumorigenesis associated with lentiviral and AAV integration in humans, assessment of viral vector integration in *in vitro* studies, animal models, and patient samples are recommended by FDA for preclinical and clinical studies and long-term follow-up studies (LTFU). AAV integration analysis presents several challenges, including the random nature of AAV integration sites and low expected integration frequency. To address this, Next Generation Sequencing (NGS) for viral vector integration analysis has been adopted as a highly sensitive assay. Traditional approaches utilize targeted enrichment sequencing with hybrid-capture or linear amplification PCR in combination with short-read sequencing technology. The addition of long-read sequencing offers the advantage of being able to identify chimeric reads with clear sequence structure

as genome-insert-genome. Here we describe multiple mixed ISA approaches in lentiviral and AAV studies, with improved sensitivity and specificity for ideal detection of viral vector integration.

893. Investigating Tropism of AAV5 and AAV3b Using Multi-Organ Human Tissue Equivalents

Ritu M. Ramamurthy[1], Aya El-Taibany[1], Janmejay Hingu[1], Jonathan H. Diaz[1], Yu Zhou[1], Sarah E. Wachtman[1], John C. Armstrong[1], Daniel Porada[1], Douglas E. Shankle[1], Meimei Wan[1], Colin E. Bishop[1], Michael Seeds[1], Christopher B. Doering[2], H. Trent Spencer[2], Hooman Sadri-Ardekani[1], Sean V. Murphy[1], Anthony Atala[1], Christopher D. Porada[1], M Graça D. Almeida-Porada[1]

[1]Fetal Research and Therapy Program, Wake Forest Institute for Regenerative Medicine, Winston-Salem, NC,[2]Department of Pediatrics, Aflac Cancer and Blood Disorders Center, Emory University, Atlanta, GA

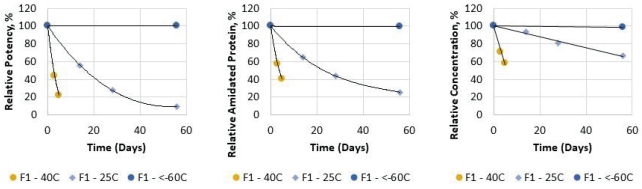
There is no definitive data concerning human tropism/cell-specificity of adeno-associated viral (AAV) vector serotypes in clinical use due to the inability to justify multiple organ biopsies in patients receiving the therapy. Thus, most clinical approaches have relied upon tissue-specific promoters to restrict transgene expression to the desired cell type *in vivo*. However, tissue-specific promoter does not limit transduction itself to a particular cell type and such an approach continues to be inherently problematic, as off-target effects are possible and binding/uptake of the vector by cells that will not contribute to transgene expression reduce the effective vector dose reaching the desired cells. Here, we used human multi-organ tissue equivalents (OTEs), which are 3D culture systems comprised of major cell types present in respective tissues and that can mimic function and histology of actual human organs to define human tropism of AAV. Two clinically-relevant AAV serotypes, AAV5 and AAV3b, encoding a GFP reporter to enable tracing of transduction were used. Transduction was performed at a dose of 2.6E10 vg/mL, predicted to be the dose circulating after administration of a total dose of 1.72E14 vg in an adult weighing 86 kg. Transduction efficiency was determined using digital PCR to quantify GFP mRNA and ELISA (n=3) to quantify protein concentration in samples collected 1 wk post transduction. Incucyte live imaging for GFP fluorescence was also performed throughout the study period. Transduction was repeated three times with biological (liver and testicular OTE) and technical (BBB and lung OTE) replicates, and a minimum of 96 OTEs were used per group for every transduction. Statistical analysis was performed using ANOVA and *post hoc* Tukey test. Live/dead imaging and maintained ATP levels 1 wk post transduction confirmed OTE viability in all groups. The presence of GFP mRNA verified transduction in all OTEs, however only BBB, testicular, and liver OTEs exhibited detectable levels of GFP protein. In the AAV5-transduced group, BBB OTEs produced the highest levels of GFP protein (27.7 ± 4.4 pg/100 µg total protein) followed by testicular (15.42 ± 3.1 pg) and liver OTEs (< 3 pg) ($p<0.05$). Similarly, in the AAV3b group, BBB OTEs produced the most GFP protein (12.28 ± 2.67 pg/100 µg total protein; $p<0.05$) followed by testicular (5.09 ± 0.8 pg) and liver OTEs (< 3 pg). Of the two serotypes, AAV5 exhibited significantly higher transduction efficiency ($p<0.05$) compared to AAV3b in BBB and testicular OTEs, while the efficiencies were comparable in liver OTEs. Transduction of testicular OTEs is of note and warrants further investigation. Studies are currently underway to investigate underlying mechanisms for the observed differences in tropism, but data thus far demonstrate the potential of human-derived OTEs to serve as a tool to determine the human tropism of AAV, accelerating preclinical studies for AAV gene therapy.

894. Deamidation Drives AAV9 Potency Loss

Austin E. Smith, Michael Murphy, Stephen Ollosi

Analytical Development and QC, Advanced Medicine Partners, Durham, NC

Recombinant adeno-associated viruses (AAV) are promising single-stranded DNA delivery vectors for treating genetic diseases. The viral capsid is formed by three proteins whose sequence and structure determine tissue specificity and vector potency: the ability to infect cells, deliver payload and induce protein expression. Successful development and storage requires understanding how the physical state of the viral proteins and resulting capsid affect potency. Here, we demonstrate that protein deamidation is a primary factor determining potency by examining three wild-type AAV9 vectors, each containing a different DNA payload. The vectors were produced in HEK293 cells, purified using ion-exchange chromatography and density-gradient ultracentrifugation and formulated in a single buffer. AAVs were stored at different temperatures and tested at various times for concentration, aggregation, protein purity, deamidation and cell-based potency focusing on function in the target cell. For all three vectors, a time- and temperature-dependent potency decrease occurred concomitant with increased deamidation. Two of the three vectors also decreased in concentration. Results from one of the model vectors, AAV9 encoding eGFP, are shown below.



These results highlight the need for manufacturing process controls that limit deamidated species and routine quality control analytical methods to quantify deamidation. These results also suggest that engineering capsids that are less prone to temperature-induced deamidation will combat loss of potency during AVV9 manufacturing and storage.

895. Peeling the Onion: A Stratified Approach to rAAV DNA Purity and Integrity Characterization

Melissa Carver, Stephen Ollosi, Austin E. Smith, Michael Murphy

Advanced Medicine Partners, Durham, NC

The previous decade has shown rapid advancement of recombinant adeno-associated viral vectors (rAAV) for the treatment of a broad range of genetic diseases. As production of rAAV products advances, so must the analytics used to assess quality attributes. There is a need in the field for robust assessment of methods characterizing DNA purity in rAAV products. One well characterized purification strategy includes a

CsCl ultracentrifugation (UC) process for the separation of functional rAAV capsids from partially filled and empty capsids. During the UC process, capsids of different densities form bands which can be extracted individually and represent differing percentages of empty, partial and full capsids. Here we describe a robust characterization of samples taken from across a CsCl density gradient. We distinguish between methods which can assess DNA content of intact capsids, extracted DNA length, and extracted DNA sequence composition. This stratified approach allows for the complete characterization of DNA purity and integrity in rAAV preparations. Methods assessing intact capsids include Analytical Ultracentrifugation (AUC), Mass Photometry and Charge Detection Mass Spectroscopy (CDMS). These methods share the benefit of limited sample preparation and the ability to assess empty capsids but cannot directly assess genome length or sequence composition. Methods directly assessing genome length include Agarose Gel Electrophoresis (AGE), Capillary Electrophoresis (CE) and Reverse Phase HPLC (RP-HPLC), however these methods cannot assess empty capsids or sequence composition. Methods assessing sequence composition include Short Read Sequencing by Illumina and Oxford Nanopore platforms. These methods provide a direct assessment of sequence composition but cannot assess empty capsids or genome length. Long Read Sequencing by PacBio is unique as it provides information on both genome length and sequence composition. The scope of this evaluation includes extracted bands within a CsCl density gradient, however the methods described here are applicable to the characterization of any rAAV purification strategy. Each method described provides a unique but incomplete view on the question of DNA purity and integrity. We assert that to fully characterize DNA purity and integrity of an rAAV product, the appropriate subset of methods described here should be performed.

896. Development of a Universal Genomic DNA Digital PCR Assay Targeting the ZEB2 Gene for the Measurement of AAV Biodistribution

Trang P. D. Nguyen, Bezawit F. Ejigu, Sungyeon Cho, Ye Liu, Olivier F. Danos, Samantha A. Yost

REGENXBIO, Inc., Rockville, MD

Biodistribution is an important measurement in gene therapy studies to determine the transduction efficiency of adeno-associated viral (AAV) vectors in target tissues. AAV biodistribution is often represented with a unit of vector genome copy (VGC) per host diploid genome (DG) or VGC per nucleus. In the literature, VGC/nucleus has been calculated in two ways: VGC per microgram template DNA divided by a theoretical correction factor based on the genome size of a species (e.g., $1.548x10^5$ DG/μg DNA for humans), or VGC divided by the host DG measured by a separate PCR assay with primer/probe targeting a host genomic DNA sequence. The former method does not account for experimental variation regarding nucleic acid extraction efficiency or resulting DNA quality. A host DG assay can mitigate these problems, leading to a more accurate calculation of VGC/nucleus and providing additional assay robustness against experimental variations. As gene therapy development moves from rodents to monkeys and finally to humans, an ideal host DG assay would be applicable across species, decreasing the cost and developmental timeline. Previously published universal primers/probes that target ribosomal genes (28S rRNA, 18S rRNA), histone genes (HIST2H4), or mitochondrial DNA (mtDNA; cytochrome b) are highly conserved but have limitations: their copy numbers are not consistent between species and tissue types, and often have many pseudogenes, impeding the accurate measurement of the diploid genome. We present a cross-species digital PCR assay targeting a highly conserved gene, Zinc finger E-box-binding homeobox 2 (ZEB2), which presents at two copies per nucleus in all mammals. A stretch of DNA in exon 2 of the ZEB2 gene was used to design a PCR primer/probe assay with no off-target binding sites predicted via Primer-BLAST for several relevant species to gene therapy research [human (*Homo sapiens*), non-human primate (NHP; *Macaca fascicularis*), pig (*Sus scrofa*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), cat (*Felis catus*), and ferret (*Mustela putorius furo*)]. ZEB2 digital PCR assay was evaluated across 109 genomic DNA samples from various tissues (e.g., liver, muscle, eye) of 5 species (human, pig, NHP, rat, and mouse) and compared to in-house historical data using species-specific digital PCR assay targeting the glucagon gene. The average ratio between ZEB2 and glucagon copy number per microgram input DNA is 1.01 with a standard deviation of 0.13, confirming the accurate measurement of 2 copies of ZEB2 per nucleus in all tested tissues and species. Adopting a universal cross-species ZEB2 digital PCR assay to measure host DG has the potential to streamline the biodistribution assay transfer from research to clinical development, reduce cost, and improve assay accuracy.

897. A Process for Identifying AAV and Transgene Integrations in Mouse and Human Genomes Using Long Read Oxford Nanopore Sequencing

Terrence Turner, Jose Rojas, Yajun Tang, Charleen Hunt, Michael Glasser, Ciro Bonetti, Gregg Warshaw, Daisuke Kajimura, Susannah Brydges, Jeffrey D. Lee, Jeremy Rabinowitz, Chia-Jen Siao, Eric Chiao, Brian Zambrowicz

Velocigene, Regeneron Pharmaceuticals, Tarrytown, NY

Adeno-associated virus (AAV) vectors and transgenesis are pivotal tools in gene therapy and genetic engineering. However, identifying precise integration sites of these genetic segments within the host genome remains challenging. This study presents a process for identifying AAV and transgene integrations in mouse and human genomes using long read Oxford Nanopore Technology (ONT) sequencing. Our method uses long read sequencing generating sequencing libraries, enriched for the regions of interest. After alignment to the reference genome, the long read capability of ONT spans entire transgene and vector sequences, providing a comprehensive view of the integration sites. Subsequent read alignment and breakpoint identification are performed using a custom bioinformatics pipeline designed to handle the unique challenges posed by long read sequencing data, such as sequencing error rates and how to interpret complex transgene rearrangements. Our results demonstrate the effectiveness of this process in accurately identifying AAV and transgene integration sites. We have also characterized tandem/inverted integrations and off target integrations of AAVs in the host genome. This method allows

for the precise and more comprehensive understanding of vector and transgene integration, an important consideration given the recent advances in AAV based gene therapies.

898. DNA Leakage of rAAV Under Freeze/Thaw Stress and Analytical Method Development for Free DNA Characterization

Shuai Li, Xiaoyan Wang, Kuan-Yu (Nick) Lai, Jonathan Wert, Li Zhi, Mohammed Shameem, Dingjiang Liu
Formulation Development, Regeneron Pharmaceuticals, Tarrytown, NY

Recombinant adeno-associated virus (rAAV) vectors are widely used to deliver therapeutic transgenes due to their superior safety, relatively low immunogenicity, and ability to target diverse tissues. Recent studies have shown that DNA leakage could be induced by freeze/thaw stress, which is concerning since rAAV gene therapy products are typically stored below -65°C and subjected to multiple freeze/thaw cycles during manufacturing and administration. DNA leakage from rAAV capsids could potentially impact rAAV product stability, induce immune responses, and compromise product efficacy, therefore it is important to characterize DNA leakage during rAAV product development. However, currently reported analytical methods used to characterize free DNA often lack the desired sensitivity and reproducibility required for reliable detection and quantitation of free DNA. In this work, we have developed a size exclusion chromatography (SEC)-based free DNA quantitation method. This method demonstrated satisfactory assay performance in specificity, accuracy, reproducibility, and linearity for quantitative analysis of free DNA. It has been applied into our current formulation development and characterization work. Thus, this SEC-based free DNA method can be used to reliably characterize the DNA leakage during rAAV formulation, product, and process development to ensure the quality and stability of rAAV gene therapy products.

899. Identification of Degradation Pathways of rAAV8 to Aid Stable Drug Product Formulation Development

Ariel Chen, Shuai Li, Marilia Barros, Xiaoyan Wang, Jonathan Wert, Kuan-Yu Lai, Li Zhi, Mohammed Shameem, Dingjiang Liu
Formulation Development, Regeneron Pharmaceuticals, Tarrytown, NY

Recombinant adeno-associated viruses (rAAVs) have emerged as promising gene therapy vectors for delivering therapeutic genes to target tissues. However, our understanding of the critical quality attributes (CQAs) of rAAVs and degradation pathways is limited. To increase our understanding, we performed a forced degradation study on rAAV8 under various stress conditions relevant to AAV drug product manufacturing and clinical use. A panel of analytical methods was applied to characterize the stressed samples. The results revealed multiple degradation pathways for rAAV8, including aggregation, release of AAV genome DNA, and post-translational modifications (PTM). After identifying the degradation pathways, a formulation optimization study was conducted, leading to a rAAV8 formulation with significantly improved stability under thermal and freeze-thaw stress conditions. This work enhanced our understanding of degradation pathways and critical quality attributes of rAAV8; the knowledge gained here can be applied to other AAV serotypes for developing stable AAV drug product formulation, robust analytical control strategy and systematic process development, potentially reducing development time and cost for gene therapy products.

900. Long-Read Sequencing of Single Stranded AAV Isolated from Crude Lysate for Qualitative Analysis, Improving Characterization, and Informing Vector Design

Russell Buchanan[1], Asher Baltzell[1], Michael Walker[1], Jennifer Israel[2]
[1]Kriya Therapeutics, Durham, NC,[2]Kriya Therapeutics, Redwood City, CA

Understanding the purity, identity, and capsid packaging of recombinant AAV (rAAV) is critical to inform future vector design and production decisions for manufacturing a safer, more effective therapy. Sequencing is the best available method to understand vector genome quality, however characterizing single-stranded AAV (ssAAV) has been hindered by challenging innate features of the viral genome along with limitations of sequencing technology. The source of rAAV being characterized can present further hurdles, such as isolating from preparations containing host genome and other DNA impurities. To address these challenges and qualitatively assess ssAAV, we developed a long-read sequencing workflow which enables the evaluation of key attributes early in development. For our process, we utilized viral particles prepared by a freeze-thaw of pelleted HEK293 cells and resuspended in lysis buffer, performed a Salt Active Nuclease (SAN) treatment, affinity purification of rAAV capsids from the lysate utilizing AAVX Magnetic Beads, and then ssDNA extraction using the viral spin columns. DNA polymerase was added to the isolated material to perform a second strand synthesis, converting ssDNA viral genome to double-stranded DNA (dsDNA) with a hairpin at the 3' ITR end. Libraries were prepared via Oxford Nanopore's Ligation Sequencing Kit V14 and sequenced using a minION Mk1b. A bioinformatic workflow for analyzing the data that separates packaged DNA reads from background impurities, separates synthesized double-stranded DNA/hybridized ssDNA reads, and re-constructs packaged DNA sequences is provided. This method produces >80% of total reads that map to the packaged vector genome, including both synthesized dsDNA and hybridized ssDNA, with the majority of remaining reads mapping to unpackaged lysate material. We conclude that this method yields enough packaged DNA data to reconstruct full ITR-ITR length sequences and shorter sequences which enables us to investigate potential sources of impurities and incomplete capsid packaging.

901. Ensuring Genome Integrity of Recombinant AAV Vectors Using Digital PCR

Domenica Martorana[1], Kevin Matthäi[1], Jonathan Thorn[1], Mario Quinto[1], Julius Albers[1], Anne Schieren[1], Anida Mesihovic[2], Andreas Hecker[2], Francesca Di Pasquale[1]
[1]R&D, QIAGEN GmbH, Hilden, Germany,[2]Product Management, QIAGEN GmbH, Hilden, Germany

Development of safe and effective cell and gene therapies is key to potentially treat a wide spectrum of diseases. Viral vectors have become powerful delivery vehicles for gene therapies. Adeno-associated virus (AAV) vectors have turned into primary modalities for efficient gene therapy applications due to their lack of pathogenicity and persistent transgene expression. Besides the requirement to accurately and reproducibly quantify viral vector genome titers, it is essential to determine the intactness of the viral vector genomes for a safe, stable and effective therapy. Errors made during the replication and packaging process of recombinant AAVs can lead to heterogenous viral vector populations with direct impact on their efficacy and safety. Current purification workflows can efficiently separate empty capsids from full capsids. However, removal of capsids carrying partial or truncated genomes as well as capsids packaged with host cell or plasmid DNA, are difficult to separate and can be present in the viral vector product after purification. Traditionally, genome integrity has been determined via agarose gel electrophoresis and southern blot. Next generation sequencing approaches have been used to characterize the capsid content as well. Nevertheless, high resolution and accuracy needed for integrity determination as well as repeatability and high-throughput capabilities remain unmet needs. Lately, multiplex digital PCR has been adopted for genome integrity analyses. Digital PCR enables absolute quantification with unprecedented precision and a higher tolerance towards inhibitors without the need for any standards. Here we layout a rapid dPCR approach for characterizing genome integrity of in-process and purified AAV samples using the same primers and probes that have been optimized for vector genome titration. The underlying Poisson distribution of dPCR enables the assessment of genome integrity over a broad dynamic range by differentiating between physically linked and unlinked targets. As the calculation estimates the concentration for all present groups of template molecules within a sample individually, it could also be used for other applications such as determination of integrity and stability of DNA and plasmids after e.g., certain processing procedures or storage. We show that up to five targets can be analyzed simultaneously once increasing precision and reproducibility of the analyses.

902. Quantification of Plasmid Copy Number of *Escherichia coli* (*E. coli)* Master Cell Bank by Digital PCR

Syam Pillai[1], Honggeun Park[2], Fernanda Vasconcelos[1], Maryam Rahimian[1], Chun-Ti Chen[1] Tatiana Nanda[1]

[1]Analytical Development, SK Pharmteco, King of Prussia, PA,[2]Testing Operations: Molecular; SK pharmteco - US, King of Prussia, PA

Accurate measurement of plasmid copy number (PCN) is highly desirable for *E. coli* master cell bank to understand the distribution of plasmids in daughter cells, and thus is crucial for the plasmid DNA (pDNA) production by upstream process. Here, we developed a fast and effortless method utilizing dual channel QIAcuity dPCR to monitor the activity of designed promotors in DH5α cells. To prevent any bias toward genomic DNA and plasmid DNA generated by DNA isolation, no extra step of DNA purification was applied in this assay. Simply boiling the bacterial cultures after a serial dilution, samples are span down to separate the supernatant (that contains entire genomic information), and cell debris. Primer-probe sets designed to target *Kanamycin* Resistance and *cysG* genes represent the plasmid DNA and the chromosomal DNA, respectively. A step of restriction digestion is included prior to dPCR analysis to facilitate the partition of template DNA which thus leads to precise quantification. Our results showed that fragmentation of the template DNA increased the plasmid/chromosome ratio at least 5 times compared to reactions without enzyme treatment, thus accurately detecting the PCN. In conclusion, we present a simple and efficient method optimized for the accurate quantification of PCN by dPCR in E. *coli* master cell bank.

903. Optimization of Sonication Linker Mediated (SLiM)-PCR for Vector Integration Sites Retrieval and Quantification

Fabrizio Benedicenti, Sergio Arevalo, Maria Luisa Dalessandro, Giulio Spinozzi, Giulia Pais, Andrea Calabria, Daniela Cesana, Alessandro Aiuti, Eugenio Montini

San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy

In gene therapy (GT) applications based on integrative vectors, the retrieval and mapping of vector integration sites (IS) in the genome of transduced cells and their progeny using specialized PCR-based techniques combined with next-generation sequencing (NGS) and bioinformatics analyses allows to obtain safety and efficacy readouts of the treatment. We developed Sonication Linker mediated- PCR (SLiM-PCR), a method for IS retrieval based on specific PCR amplification of vector/genome junctions from sheared genomic DNA and NGS. SLiM-PCR identification and quantification of IS and the downstream analyses are performed by dedicated software VISPA2 and ISAnalytics. The abundance of each IS is then obtained by counting the number of amplified DNA fragments of different lengths (depending on the shear site position) containing the same IS, which is proportional to the number of cells contributing to a specific clone, avoiding the biases introduced by PCR amplification. SLiM-PCR showed to be highly efficient in IS retrieval and allowed accurate quantification of a single lentiviral (LV) or γ-retroviral (γ-RV) IS down to 0.1% of relative abundance, when applied to an experimental framework in which DNA standards were composed of monoclonal samples carrying a defined number of IS in known positions of the genome mixed to a polyclonal sample harboring a random IS distribution. Given its accuracy and efficiency, we adopted SLiM-PCR as reference assay for IS analysis. SLiM-PCR is currently being applied on thousands of samples derived from more than 30 preclinical studies and 7 different GT clinical trials exploiting different vector types, like γ-RV, LV, Sleeping Beauty Transposons and Adeno-Associated Vectors. SLiM-PCR has been fully automated with liquid handlers able to process hundreds of DNA samples per week in 96/384-well plates. We are currently working on the miniaturization of the reactions exploiting the Acoustic Droplet Ejection technology of the Beckman Labcyte 525, an instrument capable of moving nanoliters of liquids by acoustic waves. So far, we could reduce reaction volumes by 10 times, down to 5 microliters, achieving cost reduction, higher sample throughput, and higher overall assay speed and reproducibility. We tested the miniaturized SLiM-PCR on DNA standards, using different amounts of genomic material and vector copy number (VCN), PCR amplification conditions and reagents, to understand the best balance between miniaturization and performance. Miniaturized SLiM-PCR can obtain comparable or even

better results to those obtained with standard volumes in terms of IS number retrieved from the same amount of genomic DNA, maintaining the precision in IS abundance estimation and detection limit. To reduce the number of false positives and sequence-sample misassignments caused by index hopping, we are currently comparing the emerging MGI DNBseq to the Illumina NGS technology and investigating if the lower error rate potentially provided by the rolling circle amplification instead of the conventional exponential amplification during sequencing may have a beneficial impact. Overall, our results showed that SLiM-PCR is a reproducible, accurate and versatile method that allows to perform high-throughput IS analyses, which may be further improved by the ongoing optimization process.

904. Optimizing Transgene and Integration Site Analysis with Minimal Cell Input

Maya Wright, Joost Swennenhuis, Nicole Hendriks, Georgios Georgiou, Erik Splinter, Ellen Stelloo
Cergentis, Utrecht, Netherlands

Introduction TLA, a technology invented by Cergentis, is an unmatched NGS-based technology for transgene and integration site analysis. The unique proximity ligation reaction in combination with a PCR-based target enrichment step allows for complete analysis of integrations and the surrounding sequences. While the present technology is robust and highly specific, there are certain aspects that warrant further enhancement. The minimum input required for optimal complexity is currently minimal 3 million cells. It is preferable to reduce the minimum cell input, which would also enable automation options. Additionally, there is need to address the sequence ambiguity of the at the exact restriction sites of the restriction enzyme used to generate TLA template and mitigate bias arising due to PCR efficiency of fragments of variable size and composition. To enable these improvements, a hybrid-capture TLA-based approach has been developed starting from 100,000 cells (Targeted Locus Capture, TLC). Hybrid-capture target enrichment, using capture probes e.g. specific for either the transgene or vector sequence, is done on randomly fragmented, proximity ligated DNA. The random fragmentation ensures equal distribution of the reads without restriction enzyme bias. Through proximity ligation, co-capture will occur of DNA which was spatially close to the targeted sequences in-vivo. Supported by the many reads with the vector-genome breakpoint sequence and reads on the proximal genomic regions, the improvements maintained the robust transgene and integration site analysis. **Method** As an illustrative project, we conducted a comparative analysis of both technologies on a CHO cell line (CHO DP-12 clone#1933 [CHO DP-12, clone#1933 aIL8.92 NB 28605/12] (ATCC CRL-12444)) which has been transfected with a vector designed to co-express variable light and heavy chain regions of a monoclonal antibody, anti- IL-8. The conventional TLA technology utilizing 5 million cells as input material and two primer sets targeting the vector was compared to the new TLC technology using 100.000 cells and capture probes designed from the entire vector sequence. **Results** The comparative analysis between TLA and TLC revealed a general alignment in the overall conclusions. Both technologies identified vector integration sites on chromosome 4, 5, 7 and 8. The integrations were all accompanied by genomic rearrangements. Despite identical conclusions, small variations were seen which can be ascribed to the use of the specific technology. TLC exhibits a more uniform distribution of coverage across the vector and the integration site locus due to the absence of restriction enzyme bias. Therefore, TLC was more complete in capturing sequence and structural variants, whereas TLA encountered challenges related to local coverage or restriction enzyme bias. **Conclusion** We present a robust and automatable new technology on a limited amount of input material, yielding complete and detailed data of integrated sequences. TLC has significant advances: random fragmentation eliminates the restriction enzyme bias and using capture-based target enrichment overcomes PCR bias ensuring uniform and high coverage across complete transgenes.

Wednesday Posters: Chemistry, Manufacturing, and Controls

905. Regulatory Agility: Value of CDMOs Utilizing Platform Technology

Angela Coy, Christopher J. Shilling
Forge Biologics, Grove City, OH

Bespoke gene therapies are often developed in a siloed manner, which can require a significant investment of time and resources - a challenging strategy for developing treatments for patients in nearly 10,000 rare diseases. The provisions introduced in the Consolidated Appropriations Act of 2023 aim to address this issue by defining platform technologies and authorizing FDA to expedite the development and review of a product that uses a designated platform technology. As part of the premarket notification (510k) clearance pathway for medical devices, new medical devices of a certain class can gain regulatory approval based on the 'substantial equivalence' to a previously approved device, or predicate device, thereby minimizing the amount of regulatory burden on developers of substantially equivalent devices. The legislation introduced in the 2023 Appropriations Act presents a great opportunity to allow regulators the ability to consider 'predicate' information from previously approved drug products in the review of new drug products manufactured using 'equivalent' processes. Forge participated in a Scientific Exchange with other stakeholders from industry, academia, and FDA recently to identify platform technologies in CGT that can serve as reusable predicate tools, defined as "building blocks," across development programs. Some of the proposed building blocks include starting materials (cell banks, plasmids) and entire unit operations (cell culture, sterile filtration). While many details and logistics of the building blocks concept have yet to be worked out, we propose that contract development and manufacturing organizations (CDMO) like Forge are poised to be leaders in this space. CDMOs are experienced in developing and validating a platform process, which includes reusing building blocks from one development program to another. We present here examples of how Forge Biologics is leveraging predicate information from its own building blocks to accelerate regulatory activities for developers. Forge Biologics is also experienced in documenting and sharing details of its building blocks with regulators through the use of master files. We will provide a review on the success of our master files and how they have aided in developers' regulatory agility. The principles introduced in the

Consolidated Appropriations Act are already being explored through initiatives like the Bespoke Gene Therapy Consortium, which Forge Biologics is an industry sponsor and participant. Our review suggests that the information and experiences from platform CDMOs offers a leading perspective and deep insight into the possibilities of building predicate data, which can be leveraged by future programs to address a substantial need in the CGT industry.

906. A Change is Gonna Come - Managing Process Changes Through the Product Lifecycle for ATMPs

Darius Pillsbury
Valsource Inc, New Paltz, NY

The only constant in life is change! This adage certainly applies to the development and manufacture of the many new modalities including cell and gene therapies and how an organization manages and embraces change is paramount to the ultimate success of the commercialization of these novel products for those patients in need. In this presentation we will explore how to navigate various types of changes to cell and gene therapy manufacturing processes using key enablers like knowledge management and risk management in order to ensure success. The recent draft guidance by the FDA "Manufacturing Changes and Comparability for Human Cellular and Gene Therapy Products" will be reviewed. Process changes at all stages of the product lifecycle from the manufacture and testing of initial clinical trial materials to the commercialization and long-term management of the manufacturing process based on optimization of the Process Control Strategy (referred to by the FDA as the "Cookbook") will be presented. A case study will be provided with an example of a significant process change made to an autologous gene-modified cellular therapy. The case study will encompass the initial identification of the need for a change, the supporting activities to implement the change, and the design and execution of an analytical product comparability study, based on best industry practices including the use of risk assessment and compliance with the expectations of global regulatory agencies. The challenges will be highlighted with solutions provided to overcome any barriers and challenges to implementation, as well as the follow up activities also important post-change.

907. Failure to Plan is Planning to Fail: Risk Assessment for an Early-Stage Autologous Product to Identify and Mitigate Process Hazards

Beth Fulton
ValSource, Inc., Downington, PA

Cell therapy products inherently have unique manufacturing challenges. Processes for autologous products may be limited by patient condition (e.g. low volume or titer available as a starting point). Both autologous and allogenic products may be limited by the capabilities of early stage processes (e.g. failure rate of laboratory assays, failures of equipment that may not be purpose-designed) or by logistical failures (related to tight timelines for shipment of product and materials, given the short shelf life of product/materials). These problems are not insurmountable; careful risk-based planning can allow for agile response and recovery. In this talk, a case-study is presented in which the manufacturer of an autologous product conducts a risk assessment to identify foreseeable process hazards. Examples of identified mitigations for these failures are provided.

908. The Impact of Joint Auditing with Cell & Gene Therapy

James Fries
Rx-360, Philadelphia, PA

De-risking of supply chain through effective utilization of joint audit program model. To protect patient safety by ensuring the quality of the materials within the supply chain in a scalable sustainable way, biopharma manufacturers and critical material suppliers leverage a joint audit program or model as a best practice. Utilization of the model has resulted in increased standardization of approach delivering quality oversight complimented by significant efficiencies in the process, estimated at approaching $10M in 2023 with continued growth expected as industry broadens utilization of the model. This case study jointly presented by representatives from a critical material supplier and biomanufacturer explains how the value is being recognized based on actual data.

909. HAA-Based Ion Pair Reversed Phase HPLC Method for More Accurate gRNA Purity Test

Toshifumi Kano, Masataka Shibata, Daisuke Furukawa, Yoshimi Fujii, Hideki Ihara
Sumitomo Chemical Co., Ltd., Osaka, Japan

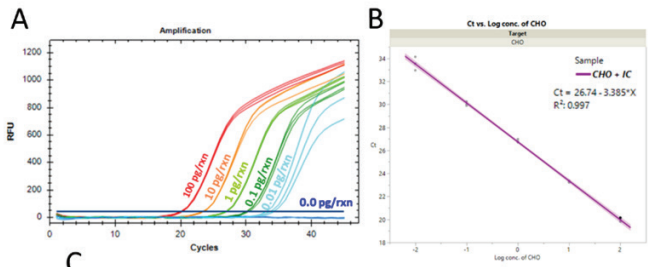
CRISPR-based gene therapy has a potential to treat unmet genetic diseases. Since gRNA is a component to specifically bind the target site, the success of CRISPR-mediated gene editing is highly dependent on the quality of the gRNA. Therefore, high-purity gRNA is critical for clinical applications, and optimization of the gRNA manufacturing process has been studied intensively in recent years. Meanwhile, there are few reports on analytical methods to determine the gRNA purity. We evaluated various analytical methods such as capillary electrophoresis and IP-RP-HPLC methods, and finally found that Hexylammonium Acetate (HAA)-based IP-RP-HPLC method showed better separation than ever. This method enabled us to achieve peak-top separation of 96-103nt single-stranded RNAs mixture. Then, we performed a comparative analysis of a commercially available gRNA between a general HPLC method and our newly developed HPLC method. The purity was about 80% by the general HPLC method, on the other hand, our new method revealed that the purity was 38%. This method will be helpful in precisely understanding the safety of gRNAs for clinical applications.

910. A Realtime PCR Assay for CHO Host Cell DNA Detection for Bioprocess QC

Nidhi Nandu, Yanhong Tong, Zhi-xiang Lu
Revvity, Waltham, MA

Therapeutic recombinant proteins and monoclonal antibodies have become pivotal and expanding categories of biologic products employed in the treatment and management of various diseases. The

presence of residual DNA derived from host cells such as CHO cell lines at elevated levels during the production of biologic products raises significant concerns due to its potential oncogenic or immunogenic risks. This highlights the essential need for analytical solutions to measure CHO residual DNA in biologic products. Here, we developed real-time PCR assay using hydrolysis probe-based method to quantify CHO residual DNA. The quantification range is wide, from 0.01 pg to 100 pg CHO genomic DNA per reaction (***Figure 1***), which meets the United States Pharmacopeial Convention (USP) General Chapter <509> "Residual DNA Testing" criteria. By using Revvity proprietary PCR formulation, the residual DNA quantification workflow allows industrial experts to simultaneously test 96 or 384 samples (including controls and standard materials) in less than 2 hours (~15minute hands-on time) from sample PCR amplification to data report.



C

		Ct					
		Target					
		CHO			IC		
Sample	Conc. of CHO (pg/rxn)	N	Mean	Std Dev	N	Mean	Std Dev
CHO + IC	100	3	19.99	0.170	3	31.87	0.157
	10	3	23.29	0.076	3	31.82	0.184
	1	3	26.83	0.137	3	31.60	0.180
	0.1	3	30.05	0.184	3	31.62	0.023
	0.01	3	33.54	0.585	3	31.84	0.199
	0	0	.	.	3	31.58	0.112

Figure 1. Standard curve using CHO genomic DNA in presence of Internal Control (IC). A. amplification curve of CHO. B. standard curve of CHO 0.01 to 100 pg/rxn. C. Ct values of samples. Std Dev. standard deviation.

911. Digital PCR Applications for Cell and Gene Therapy - High-Quality Detection of Mycoplasma Contamination

Janina Bracker[1], Julius Albers[1], Anida Mesihovic[2], Andreas Hecker[2], Francesca Di Pasquale[1]

[1]R&D, QIAGEN GmbH, Hilden, Germany,[2]Product Management, QIAGEN GmbH, Hilden, Germany

Cell and gene therapies seek to target previously untreatable diseases at their source using individualized treatments. Developing safe and effective cell and gene therapies requires strict quality controls at all stages of the development and manufacturing process. The determination of potential impurities is crucial. Manufacturers of biologics use different approaches to test for Mycoplasma contaminant throughout the production process and for final substance release. The sensitivity of mycoplasma detection is crucial for ensuring safe and high-quality ATMPs. The detection of mycoplasma contamination using Nucleic Acid Techniques (NATs) is well established and described by different Pharmacopeias worldwide. Some compendia like the European Pharmacopeia have already announced a revision of the detection method that will make it more stringent particularly for the validation step. Indeed, the qualification of the standards and the GC:CFU ratio will be probably better defined. We would like to show the advantage of using RNA instead of mere DNA to detect mycoplasma contamination in a variety of samples. Indeed, the use of RNA enables to detect a transcript with a higher copy number enhancing the sensitivity down to 5 CFU/ml. Moreover, the robust RNA detection also enables ability to process samples at different stages of manufacturing that may be more complex in terms of composition, like containing high salts or high amounts of producer cells.

912. Advancing Quality Control for CRISPR-Cas9 Systems: Separation of CRISPR Components Using Ion Exchange Chromatography

Kyle Scheel[1], Matthew Lauber[2], Rachel C. Ginther[1]

[1]MRIGlobal, Kansas City, MO,[2]Waters Corporation, Milford, MA

Recently, the first CRISPR/Cas9 edited cell therapy was approved for treatment of sickle cell anemia, with other CRISPR-based therapies in the pipeline. However, despite the growing market for CRISPR-based therapeutics, technologies for all-in-one quality control analysis of CRISPR components and ribonucleoprotein (RNP) complexes are not well-established. In order to ensure safe and effective CRISPR drug substances, thorough quality control assessment of CRISPR reagents and characterization of RNP complex formation is needed. Current methods for characterization of CRISPR/Cas components are limited and do not typically allow for simultaneous characterization of apo-Cas, apo-sgRNA, and RNP species. Here, we exploit the differing charges of apo-Cas, apo-sgRNA, and RNP in order to achieve separation of CRISPR components by ion exchange (IEX) high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection. In this work, we have applied a corrosion resistant UHPLC instrument equipped with UV detection and low adsorption flow path components. Anion exchange and cation exchange columns were coupled, allowing for simultaneous retention of Cas9 on the cation exchange column and sgRNA on the anion exchange column. A number of practical considerations for the analysis of CRISPR components by IEX chromatography were determined during method development. Considerations include UV detector flow cell/analyte compatibility, sample diluent, system suitability, choice of mobile phase buffer and ionic strength, and the impact of sgRNA sample heterogeneity. Adequately addressing these considerations will remain of critical importance as more CRISPR drug substances enter the drug development pipeline.

913. A Novel Network of Cell Processing and Cryopreservation Hubs Addressing Critical Gaps in Clinical Sample Processing Capabilities for Pharmaceutical Companies and Clinical Trial Sponsors

Sarah Alter, Andrew Larson, Priya R. Baraniak

OrganaBio, South Miami, FL

Following the global COVID-19 pandemic and due to recent advancements of mRNA vaccines for a host of diseases, the need for organizations with capabilities to support clinical trials has been increasing and is expected to continue to increase in coming

years. Clinical Research Organizations (CROs), clinical sites, and translational centers/hospitals often have the capabilities to administer drug doses to participants, perform autologous leukapheresis, and draw blood samples over a period of time. However, many of these organizations lack the laboratory infrastructure and staff to support sample processing, such as peripheral blood mononuclear cells (PBMCs) isolation and cryopreservation and cryopreservation of patient apheresis materials, a critical intermediate manufacturing step for many cell-based therapies. Samples often need to be shipped to a central lab, which adds risk for sample loss and prolongs transit time, leading to unintended functional and phenotypic changes in cells, compromising quality and data analyses. To address this issue, OrganaBio's Cell Processing and Cryopreservation Services (CPC Services) business unit has partnered with several large multinational, multibillion dollar pharmaceuticals companies (Sponsors) to support clinical studies enrolling participants in the greater Miami, FL and Irvine, CA metro regions. OrganaBio team members were trained and qualified by Sponsors within 4 weeks of initial contract execution, and a dedicated Project Manager works alongside clinical sites to effectively manage timing of sample deliveries and to ensure that the OrganaBio team is prepared to receive and process samples 5 days a week. In the first 18 months, over 1,200 samples were received and processed with >99% success. Given the team's expertise in sample and document management, it now takes merely 30 minutes on average to initiate processing of blood samples from time of sample receipt, which is critical to accurate data analysis and data integrity for the trials. In addition to cell isolation, OrganaBio cryopreserves and stores study participants' PBMC samples and retains, allowing for batch shipping to Sponsors, simplifying cold chain management, mitigating risk of loss, and keeping Sponsors' shipping costs down. In addition, add on business for new trials is experiencing contract turnaround times of less than one week, driving efficiencies on the business side as well. OrganaBio's unique Hub Processing model has proven vital to the success of Sponsors' clinical trial collection and processing functions and performs more efficiently and faster than the required four- to six-hour sample processing window, ensuring consistency and validity of sample data. CPC Services has successfully scaled processing and cryopreservation capacities to fulfill initial and ongoing requirements to cover the lifespan of the trials. OrganaBio's client-centric and data-driven approach enables seamless operation and timely processing and delivery of crucial clinical trial participant samples. This purpose-built solution has been specifically developed to provide decentralized clinical trial support, including timely sample processing and data capture that are essential to accurately gauge the outcomes of clinical trials.

Wednesday Posters: Gene Therapy Trials - In Vivo Modification

914. CRISPR/Cas13 RNA-Targeting Therapy to Treat Patients with Neovascular Age-Related Macular Degeneration

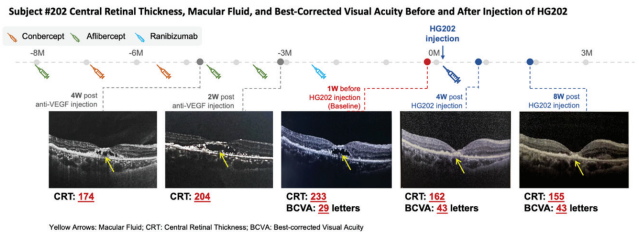
Dongjun Xin[1], Boshi Liu[1], Weiwei Liu[2], Shuaishuai Zhang[2], Yuhan Jiang[2], Xuan Yao[2], Linyu Shi[2], Hui Yang[2], Alvin Luk[3], Yiqun Yuan[2], Xiaorong Li[1]

[1]Tianjin Medical University Eye Hosptial, Tianjin, China,[2]Huidagene Therapeutics Co., Ltd, Shanghai, China,[3]Huidagene Therapeutics Inc, Clinton, NJ

Purpose: Neovascular age-related macular degeneration (nAMD) is a leading cause of blindness in the aging population, with vascular endothelial growth factor (VEGF) playing a key role. Frequent intravitreal injection of anti-VEGF therapy is the front-line treatment of nAMD; however, it is only effective for 1-2 months at a time and requires frequent re-administration. Despite favorable clinical outcomes, persistent fluid or recurrent exudation returns in numerous patients after repeated administrations which lead to serious complications and reduce patient adherence to treatment, resulting in vision loss over time. Therefore, we developed HG202, a CRISPR/Cas13-based RNA targeting therapy, to partially knock down the mRNA expression of VEGFA in the retina with a single dose, reducing the frequency of treatment and potentially improving clinical outcomes. Here, we initiated the **SIGHT-I** clinical study (NCT06031727) investigating HG202 in patients with nAMD. **Methods: SIGHT-I** is an open-label, multi-center, and dose-escalation study investigating the safety, tolerability, and preliminary efficacy of HG202, a single adeno-associated viral (AAV) vector packaging novel high-fidelity CRISPR/Cas13 RNA targeting technology, to reduce the expression of VEGFA and thus inhibit choroidal neovascularization (CNV) formation. A total of 9 nAMD patients who are either non-responsive and resistant to anti-VEGF therapies or responsive to anti-VEGF therapies but require frequent injections with Early Treatment Diabetic Retinopathy Study (ETDRS) best-corrected visual acuity (BCVA) between 73 and 23 in the study eye at Screening will be randomized to one of the three doses of HG202. The primary objectives are the safety and tolerability of HG202 at different doses. Secondary objectives include mean change in BCVA, central retinal thickness (CRT), and other efficacy endpoints. **Results:** As of Jan-2024, 5 subjects have signed the ICF and 3 subjects have been enrolled in the low-dose cohort. Subject #202, a non-responsive and resistant to anti-VEGF therapies patient, had a history of 6 anti-VEGF injections within 8 months with persistent macular fluid before the injection. Subject #202 baseline included CRT of 233 μm and BCVA of 29 letters. Retinal fluid was significantly eliminated post-injection, and CRT was recovered to 162 and 155 μm at 4- and 8-week post-injection, respectively. The BCVA improvement of nearly 15 letters was observed starting at 4 weeks and sustained at 8 weeks post-injection visit (**Figure 1**). No drug-related serious adverse event has been observed in all three treated subjects. **Conclusions:** Three subjects with nAMD had an acceptable local and systemic adverse-event profile after the delivery of HG202. Subject #202 had significant improvement in BCVA, CRT, and elimination of persistent fluid within

8 weeks after the injection of HG202. Although only a low-dose cohort completed the enrollment, this study provides the basis for CRISPR/Cas13 RNA therapy is relatively safe in humans.

Figure 1.



915. Preliminary Safety and Efficacy of HG004 Gene Therapy Trial for Leber's Congenital Amaurosis Associated with Mutations in *RPE65* Gene

Ping Fei[1], Yuan Yang[1], Jie Peng[1], Xiang Zhang[1], Yanfei Wang[2], Shuaishuai Zhang[2], Xuan Yao[2], Alvin Luk[3], Yiqun Yuan[2], Peiquan Zhao[1]

[1]Xinhua Hospital affiliated to Shanghai Jiao Tong University, Shanghai, China,[2]Huidagene Therapeutics Co., Ltd, Shanghai, China,[3]Huidagene Therapeutics Inc, Clinton, NJ

Purpose: Leber's Congenital Amaurosis (LCA) is a group of inherited retinal diseases involving retinal degeneration with abnormal eye movements (nystagmus) and severe vision loss in early infancy and childhood. One of the forms of the disease, type 2 LCA (LCA2), is associated with mutations in the retinal pigment epithelium-specific 65-kDa protein gene (*RPE65*). The safety and efficacy of adeno-associated virus (AAV) gene therapy for LCA2 have been validated in humans using AAV serotype 2 (AAV2). However, transduction of retinal pigment epithelial (RPE) cells with AAV2 has partially corrected LCA2 in animals while AAV serotype 9 (AAV9) is an ideal AAV vector to restore cone-photoreceptor function than AAV2. We, therefore, initiated the **LIGHT** (**L**eber congenital amaurosis **I**nherited blindness of **G**ene t**H**erapy **T**rial) clinical study (NCT06088992) to evaluate a single injection of HG004 (AAV9-*RPE65*) in children and adults with LCA2. **Methods: LIGHT** is an open-label and dose-escalation study investigating the safety, tolerability, and preliminary efficacy of a single subretinal injection of HG004 (AAV9-*hRPE65*). We assessed the retinal and visual function in subjects with LCA2 given 200 µL of HG004 in the worst eye at low ($6.0x10^9$ vector genomes, vg), middle ($1.0x10^{10}$ vg), and high ($2.0x10^{10}$ vg) doses. After completing the informed consent process and confirming the eligibility criteria, the eye with the worst visual function was selected for subretinal injection of one of the three doses of HG004. Subjects were evaluated before and after surgery at designated follow-up visits up to 5 years by complete ophthalmic examination. Efficacy for each treated patient was monitored with best-corrected visual acuity (BCVA), full-field stimulus threshold (FST), and kinetic visual field (KVF) assessments. **Results:** Nine adults or children (aged 8-38 years) with *RPE65*-associated LCA have enrolled in the low, middle, and high-dose cohorts. The average BCVA, FST-white light, and KVF improvements are +11 letters, -2.268 log cd.s/m^2, and +554.25 deg^2 (III4e), respectively, at 6-month post-injection in the low dose cohort, +7.5 letters, -1.413 log cd.s/m^2, and +958.30 deg^2 (III4e), respectively, at 3-month post-injection in the middle dose cohort, and +12.7 letters, -3.038 log cd.s/m^2, and +1142.77 deg^2 (III4e), respectively, at 1-month post-injection in the high dose cohort. No serious adverse event or dose-limiting toxicity has been observed. The majority of subjects after injection had at least a 2-log unit increase in pupillary light responses. Most adverse events were mild and transient which have resolved without any intervention. No signs of inflammation or acute retinal toxicity after the injection of HG004. **Conclusions:** Most of the treated subjects had at least a 2-log unit increase in pupillary light responses with durable effects during follow-up. There is no retinal detachment or drug-related serious adverse events, suggesting subretinal gene therapy at lower total volume (200 µL) is safe. These three doses (25-,15-, and 7.5-fold lower than the approved AAV2-*hRPE65* gene therapy in the Phase 3 trial) in this **LIGHT** trial showed comparable results, suggesting that HG004 may be a safer and more efficacious alternative treatment option for LCA2. Currently, HG004 is being evaluated in a multi-regional clinical trial (NCT05906953) with both Orphan Drug Designation and Rare Pediatric Disease Designation granted by the U.S. FDA.

916. PRODYGY: A First-in-Human Trial of Rod-Derived Cone Viability Factor (RdCVF) Gene Therapy in Subjects with Rod-Cone Dystrophy

James A. Gow[1], Joseph Martel[2], Laure Blouin[3], Anne-Sophie Gautron[3], Alice Le Meur[1], Nelle Messeca[3], Anne Celle[3], Daniel C. Chung[1], Isabelle Audo[4,5]

[1]SparingVision, Inc., Philadelphia, PA,[2]Department of Ophthalmology, University of Pittsburgh Medical Center, Pittsburgh, PA,[3]SparingVision, Inc., Paris, France,[4]Centre Hospitalier National d'Ophtalmologie des Quinze-Vingts, Centre de Référence Maladies Rares REFERET, INSERM-DHOS CIC 1423, Paris, France,[5]Sorbonne Université, INSERM, CNRS, Institut de la Vision, Paris, France

Purpose Rod-cone dystrophy (RCD) is a rare inherited retinal disorder leading to significant vision loss, and for which no treatment is currently available to most patients. SPVN06 is a gene-independent investigational gene therapy expressing the neurotrophic rod-derived cone viability factor (RdCVF) and the thioredoxin RdCVF-Long (RdCVFL), that aims at slowing down the progression of central vision loss in patients with RCD, regardless of the underlying pathogenic variant(s). **Methods** PRODYGY (NCT05748873) is a first-in-human Phase I//II trial enrolling subjects with advanced RCD due to a mutation in the *RHO*, *PDE6A*, or *PDE6B* gene. The study will assess the safety and tolerability of a unilateral subretinal injection of SPVN06, one year after treatment administration. Its two-step design includes an open-label dose-escalation phase (Step 1) followed by a controlled, double-masked, randomized, extension phase (Step 2). Step 1 will test 3 increasing doses of SPVN06 in subjects with severe advanced RCD, and two doses will be selected for assessment in Step 2. Step 2 will include 3 cohorts of subjects with intermediate advanced RCD. A total of 33 subjects is planned to be enrolled, of whom 27 will receive a single injection of SPVN06 in their worse-seeing eye. **Results** Six months after SPVN06 administration in the first cohort treated at the lowest dose, 7 adverse events (AEs) were reported in the treated eyes, including mild subconjunctival hemorrhage that resolved spontaneously, transient mild intraocular pressure elevation, and mild macular edema. One month after SPVN06 administration

in the last subject of the second cohort treated at the medium dose, 15 AEs were reported in the treated eyes, which included but were not limited to mild-to-moderate subconjunctival hemorrhage that resolved spontaneously; macular hole during subretinal injection that resolved without sequelae; and mild-to-moderate intraocular pressure elevation that resolved with standard treatment. Mild cells in the vitreous and anterior chamber were observed in 4 subjects (3 at the medium dose), vitreous haze was reported in 3 subjects, and anterior chamber flare in 1 subject. All findings were of Grade 1 or less and resolved spontaneously within 4 weeks after injection. A transient increase in anti-capsid antibodies, peaking at Week 2, was observed in 2 subjects who had pre-existing antibodies before injection of SPVN06. To date, no dose-limiting toxicities have been observed. An independent Data Safety Monitoring Board provided a positive recommendation to continue the trial without modifications, and to proceed with treatment administration at the highest dose in the third cohort. **Conclusions** First administration of SPVN06 to patients showed a favorable safety profile at low and medium doses, with no significant immune response.

917. Longitudinal Analysis of BCVA and Near-Field Object Recognition in Low- or High-Dose MCO-010 Mutation Agnostic Optogenetic Therapy for Retinitis Pigmentosa: 12-Month Results from a Phase 2b/3 Randomized, Sham-Controlled, Patient- and Assessor-Masked Clinical Trial (RESTORE)

Samuel Barone

Nanoscope Therapeutics, Dallas, TX

Purpose MCO-010 is a gene mutation agnostic optogenetic therapy being evaluated in an ongoing Phase 2b/3 RESTORE clinical trial in individuals with advanced retinitis pigmentosa (RP). We performed an analysis of 52-week longitudinal data to evaluate the safety and efficacy of a single administration of 2 different doses of MCO-010 therapy vs sham. MCO-010 is an AAV2-delivered multi-characteristic opsin (MCO) transgene administered by intravitreal injection. MCO-010 transduces bipolar cells to express a photosensitive opsin protein, restoring light sensitivity to the retina in patients with RP. **Methods** Subjects had an advanced RP clinical diagnosis and baseline visual acuity worse than 1.9 logMAR (CF, 20/1600 equivalent) in the study eye and no better than 1.6 logMAR in the fellow eye. Subjects (n = 27) were randomized 1:1:1 to receive a single dose of 0.9E11 gc/eye (low-dose), 1.2E11 gc/eye (high-dose) MCO-010, or sham IVT in the study eye at day 0. Visual function was assessed systematically through week 52 by best-corrected visual acuity (BCVA) using Freiburg visual acuity and a multi-luminance shape discrimination test (MLSDT). BCVA data were analyzed comparing the low- or high-dose MCO-010-treated individuals vs sham using a mixed-effects model for repeated measures with treatment, visit, and treatment-by-visit interaction as factors in the model, using the baseline score as a covariate. MLSDT data were analyzed by comparing the baseline scores of the low-dose, high-dose or sham vs Week 52 scores using a paired samples t-test. **Results** MCO-010 patients had mean baseline BCVA scores of 2.207 ± 0.105 and 2.250 ± 0.00 logMAR (low- and high-dose, respectively), and the sham patients had a mean baseline BCVA of 2.172 ± 0.1342 logMAR. The following BCVA results are reported from weeks 16, 24, 36, and 52. Compared to sham, low-dose MCO-010 patients had a mean improvement of 0.160, 0.192, 0.431, and 0.336 logMAR (P = 0.2780, 0.1958, 0.0060 and 0.0300 respectively). High-dose MCO-010 patients had a mean improvement of 0.022, 0.197, 0.214, and 0.287 logMAR (vs sham; P = 0.8392, 0.0765, 0.0544 and 0.0103, respectively). MCO-010 patients had mean baseline MLSDT scores of 1.44 ± 0.67 and 0.22 ± 0.15 (low- and high-dose, respectively), and the sham patients had a mean baseline MLSDT score of 1.67 ± 0.62. The following MLSDT results are reported as the mean change from baseline for weeks 16, 24, 36 and 52. The low-dose MCO-010 patients had mean scores of 0.22, 0.00, 1.22, and 1.33 (P = 0.8337, 1.000, 0.2489, and 0.2721, respectively). High-dose MCO-010 patients had mean scores of 1.57, 0.78, 1.00, and 1.89 (P = 0.0914, 0.2594, 0.1599, 0.0265, respectively). Sham patients had mean scores of -0.25, 0.33, -0.11, and 0.22 (P = 0.7486, 0.6666, 0.5943, and 0.8032, respectively). MCO-010 was well tolerated with no serious adverse effects observed through week 52. **Conclusion** BCVA assessed with Freiburg visual acuity can objectively quantify visual acuity for low-vision patients on a logMAR scale and has been recommended for clinical trials in individuals with low vision, including optogenetics, by the International HOVER Taskforce (Harmonization of Outcomes and Vision Endpoints in Vision Restoration Trials). The RESTORE data further support the validity of using Freiburg visual acuity for low-vision patients as an efficacy endpoint. RESTORE data demonstrate MCO-010-treated patients improved in BCVA in both dose groups, compared to sham-treated patients, where statistically significant improvements in visual acuity were observed at week 36 and was maintained through the 52-week study. Furthermore, a dose-dependent improvement in near-field object recognition was observed in the MCO-010-treated patients; the high-dose patients statistically improved in MLSDT at week 52 compared to baseline.

918. Preliminary Safety and Efficacy Results from an Open-Label, Dose-Escalation Pilot Gene Therapy Study of FT-002 in Subjects with RPGR Gene Mutation Associated X-Linked Retinitis Pigmentosa (XLRP)

Jihong Wu[1], Ruifang Sui[2]

[1]Eye and ENT Hospital of Fudan University, Shanghai, China,[2]Peking Union Medical College Hospital, Beijing, China

Background: X-linked retinitis pigmentosa (XLRP) is a severe form of retinitis pigmentosa (RP) with impaired night vision and constriction of the peripheral visual field, which leads to visual impairment or even functional blindness. The mutation of the RPGR gene accounts for a majority of XLRP cases. Although there is still no effective treatment for XLRP due to defects in the RPGR gene, gene therapy has the potential to prevent and halt the progression of the disease, or even revert the pathological phenotype, constituting a long-lasting cure. FT-002, a novel AAV5 gene therapy vector introducing the codon-optimized $hRPGR^{ORF15}$ gene into retinal photoreceptor cells by subretinal injection to express active RPGR protein which participates in photoreceptor ciliary transport function, rescues photoreceptor cell loss due to RPGR gene mutation and improves visual function or delays the progression of visual impairment of XLRP patients. **Methods and Results:** A pilot clinical study was launched to investigate the safety, tolerability, and

preliminary efficacy of FT-002 via subretinal administration in male patients with RPGR mutation-associated XLRP (NCT05874310). In this first-in-human dose escalation study, dose levels of 5 x 10^{10} vg/eye, 10 x 10^{10} vg/eye, and 20 x 10^{10} vg/eye were evaluated, with 6 subjects enrolled in each dose group from two investigational sites in China. All 18 subjects received one-time FT-002 in the study eye via subretinal injection, with the safety, efficacy, immunogenicity, and virus shedding being evaluated up to 1 year, with a total follow-up of 5 years. FT-002 is well tolerated in all 18 subjects, with no DLT or FT-002 related SAE observed. Most AEs are related to the surgical procedure, and signs of FT-002 related ocular inflammation were observed in some subjects but controllable via the topical use of steroids. Improved retinal sensitivity and visual function were seen in the medium and high dose groups assessed by microperimetry, full-field stimulus threshold (FST), or mobility test. Notably, 4 subjects were with 28 loci, 24 loci, 21 loci, and 28 loci ≥5 dB improvement, and with 6 loci, 15 loci, 4 loci, and 22 loci ≥7 dB improvement, respectively, among the 68 loci of the whole grid in microperimetry evaluated up to the most recent visit (Day 56-168). **Conclusion:** XLRP due to defects in the RPGR gene is a rare disease that causes patients to go blind. Effective treatments remain a critical unmet need. The novel AAV-based gene therapy drug FT-002 shows favorable therapeutic effects with a good safety profile in XLRP male patients; although more clinical data are yet to be collected from the longer follow-up visit, FT-002 targeting RPGR is a potentially effective cure for XLRP due to defects in the RPGR gene.

920. Clinical Periodic Study Report on Safety and Efficacy of NGGT001 for Treating Bietti's Crystalline Dystrophy

Xiuju Chen[1], Xiao Liu[2,3,4], Shihe Cui[5], Gang Wang[5], Yiting Liu[6], Guang Qu[5,6], Lixin Jiang[5,6], Yong Liu[2,3,4], Xiaoxin Li[1]

[1]Xiamen Eye Center of Xiamen University, Xiamen, Fujian, China,[2]Ophthalmology, Southwest Hospital, Army Medical University, Chongqing, China,[3]Key Lab of Visual Damage and Regeneration & Restoration of Chongqing, Chongqing, China,[4]Jinfeng Laboratory, Chongqing, China,[5]NGGT (Suzhou) Biotechnology Co., Ltd., Suzhou, Jiangsu, China,[6]NGGT, Inc, Walnut Creek, CA

Introduction: Bietti's crystalline corneoretinal dystrophy (BCD) is caused by mutations in the cytochrome P450 (CYP) family 4 subfamily V member 2 gene (*CYP4V2*) encoding a polyunsaturated fatty acid hydroxylase which is dominantly expressed in RPE cells in retina. There has not been any treatment available for BCD. NGGT Biotechnology has developed NGGT001, an rAAV2-based vector expressing codon optimized human CYP4V2 for treating BCD. A two-site investigator-initiated trial (IIT) study is conducted to assess the safety and the efficacy of NGGT001 in adult patients with BCD. **Methods:** Twelve adults, aged 30 to 60 years old with BCD received a unilateral subretinal injection of NGGT001 in the poorer-seeing eye, at 2 dose levels of 1.5X10^{11} or 3X10^{11} vg/eye with 6 subjects in each cohort. As of data cutoff, patients have been followed up for 6 to 9 months after treatment. The primary safety measures were ocular and non-ocular adverse events (AEs). Exploratory efficacy included changes in best-corrected visual acuity (BCVA) assessed by ETDRS charts and patient daily life quality self-report. **Results:** All patients tolerated subretinal injections of NGGT001 and there were no treatment-related serious adverse events (sAEs). A total of 6 transit AEs were reported in 5 participants, such as calcaneal stress fracture and eye conjunctival or eyelid edema, which were associated with the surgical procedures and mostly recovered without any medications. In 11 patients completed the 6-month follow-up, 55% of them had a ≥10-letter and 36% had a ≥15-letter improvement in ETDRS letter counts, respectively. At 6 months, the improvement was 12.36 ± 3.47 (Mean ± SEM, n=11) letters from baseline in the treated eyes and remained stable in patients who completed 9-month follow-ups (n=5). The treatment effect of NGGT001 reached statistical significance when compared to baseline (p=0.0019). In untreated eyes, an increase of 6.64 ± 1.75 (Mean ± SEM, n=11) letters was also observed at the 6-month follow-up. 11 out of total 12 patients reported improvements in visual functions and daily life activities. **Conclusions:** These results of the IIT study demonstrated the safety of NGGT001 and highlighted that NGGT001 achieved preliminary efficacy in adult BCD patients.

921. Efficacy and Safety of Intravitreal rAAV2-*ND4* Therapy for Leber's Hereditary Optic Neuropathy

Wenbin Wei[1], Libin Jiang[1], Sitong Guo[2]

[1]Beijing Tongren Hospital, CMU, Beijing, China,[2]Beijing Friendship Hospital, CMU, Beijing, China

Objective: To evaluate the safety and efficacy of a recombinant adeno-associated virus serotype 2 (rAAV2) carrying normal *ND4* (rAAV2-*ND4*) (NR082) in participants with visual loss from Leber hereditary optic neuropathy (LHON) carrying the m.11778G>A mutation, and explore a safe dose of NR082 intravitreal injection. Methods: 12 participants with the m.11778G>A mitochondrial DNA mutation and vision loss more than 6 months from onset in both eyes were included in this prospective open-label trial. They were treated with intravitreally injection unilaterally, the first 6 participantswere treated at the initial dose of vector (1.5*109 vg, 0.05mL) (Group I), and the other 6 participants weretreated at the high dose (4.5*109 vg, 0.05mL) (Group II).Treated participants were followed for 52 weeks and underwent ocular and systemic safety assessments along with visual structure and function examinations.Results: A total of 12 participants were included in this study, 5 male subjects and 1 female in Group I with a mean age of 19.0 ± 1.55 years and 21.8 ± 5.27 years in Group II. The baseline BCVA in the injected eyes in Group I was 1.89 ± 0.36 logMAR, which improved to 1.59 ± 0.10 logMAR at week 52nd after intravitreal rAAV2-ND4. In Group II, the baseline BCVA in the injected eyes was 2.20 ± 0.15 logMAR, and BCVA at week 52nd after treatment was 1.92 ± 0.32 logMAR.There were three and four injected eyes improved significantly after 52-week's follow-up in Group I and Group II respectively. There was no serious ocular or systemic adverse events or dose-limiting toxicity in all participants. The adverse events possibly related to thetreatment included uveitis,vitreous opacity and keratic precipitates.Conclusion: No serious safety problems were observed in the 12 participants enrolled in this phase 1/2 trial of virus-based gene transfer in this mitochondrial disorder. Additional study follow-up of these and additional participants is needed to confirm these preliminary observations. Key Words: safety, efficacy, gene therapy, rAAV2-*ND4*, leber's hereditary optic neuropathy

922. Honoring the Generizeables

Rafael D. Escandon

Rafael Escandon, Bainbridge IS, WA

To date, since ASGCT's 26th Annual Meeting in May of 2023, six gene replacement and gene editing therapies in inherited, monogenic conditions have been approved in the USA. Many of these programs employed dose-escalation designs in attempts to rapidly translate a preclinical dose into a safe and efficacious dose in humans. Unlike with drugs and biologics, risks of gene therapies are not limited to unknown and untoward effects, it is also the permanence of a limited or a lack of efficacy due to underdosing that distinguishes early clinical gene therapy development from that of traditional drugs and biologics. Gene therapies cannot currently be turned up, turned down, or turned off. To conduct research ethically, participants and guardians are informed that one of the exchanges for a research participant's possible lack of direct benefit is the possibility of providing generalizable knowledge that may be useful to future participants in research, and/or future patients with the genetic condition. As safe and effective doses are sought, most gene therapy programs, whether approved or in development, have created and continue to create groups of research participants who were under-dosed, or dosed outside of an optimal window of opportunity to halt or reverse progression of disability. Since re-dosing of vector-based gene therapies is not feasible for myriad reasons, and participation of prior gene therapy recipients is often excluded from clinical trials of other interventions, options for these patients are limited. While creation of such a population was unintended, it continues to grow. It is, therefore, reasonable to ask whether the scientific community is appropriately honoring such altruism, and if not, how and in what ways it may do so in the future.

923. AAV Therapeutic Windows by Route of Administration: A Meta-Analysis Covering 20 Years of Clinical Dosing Data from 154 AAV Trials

Gregg Wagner, Chris A. Learn

Cell & Gene Therapy Center of Excellence, Parexel, Durham, NC

Introduction: Summary data guiding clinical dose selection for adeno-associated virus (AAV) gene therapy investigational products have largely been underreported. The high homology of AAV product classes suggest that their pharmacologic properties, including therapeutic window, may be comparable by route of administration (RoA) irrespective of capsid serotype, expression construct, and target indication. Here, we collected and analyzed dosing information from 154 AAV clinical trials to estimate the therapeutic window of AAV products by RoA to demonstrate the potential importance of mining public databases for 1) clinical trial design and 2) to illustrate safety considerations on dose to further inform clinical practice. *Methods:* We collected dosing information and other reported metadata (e.g., RoA, indication, etc.) from 154 AAV clinical trials over 20 years on ClinicalTrials.gov. The dataset contains 50 phase 1 trials, 76 phase 1/2 trials, 12 phase 2 trials, 5 phase 2/3 trials, and 11 phase 3 trials. Of the trials surveyed, 53% (82/154) reported dose information. This data was subsequently stratified by RoA and, for trials reporting multiple doses, the highest dose was selected for analysis. Using this strategy, we determined the clinical dosing range for six RoAs: intravenous (IV), intracoronary, intramuscular, intra-articular, intravitreal (IVT), and subretinal. AAV trials using an IV RoA were further stratified into hepatic and non-hepatic indications to consider the effect of AAV hepatotropism on dose selection. Considering the data were collected from clinical studies in which the optimal dosing risk: benefit profile had yet to be determined, we accept that a subset of lower doses reported may lack efficacy and a subset of higher doses may show dose-limiting toxicity. Accordingly, we excluded the first and fourth quartile of the clinical dosing range and define the interquartile range (IQR; middle 50%) as the estimated therapeutic window for each RoA. In addition, we performed exploratory analyses of AAV doses and stratified with trial metadata to uncover novel trends. *Results:* The estimated therapeutic window as defined by the interquartile range for each RoA is as follows: IV-hepatic indication, 6E12-2E13 gc/kg; IV-non-hepatic indication, 9.75E13-1.85E14 gc/kg ; intracoronary, 1E13 - 1.9E14 gc; intramuscular, 3.5E12 - 3.5E13 gc/kg; intra-articular, 1.1E13 - 6E13 gc/joint; subretinal, 4.6E10 - 2.25E11 gc/eye; IVT, 1.8E11 - 4.1E11 gc/eye. Exploratory analyses indicate a higher IV dose range in pediatric and infant patient populations relative to adult populations. In addition, the results show an increase, on average, in AAV doses over time for IV, intracoronary, and intra-articular RoAs while subretinal and IVT RoAs exhibit a decrease over time. We acknowledge that other factors may influence dose (e.g., full/empty ratios) but these data have either not been collected or reported. *Conclusions:* These findings support the notion that the therapeutic windows of AAV products administered via the same RoA are comparable despite different target indications, capsid serotypes, and expression constructs. In doing so, the data provide preliminary guidance to sponsors regarding safe and efficacious clinical dosing windows for AAV products by RoA. Differences in AAV doses over time and across ages may signify an evolving understanding of the risks and benefits of AAV gene therapy products, considering RoA, target population, and disease severity. This work collectively provides early proof-of-concept for estimating therapeutic windows using publicly available data - a strategy to guide clinical dose selection in AAV trials. Broadly, this effort highlights the potential of focused mining of public databases to inform trial design, with potential implications for the clinical testing of adjacent advanced therapy product classes, such as lentiviral and CAR-T cell therapy.

924. Recommendations for rhe Safe Handling of Gene Therapy within Institutions

Estela Rodriguez Garcia[1], Kevin Zinchuk[2], Jon Silverman[2], John Ryan Stackhouse[2]

[1]University of Navarra, Pamplona, Spain,[2]Brigham and Women´s Hospital, Boston, MA

With an increasing volume of viral vector-based gene therapies on the horizon, ensuring the safety and protection of healthcare workers handling these agents of primary importance. Staff who prepare and administer these agents, as well as those caring for patients should be considered in the developing safe handling policy and procedures along with institutional occupational health, quality and safety administrators. Establishing and operationalizing standardized gene therapy handling practices presents a challenge. As these agents have specific handling requirements based on safety designations not typically used in clinical care settings. However, regulations governing their handling in a consistent manner in these settings are not available for reference.

Although the last edition of the Biosafety in Microbiological and Biomedical Laboratories ("BMBL") and the National Institutes of Health Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules ("NIH Guidelines") outline best practices for handling biohazardous agents and recombinant DNA product in a laboratory research setting, laboratory guidance is not easily translatable to the handling considerations that healthcare workers take when caring for patients. Specific guidelines need to be established to assist institution based healthcare workers in assessing designated risk groups, biological safety level and other various product characteristics. In addition, standardized requirements for storage and handling of viral vector-based therapies, like those outlined in USP <800>, must be designated. Only after an objective and clearly defined process is developed and implemented will institutions be able to control and mitigate the occupational risks associated with providing gene therapy agents to an institution's patient population. In the absence of recent national or international guidance, each clinical facility or institution must develop its own standard operating procedures based on historical guidance with careful considerations of current legislation and regulations in their country, state and local municipality. Appropriate policies and procedures, guidelines and best practice recommendations should be developed and implemented in advance of these products being needed at clinical sites. Existing Biosafety guidance and oversight bodies, usually in the form of an Institutional Biosafety Committee ("IBC"), need to evaluate and adjust their committee membership to ensure that clinical staff are appropriately represented. Institutions should also consider establishing a "Clinical Biosafety Committee" to fulfill the duties of an IBC but for marketed products. With continuing advances in the field of gene therapy, the institutional oversight structure needs to evolve to address the necessary changes in order to deliver safe practice guidance for healthcare workers, researchers and patient caregivers. Once clinical environments are able to standardize guidance for working with viral vector-based gene therapy products, a comprehensive risk assessment framework is needed to ensure that each new agent is reviewed and identified such that caregivers know what safeguards to apply. These safeguards alert institutional staff to the specific requirements for each product's storage, handling, administration, and disposal. The guidelines described here will help direct healthcare institutions in the operational management of vector-based gene therapies, define risk levels, identify necessary infrastructure, and develop suitable policies and protocols, to protect workers and caregivers handling and administering these therapies.

925. Delivering on the Ethics of Gene Therapy Trials

Carolyn R. Chapman

Multi-Regional Clinical Trials Center of Brigham and Women's Hospital and Harvard, Boston, MA

The tremendous excitement about the vast potential for gene therapies, including genetically modified cell therapies, is justified. As of January 2024, the FDA has already granted marketing authorization to 17 such therapies and hundreds more are in various stages of research and development. This new class of therapeutics targets a wide variety of diseases and disorders, including those with significant unmet medical needs, creating expectations but also raising the stakes. This presentation will provide an overview of the complex and unique ethical issues arising in the specific context of gene therapy research and development. The discussion will be grounded on ethical principles that undergird all clinical research. We will discuss the ethical implications of study design choices, such as whether and which controls are included, how and what doses are chosen as starting points, which endpoints/outcomes are studied, which participants are included and excluded, what supports are provided, and how long participants are followed. We will consider how the analysis and resolution of ethical challenges depend on facts that are specific to each disease context at a given point in time. Finally, we will illustrate how different stakeholders in the clinical trial ecosystem, such as patients, caregivers and families, investigators, companies, and regulators, have both convergent and divergent interests that must be balanced to support the ethical conduct of gene therapy research. Ways to respect and engage affected communities, as well as prospective and enrolled research participants, will be discussed.

926. AAV Mediated Gene Therapy Restores Hearing in Patients with DNFB9 Deafness

Shanzhong Zhang, Jieyu Qi, Fangzhi Tan

Suzhou Otovia Therapeutics Co., Ltd., Shanghai, China

Mutations in *OTOF* lead to the autosomal recessive auditory neuropathy DFNB9. The efficacy of adeno-associated virus (AAV)-mediated *OTOF* gene replacement therapy has been extensively validated in *Otof*-deficient mice. However, the clinical safety and efficacy of AAV-*OTOF* have not been reported. Here, we generated AAV-*OTOF*, using good manufacturing practice and validated its efficacy and safety in mouse models and non-human primates in order to determine the optimal injection dose, volume, and administration route for clinical trials. Subsequently, AAV-*OTOF* was delivered into one cochlea of 5-years-old and into bilateral cochleae of 8-years-old deaf patients with *OTOF* mutations. Obvious hearing ability improvement was detected by the click and pure-tone auditory brainstem response (ABR) and the pure-tone audiometry tests (PTA) in these two patients. Hearing in the injected ear of the 5-years-old patient could be restored to the normal range at 1 month after AAV-*OTOF* injection. And the 8-years-old patient reached maybe 50%-70% of normal hearing. Most importantly, the 5-years-old patient could hear and recognize speech only through the AAV-*OTOF*-injected ear. Our study is firstly to demonstrate the safety and efficacy of AAV-*OTOF* in patients and expands and optimizes current *OTOF*-related gene therapy, which provides valuable information for further application of gene therapies for deafness.

Thursday Posters: Viral Vectors

927. Addressing the *Ex Vivo* Lentivirus Black Box Warning: Reducing Risk through Engineering

Matt P. Ercolino[1], Jade Bose[1], Jonathan Morgan[1], Shangzhen Zhou[2], Lucia Fernandez[1]

[1]Process Development and Manufacturing, VintaBio, Philadelphia, PA,[2]Research Vector Core, VintaBio, Philadelphia, PA

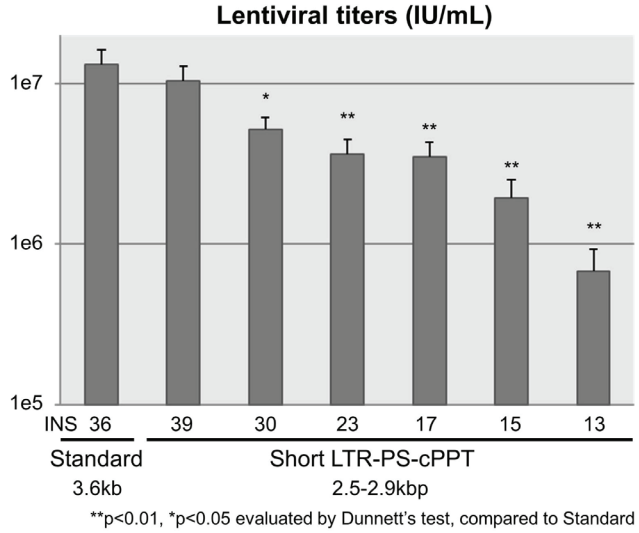
Lentivirus (LV) vectors are commonly used in cell therapy applications as a reagent used to produce the final drug product. Recently, LV has seen an uptick in use for *in vivo* transduction. LV production has been performed using transient transfection methods in HEK293T cells to produce high titer viral stocks. HEK293T cells were generated by transforming HEK293 cells with a temperature stable form of SV40 large T-antigen (Tag, a known risk factor) enabling cells to express viral proteins at high levels. Given the secondary cancer concerns with CAR-T therapies (FDA just required all 6 CAR-T therapy products to add a black box warning) the removal of Tag in the production process could help to reduce risk and safely expand LV vectors' usability for *in vivo* gene therapies. There is known productivity per cell gap between the titers achieved with HEK293T vs HEK293 cells. Through process engineering this gap can be significantly reduced if not effectively closed. This enhanced process can be achieved through improved transfection methods and effective scaling into fixed-bed bioreactors with high surface areas. LV production has broadly moved from adherent to suspension-based processes. The shift was prompted by concern over the lack of scalability of adherent processes. However, the suspension processes face challenges such as low productivity per cell, and increased pressure on downstream purification process. Our goal in this work is to compare the production of LV in proprietary HEK293 cells and an HEK293T cell line through the following aims: • Aim 1: Examine the production gap between HEK293 and HEK293T with improved transfection methods and our proprietary HEK293 cells vs prevailing methods in HEK293T. • Aim 2: Assess LV production in the HEK293 adherent system vs a commercially available HEK293T suspension system as well as production system impact on process characteristics including impurity profiles • Aim 3: Investigate the transduction efficiency of the virus produced with different modalities. In our experiments HEK293T cells indeed produce higher titer LV stocks than HEK293 cells operated under the same conditions. High titer LV production is possible with HEK293 cells when used with improved transfection methods and our proprietary transfection reagent, narrowing the production gap to less than a 1-log decrease versus HEK293T cells. HEK293 adherent cultures demonstrate similar productivity to second-generation HEK293T suspension processes while using less nutrient medium per cell. The HEK293 cell in general exhibit more consistent and predictable growth profiles in their native state of adherence. This native state results in a more reliable cell behavior allowing for more control in the industrial setting. Adherent processes offer the ability to manipulate cell attachment surfaces allows for fine tuning of the cellular process. LV produced in both adherent and suspension modalities display similar transduction efficiencies. Adherent systems also offer advantages in terms of downstream processing. Harvesting and purification of viral vectors from adherent systems are often less challenging due to the reduced presence of cellular debris and impurities indicated by the reduced presence of host cell protein (HCP) and host cell DNA (hcDNA). Harvest streams with less process impurities contribute to higher vector purity and facilitates higher recoveries across subsequent purification steps. Although HEK293T still retain higher productivity at per cell level, one can achieve commercially viable scale production using HEK293 cells; hence improve the safety profile for the final drug product with the removal of the SV40 Tag.

928. The Inclusion of HIV-1 Inhibitory Sequences Improves the Minimal Size of Lentiviral Vectors for Hemoglobinopathies

Naoya Uchida[1], Anh Le[1], Avery Bradley[1], Julia Ball[1], Selami Demirci[1], Xiong Liu[1], Matthew M. Hsieh[1], Takashi Okada[2], John F. Tisdale[1]

[1]National Institutes of Health, Bethesda, MD,[2]The University of Tokyo, Minato-city, Tokyo, Japan

Hematopoietic stem cell (HSC) gene therapy is curative for various genetic diseases, including sickle cell disease, and HIV-1-based lentiviral vectors are utilized for therapeutic β^{T87Q}-globin gene delivery to HSCs. However, the lentiviral gene-packaging capacity (~10kb) limits this application, and thus, size reduction of the lentiviral backbone remains crucial. Previous attempts for size reduction resulted in decreased titers and transduction efficiency; therefore, in this study, we sought to generate small-size lentiviral vectors while retaining their titer and transduction. We designed a range of smaller sizes of MSCV promoter-driven *EGFP*-expressing lentiviral vectors by using only the core sequence of essential fragments, including long terminal repeats (LTR), packaging signal (PS), rev response element (RRE), and central polypurine tract (cPPT). We evaluated lentiviral titers and transduction efficiency in human CD34+ cells at a multiplicity of infection 50. Switching to the core of each essential fragment gradually decreased vector sizes and lentiviral titers (short LTR-cPPT, or further deletions, $p<0.05$). Transduction efficiency in CD34+ cells was reduced by the use of core fragments as well as the splicing donor mutation (Short LTR-PS-cPPT, or further deletions, $p<0.01$), likely due to less efficient lentiviral integration. These data suggest that some key sequences for transduction are included in various locations in the lentiviral vector backbone. HIV-1 nuclear export is restricted by their inhibitory sequences (INS) and allowed by rev-RRE interaction; therefore, we hypothesized that the inclusion of HIV-1 INS is important for lentiviral integration and thereby, required for small-size lentiviral vectors with high titers and efficient transduction. We designed ~1kb-shorter LTR-PS-cPPT vectors including escalating amounts of INS motifs (13-39 INS), compared to the full-size standard vector (36 INS). As expected, lentiviral titers decreased in response to reducing INS numbers in the small-size vectors ($p<0.05$) except for that including maximal 39 INS (Figure). We then designed a small-size β^{T87Q}-globin vector using our forward-oriented construct with β-globin promoter/enhancers (Nat Commun. 2019) and the ~1kb-shorter lentiviral backbone with additional INS. When using *EGFP* instead of the β^{T87Q}-globin, the small-size vector with additional INS (50 INS) allowed for 1.7-fold higher titer and 1.2-fold more efficient transduction in CD34+ cell-derived erythroid cells ($p<0.01$), compared to a small-size vector without additional INS (32 INS), at similar levels to a full-size backbone vector (50 INS). The small-size β^{T87Q}-globin-encoding vector with additional INS (61 INS) resulted in a similar titer, 1.2-fold more efficient transduction, and 1.4-fold higher β^{T87Q}-globin production at the protein level, compared to that without additional INS (47 INS), which are comparable to a full-size backbone vector (58 INS). In summary, we demonstrated that the inclusion of HIV-1 INS motifs is important to maintain lentiviral titers when reducing the vector size. The additional INS allowed for more efficient transduction in CD34+ cells with small-size vectors. These findings would allow for further development of gene therapy applications.



929. Post-Natal Maturation of Myocardium Likely Causes a Post-Entry Block of Transduction by Lentiviral Vectors

Ruud N. Visser[1], Vincent Portero[2], Niels Harlaar[2], Jianan Wang[1], Timo Jonker[1], Max Medina-Ramirez[1], Harsha Devalla[1], Mischa Klerk[1], Arnie R. Boender[1], Klaus Neef[3], Hanno L. Tan[1], Antoine A. F. de Vries[2], Vincent M. Christoffels[1], Gerard J. J. Boink[1]

[1]Amsterdam UMC, Amsterdam, Netherlands,[2]Leiden University Medical Center, Leiden, Netherlands,[3]University Medical Center Utrecht, Utrecht, Netherlands

Introduction In cardiac gene therapy, two viral vectors are typically used for long-term transgene expression, i.e. lentivirus (LV) and adeno-associated virus (AAV) vectors. Compared to AAV vectors, LV vectors have a larger insert capacity and potentially confer permanent transgene expression integrating the vector genome in that of the host cell. However, standard VSV G protein-pseudotyped LV vectors (VSVg-LV) have shown suboptimal transduction of cardiomyocytes *in vivo*. In this study, we aimed to improve LV-mediated transduction of cardiomyocytes by evaluating LV vectors carrying various different envelope proteins and compared the most potent variants with AAV vectors. **Methods** We made a panel of 76 LV vectors pseudotyped with envelope proteins from various viruses carrying an expression cassette for enhanced green fluorescent protein (eGFP). Transduction efficiencies in neonatal rat ventricular myocytes (NRVMs), human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) and cardiomyocyte progenitor cells (CMPCs) were determined by fluorescence-activated cell sorting as fraction of eGFP-positive cells. 7-weeks old male Wistar rats were injected directly into the left ventricle with 200,000 IU of vector. One control rat followed the same procedure but instead of LV, it was injected with 5.8 x 10^{11} genomic copies (gc) of AAV9.45 capsid pseudotyped AAV2 (AAV9.45). VSVg-, Cocal virus (CoCV)- and Foamy virus (Foamy)-LV vectors were produced carrying an expression cassette to allow co-expression of ReaChR, a light-gated ion channel used in optopacing, and GFP. An AAV9.45 vector was produced with a corresponding transene cassette. LVs were functionally titrated in NRMVs. Four weeks after the surgery, optopacing responses were evaluated *in vivo* and *ex vivo*. Transduction efficiency was evaluated by immunohistochemistry on GFP. Human neonatal and adult heart slices were cultured and transduced with VSVg-LV and AAV DJ capsid-pseudotyped AAV2 (AAV-DJ) vectors as a control. Vectors carryied an expression cassette for eGFP under a cardiomyocyte promoter. Immunohistochemistry was performed to visualize GFP. **Results** Of the pseudotyped LVs, none outperformed VSVg-LV vectors, which reached transduction efficiencies *in vitro* of 58% in NRVMs to 96% in hiPSC-CMs and 100% in CMPCs. In addition to VSVg-LV, the second (CoCV-LV) and third (Foamy-LV) best performers were included for *in vivo* testing. While the heart transduced with AAV9.45 showed consistent left ventricular optical pacing, none of the three tested LV pseudotypes caused enough ReaChR expression to sustain optical pacing. These results were confirmed via immunohistochemistry, where only the AAV9.45 injected tissue was positive for GFP. To investigate the impact of tissue maturity on transduction, we proceeded testing with the transduction of human neonatal and adult cardiac slices. These experiments indicated absence of substantial transduction by VSVg-LV in human adult cardiac tissue slices, which was in contrast to the potent transduction realized by AAV-DJ in neonatal and adult tissue. **Conclusion** *In vitro* screening of a panel of pseudotyped LV vectors showed standard VSVg-LV vectors we most effective. Despite encouraging *in vitro* results and previously reported findings, LV vectors did not provide robust *in vivo* transduction. The observation that VSVg-LV was able to transduce NRVMs but not adult human cardiac tissue slices suggest the emergence of a post-entry block could be a critical limiting factor in the transduction of adult tissue, both *in vitro* as well as *in vivo*.

930. Lentiviral Gene Therapy Rescues a Novel Inducible Codanin-1 KO Mouse Model of Bone Marrow Failure

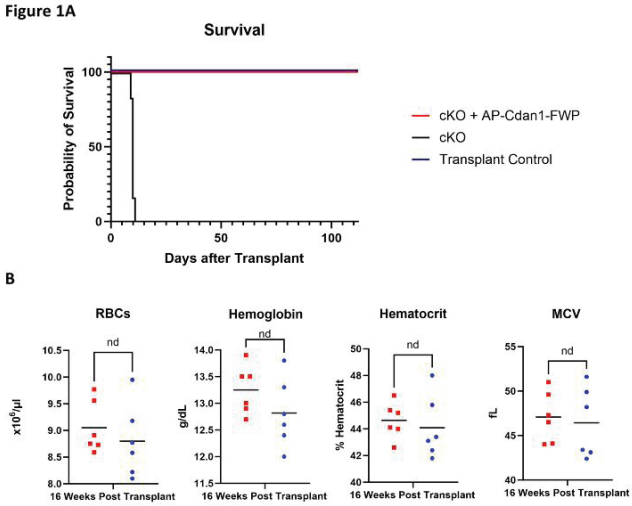
Perry Demsko[1], Laura Breda[1], Carlo Castruccio Castracani[1], Ariel Rivera[1], Tyler Papp[2], Yink Tam[3], Barbara Mui[3], Megan Fedorky[1], Hamideh Parhiz[4], Stefano Rivella[1]

[1]Children's Hospital of Philadelphia, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, Philadelphia, PA,[3]Acuitas Therapeutics, Vancouver, BC, Canada,[4]University of Pennsylvania, Philadelphia, PA

Congenital Dyserythropoietic Anemia 1 (CDA1) is an inherited blood disorder caused by biallelic mutations in Codanin-1 (Cdan1), a protein of uncertain function. CDA1 is characterized by macrocytic anemia, multinucleated erythroid precursors, and characteristic 'spongy' chromatin morphology under electron microscopy inspection. CDA1 manifests with moderate to severe anemia, skeletal deformities, and reduced stature. Complications from CDA1 are primarily derived from deficient erythropoiesis and include splenomegaly, hemochromatosis, and hyperbilirubinemia, which are further exacerbated by chronic transfusion dependence. Currently, no cure is available, and the lack of a viable CDA1 mouse model has prevented attempts to develop treatments. No homozygous-null mutant patients have been documented, and *Cdan1*-KO mice abort early in embryogenesis, generally by E7. Murine embryos with erythroid-specific KO of Cdan1 are viable until E14.5 when they succumb from pronounced anemia,

informing the need for an inducible KO system to generate an adult model of CDA1. We have developed two CDA1 models wherein a floxed Cdan1 allele is excised either by Mx-Cre after induction with a dsRNA analog (Cdan1$^{Mx\text{-}cre}$-KO) or via Cre mRNA containing lipid nanoparticles targeted to hematopoietic stem cells (Cdan1^{LNP}-KO). We found that biallelic loss of *Cdan1* is incompatible with life as both models are fatal upon induction (**Figure 1A**). The fatal phenotype can be reversed by rescuing the model by delivering the human CDAN1 gene via lentiviral gene transfer (AP-CDAN1-FWP). All surviving KO animals show normal erythropoiesis and complete blood counts (**Figure 1B**). Additional studies are underway to analyze lineage-specific hematopoiesis in the bone marrow and as colony assays. Without any known dominant negative effect for Cdan1 mutations, this directly demonstrates the applicability of a gene addition-based strategy for treating CDA1. Since hematopoietic stem cells lacking *Cdan1* are not viable, we generated mice KO with transgenic *Cdan1* using vectors expressing known hypomorphic human CDAN1 mutations. The goal is to explore the function and pathophysiology of mice expressing mutant CDAN1 alleles. In early studies, and similarly to human CDA1 patients, the mice transduced with mutated CDAN1 alleles exhibit variable decreases in hemoglobin and red blood cell count. In particular, the anemic state is more prominent up to 4 weeks following transplant or a challenge bleed, with CDAN1 mutant mice exhibiting a protracted recovery compared to WT controls. Additional experiments are underway to characterize erythropoiesis and iron metabolism in these animals.In summary, these data indicate that our novel vector has the potential to cure CDA1 and further characterize this disease.

Figure 1A



931. Envelope Protein Specific B Cell Receptors Direct Lentiviral Vector Tropism *In Vivo*

Koki Morizono[1], Kari-Ann Takano[1], Anita A. L. Wong[1], Rebecca Brown[1], Kathy Situ[1], Bernadette A. Chua[1], Angel E. Abu[1], Truc T. Pham[1], Glania C. Reyes[1], Sangeetha Ramachandran[1], Masakazu Kamata[2], Melody M. H. Li[1], Ting-Ting Wu[1], Dinesh S. Rao[1], Vaithilingaraja Arumugaswami[1], Kenneth Dorshkind[1], Steve Cole[1]

[1]UCLA, Los Angeles, CA,[2]University of Alabama at Birmingham, Hoover, AL

While studying transgene expression after systemic administration of lentiviral vectors, we found that splenic B cells are robustly transduced, regardless of the types of pseudotyped envelope proteins. However, the administration of two different pseudotypes resulted in transduction of two distinct B cell populations, suggesting that each pseudotype uses unique and specific receptors for their attachment and entry into splenic B cells. Single-cell RNA sequencing analysis of the transduced cells demonstrated that different pseudotypes transduce distinct B cell subpopulations characterized by specific B cell receptor (BCR) genotypes. Functional analysis of the BCRs of the transduced cells demonstrated that BCRs specific to the pseudotyping envelope proteins mediate viral entry, enabling the vectors to selectively transduce the B cell populations that are capable of producing antibodies specific to their envelope proteins. Lentiviral vector entry via the BCR activated the transduced B cells and induced proliferation and differentiation into mature effectors such as memory B and plasma cells. BCR-mediated viral entry into clonally specific B cell subpopulations raises new concepts for understanding the biodistribution of transgene expression after systemic administration of lentiviral vectors and offers new opportunities for BCR-targeted gene delivery by pseudotyped lentiviral vectors.

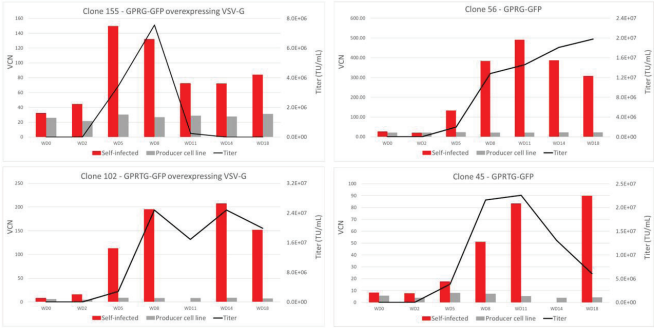
932. Challenges in Upstream Lentiviral Vector Manufacturing: Assessing VSV-G Mediated Self-Infection of Stable Suspension Producer Cells

Christin Obert[1], Parameswari Govindarajan[1], Holger Laux[1], Silke Wissing[1], Andreas Gille[2], Monica Terrao[1]

[1]CSL Behring Innovation GmbH, Marburg, Germany,[2]CSL, Pasadena, CA

Introduction Lentiviral vectors (LVVs) are a frequently used resource for gene transfer in the field of cell & gene therapy, and their high demand raises the need for highly concentrated vector material that can be produced robustly, efficiently, and on a larger scale. The manufacturing process faces different challenges in this regard, which include the possibility of the produced LVV to transduce the producer cell lines ("self-infection"). Due to the binding affinity of the lentiviral envelope protein VSV-G to the LDL receptor on the surface of the producer cells, lentiviral particles might enter the producer cells shortly after their assembly and before harvesting. A high "self-infection" of the producer cells in turn might lead to an increased cytotoxicity, cellular detachment, and a reduced virus yield, affecting the process and titer of the manufactured material. The assessment

of the degree of "self-infection" under different conditions and its putative correlation to cell toxicity and impact on the LVV yield is therefore of high interest. **Methods** To assess the correlation between the VSV-G levels in the surface of lentiviral vectors and the degree of "self-infection" during the LVV production, two suspension-adapted stable producer cell lines based on the GPRG and GPRTG packing cells were transfected to overexpress the VSV-G protein. After selection and single-cell cloning, one clone for each packing cell line together with its progenitor was used to produce lentivirus in bioreactors in a process extended up to 18 days. GPRTG clone 45 and GPRG clone 56 with normal levels of VSV-G expression, and GPRTG clone 102 and GPRG clone 155 overexpressing VSV-G. A portion of the producer cells was removed from each bioreactor and its genomic DNA was purified and digested. Primers and probes specific to regions outside and inside of the lentivirus genome were designed to differentiate the amplicons originating from the lentivirus or the producer cell lines. The amount of "self-infection" was quantified using an assay based on ddPCR during the LVV production process on days 0, 2, 5, 8, 14 and 18 post bioreactor inoculation and displayed in virus copy number (VCN)/cell. The LVV functional titer was determined by transducing HEK293T cells with a subsequent flow cytometry readout. **Results and Conclusion** A high "self-infection" level was observed in all clones with a maximum of 469 VCN/cell in GPRG clone 56 on working day 11, which poses a 20-fold increase compared to the pre-inoculation condition. The levels of "self-infection" increased in all tested clones during the vector production reaching a plateau mostly around day 8. Within these four clones, two clones showed a decreased titer at later harvest stages, which correlates with a decreased viability seen in the bioreactors at later time points. The viability might have been influenced by the high levels of "self-infection" as well as a side-effect of the bleeding process of the bioreactor. Overall, there was no distinct correlation between the functional titer and the measured self-infection for the present clones. A preliminary calculation based on the assumption of titer loss due to the high VCN/cell caused by "self-infection" suggested a total loss of roughly 95 % at the peak time point. To conclude the assessment, the analysis showed that indeed "self-infection" is a relevant topic in the production of lentiviral particles and should be further investigated with the impact on the manufacturing process in mind.



933. Enhancing CAR-Expressing Lentiviral Vector Yield Using the V-Pac System

Jiali Yang, Liping Zhang, Jia Xu, Hua Gao, Jing Bi, Houliang Wei, Guodong Jia, Weiran Shen, Xinglin Yang
OBiO Technology (Shanghai) Corp., Ltd., Shanghai, China

Lentiviral vector transduction is the preferred approach for delivering Chimeric Antigen Receptor (CAR) products. However, challenges in packaging arise due to the unique characteristics of the CAR sequence and its expression. Ubiquitous promoters such as CMV, CAG, and EF1a commonly used for CAR expression face resistance during vector packaging. To overcome this challenge, we implemented the V-Pac system for lentiviral (LV) production associated with CAR sequences. The V-Pac system integrates a concise CuO regulatory element positioned upstream of the CAR coding region and involves the expression of the CymR protein in the packaging cell line. CymR binds to CuO, thereby inhibiting CAR expression. The LV vector efficiently expresses CAR upon cell transduction in the absence of CymR. In this study, we systematically examined multiple CAR sequences, including plasmid construction, vector manufacturing, and gene expression. A comprehensive side-by-side comparison demonstrated that the V-Pac system significantly enhanced vector yield by 3~18 times, with no adverse impact on CAR expression levels. Consequently, the V-Pac system emerges as an excellent choice for efficiently packaging challenging viral CAR genes.

934. Development of the Asimov LV Edge Packaging Cell Line and Upstream Process

Omar El-Ghouch, Michelle M. Chang, Jeremy J. Gam, Paulena L. Lieske, Micheal R. Leahy, Jeffrey McMahan, Dan Weaver, Martín Cárcamo, Kevin D. Smith
Asimov, Boston, MA

Success of chimeric antigen receptor T-cell (CAR-T) therapies requires improved lentiviral vector manufacturing to meet demand and overcome the challenges of traditional production methods. Fully transient transfection systems encounter scalability and batch reproducibility limitations, and require large-scale GMP grade DNA for multiple plasmids. The Asimov LV Edge Packaging System has been engineered to overcome these challenges and achieves >1E8 TU/mL titers at harvest. Our clonal suspension HEK293T-based cell line has the necessary lentiviral packaging elements stably integrated into the genome, only requiring transfection of the transfer plasmid and induction of the packaging elements for lentiviral vector production. Further, by focusing on three distinct phases during the production run, we have generated a robust and scalable upstream process that can be transferred as part of the LV Edge packaging system. Our optimization approach encompasses three distinct phases. In the initial phase, growth characterization, the packaging cell line's growth trends and viability were assessed to establish optimal conditions. Bioreactor parameters, seed densities, and production media were evaluated and optimized, laying the foundation for the subsequent two phases. In the second phase, production characterization, the focus shifted to refining the transfer plasmid transfection process. This involved optimization of the transfection mix, timing of transfection, and incorporation of enhancers. The final phase, production optimization, focused entirely on the production phase of the process where bioreactor parameters

and process duration adjustments were explored to maximize overall productivity. Leveraging the ambr250 bioreactor system with integrated metabolite analysis and data-driven models enabled systematic, rapid data generation and iterative optimization throughout all phases of development. Put together, Asimov's LV Edge packaging platform achieves >1E8 TU/mL titers at harvest with a 4-day bioreactor process for multiple CAR transgenes. The platform exhibits high infectivity (TU/pg p24) and similar productivity levels compared to established transient systems, presenting a reliable, scalable, and robust alternative for lentiviral vector production.

935. Env-Y, a Novel Recombinant Protein to Improve Lentiviral Yield and Quality in Production and Purification

Ellen A. Otte[1], Alex Petzke[1], Yan Wei[1], Anup Gopinathan Nair[1], Ryszard Braczkowski[1], Jo Leen Lim[1], Melina Braeuer[2], Holger Laux[2], Parameswari Govindarajan[2], Tobias Brandt[2], Jessica Vogel[2], Louis Fabris[3], Andreas Gille[4]

[1]CSL, Melbourne, Australia,[2]CSL Behring Innovation GmbH, Marburg, Germany,[3]Seqirus, Holly Springs, NC,[4]CSL, Pasadena, CA

Lentiviral vectors (LVVs) play a crucial role in cell and gene therapies, exhibiting the capability to insert relatively large genes into the chromosomal DNA of various cell types with high efficiency. However, despite their numerous benefits, the clinical deployment of LVVs is hindered by substantial challenges, particularly in the upstream and downstream aspects of their production process. LVVs pseudotyped with vesicular stomatitis virus glycoprotein G (VSV-G) target the low-density lipoprotein receptor (LDLR) for cellular binding and uptake. The broad expression of this receptor significantly enhances the versatility of these vectors. However, it also poses a challenge when LVVs are expressed in cells that express LDLR, leading to (self-) infection of these cells. This situation results in diminished productivity, impaired viability of the cell culture, and consequently, lower yields of LVVs. Downstream, the fragile nature of LVVs, coupled with the stringent purification conditions, poses further challenges resulting in diminished recovery and quality. These challenges overall contribute to suboptimal and variable quality and the high cost of LVV. We have developed a recombinant envelope-binding protein to address both issues, which we have dubbed Env-Y. Env-Y is a fusion protein of Fc with the VSV-G binding domain of LDLR, which can bind and neutralize lentiviral VSV-G, thereby preventing transduction and enabling selective purification via protein A. The binding is reversible at pH <6.2, at which VSV-G changes reversibly from its pre- to a post-fusion conformation, or, by removal of calcium, allowing for gentle recovery of functional LVV particles. Here we present the molecular design and characterization of this protein. Binding of Env-Y to VSV-G was assessed by surface plasmon resonance (SPR), which determined an apparent binding affinity of $K_D \approx 1$ nM with small differences for different versions of the molecule. Dissociation at pH 6.0 was also observed by SPR, and no binding of VSV-G to the Env-Y proteins was observed in the absence of calcium ions. Binding of Env-Y to VSV-G expressing cells was assessed using an anti-Fc fluorescent antibody detected by flow cytometry. These experiments documented binding of Env-Y to VSV-G expressed on cell membranes, as well as dissociation on exposure to pH 6 and 10 mM EDTA. Importantly, subsequent re-exposure of these cells to Env-Y at pH 7.4 showed binding at >95% of the level observed prior to dissociation, demonstrating that the VSV-G returned to pre-fusion conformation. Env-Y was able to neutralize LVV in an infectivity assay, reducing transduction of HEK293T cells in a concentration-dependent manner up to 85%. Lentiviral purification of Env-Y-bound LVV using protein A capture and mildly acidic elution condition (pH6) and EDTA was shown in principle and requires further optimization. These data show promise for the concept of the application of Env-Y in in upstream and downstream LVV production process, to increase yield and quality of LVV and reducing cost of LVV manufacturing.

936. Introducing the CAZL Element: A Minimal 3' UTR Sequence That Imparts Enhanced Expression to Transgenes in Target Cells

Cristina Nogueira[1], Jordan Wright[1], Bozhena Caratti[1], Lorena Martinez Quiles[1], Ben M. Alberts[1], Alissa S. Bray[1], Kyriacos A. Mitrophanous[1], Nicholas G. Clarkson[2], Dan C. Farley[1]

[1]Innovation Group, Oxford Biomedica, Oxford, United Kingdom,[2]Process Development, Oxford Biomedica, Oxford, United Kingdom

Expression cassettes within gene therapy vectors are usually engineered to produce optimal expression in target cells. These expression cassettes have some common features including an enhancer-promoter to drive mRNA synthesis in a constitutive or tissue-specific manner, the use of 5' introns to increase mRNA abundance/stability, sequence-optimisation of the open-reading frame (ORF), and 3' UTR enhancer sequences to improve polyadenylation efficiency or polyA tail length and/or composition. There is an inverse correlation between 5'/3' UTR length and gene expression; higher expression is associated with 5' and 3' UTRs of <200 and <800 nucleotides respectively, likely due to the absence of secondary structures and microRNA target sites. For viral vectors, another pressure exists to keep these sequences short: vector genome packaging limit. For ssDNA-based AAV vectors, this limit is a 'hard' one, with packaging efficiency of intact genomes reducing substantially as the length increases above ~5kb. For lentiviral vectors, the efficiency of vector genomic RNA (vRNA) packaging (and ultimately titre) is related to vRNA steady-state abundance, which is also inversely correlated to RNA length, and tends to reduce in a semi-linear fashion over the 'softer' ~10kb limit. The decision to include *cis*-elements employed within UTRs of the transgene cassette is therefore one of 'cost-benefit', related to output titre versus transgene protein per target cell. Given the stricter packaging limit for AAV, this often means that 3' UTR enhancers are not utilised, perhaps lowering product potency. Viral post-transcriptional regulatory elements (PREs), such as from the Woodchuck Hepatitis Virus (wPRE; ~600 nucleotides), are widely used in LVs to increase expression, although this can be dependent on the promoter employed. The wPRE has been shown to promote mixed polyA tailing of mRNAs by recruitment of a TENT4-ZCCHC14 complex via a minimal stem loop. Others have identified a mammalian consensus cytoplasmic accumulation region element (CAR-E; sequence CCAGATCCTG) derived from intronless cellular transcripts that improve export of heterologous mRNAs when tandemised in the 5' UTR. Here we generate a novel composite 3' UTR enhancer element called 'CAZL', wherein a tandemised CAR-E consensus is fused to a minimal TENT4-ZCCHC14 stem loop. The

magnitude of increase in gene expression can be fine-tuned by altering the number of CAR-E consensus repeats, giving a range of sizes from just 60-to-200 nucleotides. We show that CAZLs can replace the wPRE and can be used in the context of different promoters with or without 5' introns. Being small, CAZLs are ideal tools for AAV-based products and for LVs with larger payloads.

937. Orthogonal Characterization of Lentivirus for Size, Charge, Titer, and Stability Using Multiple Malvern Panalytical Technologies (DLS, MADLS, ELS, NTA)

Jonathan Mehtala

Malvern Panalytical, Westborough, MA

Compared to smaller viral vectors, such as the Adeno Associated Virus (AAV), Lentiviral vectors are larger and more complex. Whereas AAVs are 25 nm and consist of primarily two components: a protein capsid shell and a transgene cargo, Lentivirus is 120 nm and consists of at least 7 components: an outer envelope membrane, membrane-embedded proteins, matrix proteins, protein capsid, nucleocapsid, transgene cargo, and other free proteins and enzymes. As a result of the complex Lentiviral structure, it is essential to have robust analytics that can measure Lentiviral properties that predict efficacy and stability. In this study, we characterized Lentivirus samples using four Malvern Panalytical technologies: Nanoparticle Tracking Analysis (NTA), Dynamic Light Scattering (DLS), Multi Angle Dynamic Light Scattering (MADLS), and Electrophoretic Light Scattering (ELS). High resolution Lentivirus size was performed using NTA. Size and aggregation measurements were performed using NTA, DLS, and MADLS. Charge measurements were performed using ELS. Thermal aggregation temperature was measured using DLS and NTA over the course of a thermal ramp. Lentiviral titer and titer linearity were measured using MADLS and NTA. Long term stability experiments were performed over 7 days for Lentivirus samples stored at -80, 4, and RT. Additionally, Lentivirus stability in a high salt buffer was also investigated.

938. Overview of CAR-T Cell Generation Using Optimized Plasmid Design and Lentiviral Vector Production

Enzo Bazzaro[1], Maxime Dumont[2], Mélodie Seiler[1], Léah Lecornez[1], Marine Guise[3], Lucie Klughertz[1], Claire Gueguen[1], Sylvain Julien[1]

[1]Polyplus, Illkirch, France,[2]Polyplus-Transfection, Illkirch, France,[3]Polyplus-Transfection, Illkirch Graffenstaden, France

Gene modified cell therapy such as CAR-T is one of the most promising advanced therapy medicinal products (ATMP) to fight against cancer. These therapies typically use lentiviral vectors to transfer a gene of interest and modify patient's cells. All the raw material used during the lentiviral vector manufacturing must be carefully selected based on different parameters such as quality, performance, supplier, etc. In this project, we generated CAR-T cells based different plasmid construct expressing a fusion protein CAR to track transduction efficiency. We first built different plasmid constructs using the plasmid engineering service from Polyplus, then encapsulate this gene of interest using a lentiviral vector produced with FectoVIR®-LV, transduce CD4+ CD8+ T cells from peripheral blood with this lentiviral vector, expand them for 14 days and finally test the efficiency of the transduced CAR-T cells using a killing assay.

939. Investigating the Relation Between Lentiviral Particle Functionality and the Design of UbC and EF1α Promoter Variants

Cordula Pertl[1], Bianca Volkmann[2], Viola Haeusler[3], Sebastian Fladerer[1], Jana Bauer[3], Irene Ferreira[2], Georg Huber[1], Carl Christel[4]

[1]Vector Development Department, Revvity Gene Delivery (SIRION Biotech Legacy), Munich, Germany,[2]Platform Innovation & Management Department, Revvity Gene Delivery (SIRION Biotech Legacy), Munich, Germany,[3]Quality Control Department, Revvity Gene Delivery (SIRION Biotech Legacy), Munich, Germany,[4]US Site Sales Department, Revvity Gene Delivery (SIRION Biotech Legacy), Munich, Germany

In the last decades, lentiviral vectors of the 3rd generation plasmid system demonstrated an excellent safety record and allowed to shift the attention to the detailed vector design to optimize the manufacturability of LV particles and to improve LV vector titers. With the fast evolution of the cell- therapy field, new demands emerged to intensively characterize the impact of the vector design on LV particle functionality. In this study, we investigate two constitutive promoters, elongation factor 1α (EF1α) and Ubiquitin C (UbC), that are commonly used for lentiviral gene transfer. Our analysis was driven by the hypothesis, that the promoter design alters the activation of splice sites and can lead to truncated LV genomes during production und subsequently to low functional titers. Therefore, we characterized LV particles generated from intron-containing promoters UbC and EF1α and their respective intron-free variants with two separate metrics: particle functionality, defined as total number of infectious LV mRNAs successfully expressed in the test cell by quantifying viral mRNA transcripts , and promoter activity, defined by the mRNA expression level for a specific transgene. We were able to prevent the splicing of the UbC-intron out of an intron-containing UbC promoter-driven GFP construct by a reverse cassette design. However, the particle functionality decreased by 10-fold. This can be caused by the formation of dsRNA initiating degradation by the endogenous defense mechanism against viral pathogens. Excluding the intron completely from the UbC promoter sequence rescued particle functionality and increased the mRNA expression level of GFP by 2-fold compared to the original UbC promoter design. In a second study investigating the impact of an intron-containing EF1α promoter, regulating the expression of a therapeutic Cas9 construct, the same strategy of excluding the intron lead to an 28-fold increase in particle functionality and a 1,3-fold increase in Cas9 mRNA expression level. This increase might be linked to a decrease in size in possible combination with an impact on the activity of splice sites and thus vector genome integrity. In summary our analyses support the hypothesis that, apart from the overall vector size, particle functionality is influenced by the promoter design as it is connected to the splice site landscape of a LV vector. However additional mechanisms have to be considered. As a next step, the composition and integrity of the vector genomes need further qualification, to investigate the impact of promoter-choice on the RNA sequence packed into LV particles.

Moreover, a deeper characterization of vector genomes is the basis to investigate the safety risk that arises from products of these truncated LV RNAs and their potential toxic or harmful clinical effects.

940. Lentiviral Vector Cargo Gene Silencing During Manufacturing

Sherin Parokkaran Johny[1], Maria Ababi[1], Harry Jenkins[1], Weimin Valenti[1,2], David Chang[1,2], Keith Meaney[1], Qian Liu[1], Maria Patrício[1], Matthew Tridgett[1]
[1]OXGENE, Oxford, United Kingdom,[2]WuXi Advanced Therapies, Philadelphia, PA

Lentiviral vectors (LVV) are commonly used for cargo gene delivery in cell and gene therapy. However, although constitutive cargo gene expression in the target cells is typically desired, constitutive cargo gene expression during LVV production is often problematic for two reasons: 1) Production titre can be impacted by cargo gene expression either through the additional metabolic burden placed upon the production cells or by direct inhibition of essential viral processes; 2) Membrane-displayed gene products, e.g. chimeric antigen receptors, can be presented on the LVV surface since its envelope is derived from the production cell membrane. This could have implications for downstream processing, transduction efficiency, and immunogenicity. An ideal LVV production system would silence cargo gene expression without negatively impacting titres. Crucially, to ensure optimal vector production, the packaged viral RNA must not be damaged or have its generation by transcription inhibited. However, since the viral RNA contains the entire sequence of the cargo gene mRNA, typical gene silencing approaches would disrupt vector production, thus limiting titres. To address this issue, we have engineered a distinction between the LVV viral RNA and the cargo gene mRNA, enabling selective silencing of the cargo gene while leaving titres and cargo gene expression in target cells unaffected. This system will enable the use of consistent upstream and downstream processes with different cargo genes, thus expediting, simplifying, and reducing the cost of therapeutic development. Furthermore, since gene product display on the LVV surface is reduced, the LVV safety profile will be improved.

941. Single-Chain Variable Fragment-Based Cell Surface Marker-Targeting by Sindbis-Pseudotyped Retrovectors

Makoto Sato[1], Alexander Cunha[1], Noriyuki Kasahara[2], Cecilia Roh[1], Robert G. Johnson[1]
[1]GenVivo Inc, San Marino, CA,[2]University of California, San Francisco, San Francisco, CA

Advances in genetic engineering and cGMP manufacturing have enabled high-dose intravenous delivery of murine leukemia virus (MLV)-based retrovectors for prodrug activator gene therapy and immunotherapy in cancer patients in recent clinical trials (GenVivo IND# 29718). More efficient delivery to cancer cells and tumor tissues could be achieved by altering biodistribution through physical re-targeting of intravenously administered retrovectors, but this remains a challenge. Here, we have developed a modular platform for generating retrovectors pseudotyped with engineered Sindbis Virus envelopes (SVE) for selective binding to therapeutically relevant receptors. First-generation SVE-pseudotyped retrovectors were produced by engineering the 'universal' immunoglobulin (IgG)-binding domain from Staphylococcal Protein A into the SVE gene. These vectors were tested on PiT2-KO A375 human melanoma cells, which lack the phosphate transporter (PiT2) that serves as the natural receptor for amphotropic MLV envelope. As expected, parental amphotropic MLV vectors were unable to transduce these cells. However, an optimized version of this first-generation vector conjugated with anti-HLA IgG antibody showed successful transduction of PiT2-KO A375 cells. Furthermore, in HCT-15 cells (which lack HLA expression), the anti-HLA IgG-conjugated vector showed minimal transduction, while a vector conjugated with anti-EGFR IgG showed robust transduction. Next, second-generation SVE-pseudotyped retrovectors were developed by direct incorporation of single-chain antibody (scFv) sequences into the structure of the envelope itself. This was accomplished by first restoring a furin cleavage site between the E3 and E2 domains of SVE, which enhanced the transduction efficiency of SVE-pseudotyped MLV by up to 20-fold without loss of specificity, followed by incorporation of scFvs into the modified E2 region. While there have been previous reports of similar constructs, no prior publication has studied the relationship between the targeting motifs' structures and transduction efficiencies of pseudotyped vectors. Fourteen linker variants were created of SVEs bearing an anti-CD47 scFv. These variants were comprised of three sections: two "base" linkers that link E2 and scFv domains; and, one "mid" linker between VH and VL. We selected four "base" and two "mid" linkers to construct seven such variants and tested the VL in both relative orientations. As expected, constructs with "reverse" VL were not functional; the other seven showed transduction at different levels. Based on the results, we selected AAGHVG as "base" linkers and a longer 18-amino acid "mid" linker for further SVE-scFv constructs.We then constructed and tested SVE-scFv targeting HER2, CD47, EGFR, or Nectin-4. These targeting vectors exhibited excellent cell-specificity and transduction efficiency. For instance, the Nectin-4-targeting scFv vector efficiently transduced MDA-MB-468 cells, which show high levels of Nectin-4 (95.5%), but significantly reduced transduction of PC-3 cells, which show much lower levels of the target (13.8%). The SB-scFv vector targeting EGFR transduced most cancer cell lines. Two types of HER2-targeting vectors were also developed: scFv and diabody (DB) vectors, which exhibited excellent HER2 expression-dependent transduction. Transduction efficiency of these vectors was high in HER2-overexpressing cells such as SKBR3 and SKOV3 but minimal in HER2-null cells such as MDA-MB-468.In summary, the stepwise evolution of our SVE-based targeting vectors, from Ab-conjugated SVE to scFv- or DB-engineered SVE-pseudotyped retrovectors, has culminated in the development of a reliable and modular targeting system that may contribute to improved gene therapy for cancer and other indications.

942. Scalable Manufacturing and Qualified Assays to Generate High Quality Lentivirus for Consistent Generation of CAR-T Cells

Wesley Williams, Jennifer Kandell, Kyle Sylakowski, Md Nasimuzzaman, Venkatesh Sivanadam, Henry Rupp, Sergio Salazar, Anthony Kupchek, Sergio Ita, Olivia Welch, Allison Kifer, Ilena Zavala, Uma Lakshmipathy
Thermo Fisher Scientific, San Diego, CA

As advanced therapies continue to be a promising field for therapeutics, viral vectors have proven to be a critical component, notably in the generation of lenti-modified CAR-T cells for treatment for various types of immune oncology targets. The complex biological manufacturing systems for viral vector production have the inherent challenge of establishing and maintaining product consistency throughout development while allowing for flexibility required at an early stage. Here, we utilize a scalable manufacturing process and have established robust assays to generate lentivirus at a target titer that is critical to achieve consistent CAR-T production from different donor cells. Using a suspension culture system, virus was consistently produced in 1 - 10 L scale in both shake flash and stir tank bioreactors. Additional, downstream purification was scaled based on the starting volume without compromising yield or functionality. A qualified universal infectious titer assays with ddPCR and orthogonal flow cytometry was established. These systems together were used to generate 4 batches of CD19 CAR lentivirus with titers at or above the target of 1E7 infectious units per milli liter. The resulting lentivirus was tested with 4 different donors and, split collection of one of the donors leukopaks compared as frozen versus fresh. Results indicate that inherently donor variability results in different lenti transduction efficiency ranging from 10-30%, albeit highly dependent on the quality of lentivirus. However, no significant difference was observed between transduction efficiency of T cells from fresh or frozen leukopak from a single donor with the same lentivirus lot. Our approach to implementing controlled methods for the generation and characterization of lentivirus minimizes variability introduced during viral modification of cells from different donors. This capability offers an improved understanding of variable factors that can be tightly controlled early on during the development of manufacturing process.

943. Lentiviral Vector Production in Adherent and Suspension Platforms

Olga Makovich, Kavita Sapru, Saya Vijily, Janna Q. Wisniewski, Elena Prikhodko

Upstream Process Development, Catalent Cell & Gene Therapy, Baltimore, MD

Lentiviral vector (LVV) characteristics were compared between scale down production in three types of bioreactors. iCELLis™ Nano, 2.46 m^2 fixed-bed (PALL Corp.), Scale-X Hydro™, 2.40 m^2 fixed-bed (Univercells SA), and UniVessel™ SU stirred-tank reactor (STR), 2.0 L vessel (Sartorius AG) were inoculated with HEK293 T-cells and used for LVV expression and packaging. Cells were transiently transfected with four plasmids encoding components of the LVV using PEIpro™ transfection reagent, and cell-free conditioned medium was harvested on day 3 post-transfection. Vector titers were measured by p24 ELISA. In addition, the titers of virions with capsids, virions without capsid, virion aggregates, encapsidated p24, and soluble p24 were analyzed by the Leprechaun system (Unchained Labs). Residual host cell protein concentration was evaluated by HEK293 Host Cell Protein assay (ELISA). Under the conditions tested, the highest LVV yields were obtained in the Scale-X Hydro and UniVessel SU bioreactors, both measuring ~2.1-fold greater than the iCELLis Nano yield based on p24-specific ELISA. The concentration of host cell protein was the lowest in the conditioned medium from the iCELLis Nano, less than half the levels measured in the UniVessel SU. Characterization of the LVV structure by Leprechaun in the conditioned media obtained in all bioreactors demonstrated that only a fraction of virions was "full" and contained an enveloped capsid. Data highlighted potential differences in levels of virions with capsids between each platform under the conditions tested. Soluble p24 protein concentration was lower in the samples with high titers of LVV with capsids and higher in the samples with low titers of "full" LVV. High linear liquid speed in both fixed-bed bioreactors and high impeller speed of the stirred-tank reactor may have caused altered capsid and virion assembly. Data suggests that bioreactor type, agitation, and linear speed conditions impact quality attributes during LVV production.

Thursday Posters: AAV Vectors - Virology and Vectorology

944. Influence of Species Differences of the AAVR Receptor Sequence on AAV Transduction Efficiency Among Mice, Pigs, Nonhuman Primates and Humans

Slawomir Andrzejewski, Jenifer Vasquez, Neil R. Hackett, Ronald G. Crystal

Genetic Medicine, Weill Cornell Medical College, New York, NY

Adeno-associated virus (AAV) gene therapy vectors are routinely evaluated in experimental animals to assess the level of gene expression in target organs. However, the level of gene expression differs among different species, despite use of the same AAV vector administered by the same route and same dose (adjusted by size of the experimental animal). Based on the knowledge that cell surface receptors used by AAV play a critical role in the ability of vector to genetically modify a target organ, and that AAVR, the product of the KIAA0319L gene, is the primary receptor for most AAVs, we hypothesized that the differences in transduction mediated by the same AAV vector in different experimental animals could be due, in part, to differences in the AAVR sequence among different experimental animals. Compared to the amino acid sequence of human AAVR, rhesus macaque AAVR has 98.6% identify, pigs have 90.3% and mice 84.0%. To test whether these differences affect the efficiency of transduction by AAV vectors, *in vitro* studies were carried out to assess the level of luciferase reporter gene expression resulting from AAVrh.10Luc-mediated gene transfer in HeLa AAVR knockout ($AAVR^{-/-}$) cells that had been transfected with human, rhesus macaque, pig or mouse AAVR. Transfection efficiency was based on transgene expression with human AAVR set as 100%. The same dose of AAVrh.10Luc mediated 76.4 ±5.3% with AAVR from NHP ($p<0.005$ compared to human), 30.3 ± 7.4% with AAVR from pig ($p<0.0001$ compared to human) and 45.3±2.4% with AAVR from mouse ($p<0.0001$ compared to human). This pattern mediated by AAVR species differences was dose and transgene independent. To assess whether these differences in species-specific AAVR-mediated gene transfer were unique to AAVrh.10 (clade E), the same assay was carried out with other naturally occurring AAVs including AAV9 (clade F) and AAV32.33 (unique clade, AAVR independent) and multiple novel variants of AAVrh.10 with peptide insertions in loop IV and substitutions in loop VIII including AB3 (loop IV GVSLKQG);

NY2 (loop IV DGNNTRK); 36.10 (loop IV IDGAATK; loop VIII ENHTKND) and 9D (loop VIII TTRGDRP). With AAV9, AB3, NY2 and 9D, the same species variation was observed as with wildtype rh.10 with reporter gene expression from human AAVR>rhesus>mouse>pig. In all cases pig AAVR mediated <15% expression of that derived from human AAVR. But interestingly, for 32.33 and 36.10, two AAVR independent capsids, the species origin of AAVR had no effect on AAV-mediated transduction with pig AAVR providing >90% of expression level compared to human AAVR. We conclude that, for many serotypes, cross-species comparisons are complicated by variations in the interactions between AAV with the AAVR primary receptor. These observations of AAVR species variation have implications for making decisions regarding the AAV capsid choice and using experimental animals to predict human AAV vector biodistribution.

945. Plasmid Engineering to Improve AAV Productivity and Packaging Efficiency

Chien-Ting Li[1], Brian Carpenter[2], Peng Wang[1], Lisa Prendergast[3], Dan Cohen[2], Divakar Rajeswaran[2], Paul Morin[2], Glen B. Banks[2], Bingnan Gu[1]

[1]R&D, Lonza Cell and Gene, Houston, TX,[2]BMS, Princeton, NJ,[3]Licensing, Lonza Cell and Gene, Houston, TX

The genome packaging efficiency (full to total capsid ratio, or F/T ratio) for Adeno-Associated Virus (AAV) is a critical quality attribute for the manufacturing and commercialization of AAV-based gene therapies. A higher F/T ratio has the potential for reducing dosage and eventually the cost of goods (COGS). In this study, we present design improvements in pHelper and pRepCap plasmids that can support increased AAV titers, whilst improving F/T ratio in upstream crude harvest using a triple-plasmid transfection process. Performance was measured both at Lonza and at BMS R&D labs independently. Both scenarios show significant increments in full ratio. A tentative plan to further improve productivity will also be shared. In summary, this study demonstrates that we can significantly increase the AAV productivity and packaging efficiency for various AAV serotypes and client-specific Gene of Interest (GOIs) at the upstream production stage through plasmid engineering approaches.

946. A Versatile Cell Line for Establishing Potency of Cell Type-Specific AAV Transgenes

Refugio A. Martinez, John K. Mich, Yemeserach M. Bishaw, Meagan A. Quinlan, Ed S. Lein, Boaz P. Levi

Allen Institute for Brain Science, Seattle, WA

Potency assays are essential for AAV therapeutic development allowing simple and quantitative assessments of therapeutic activity, target engagement, vector stability, and activity per batch. Ideally, potency assays are conducted in a simplified system with homogeneous culture cells as substrates. However, robust cell culture-based potency assays have been hampered by two limitations: 1) Commonly used cell lines have low AAV transduction efficiency due to their lack of appropriate surface receptors; and 2) Cell type-specific AAV transgenes are often not expressed in cell lines because their regulatory elements (enhancers, promoters, etc.) may not be active in the cell lines. A more receptive cell line to common AAVs, and one that would express any transgene regardless of enhancer or promoter, would be a major advantage to developing potency assays for future cell-type selective therapeutic AAVs. In this work, we overcome both hurdles by creating a homogeneous cell line that expresses an AAV receptor to increase transduction efficiency, as well as a transactivator to drive ectopic expression of AAV-delivered transgenes, regardless of enhancer or promoter used. We used the PinPoint-FC platform to deliver a transgene expression cassette containing a guide RNA targeting the AAV genome, a strong dSpCas9-VPR transactivator, and the AAVR cell surface receptor, to a single insulated genomic site. The result is an engineered HEK293T-derived cell line CRAAVR (CRISPRa-AAV-Receptor) that maintains expression and AAV transducibility over several passages. We show that CRAAVR cells exhibit significantly improved AAV transduction efficiency compared to the parental cell line. Moreover, high transduction rates in CRAAVR cells are achieved using orders of magnitudes fewer virus particles than in parental cells. Using enhancer-AAVs with different neural cell-type specificities targeting oligodendrocytes or astrocytes, and not predicted to be active in HEK293 cells, we demonstrate the ability of the dSpCas9-VPR transactivator to drive ectopic expression of AAV-delivered cargos. Last, we demonstrate the utility of CRAAVR cells as a tool for use in quantitative potency assays by transducing them with an AAV-delivered SLC6A11 transgene encoding the GABA transporter, GAT-3, and measuring their ability to transport GABA. We show an AAV dose-dependent relationship to GABA transport specific to the transgenic GAT-3, as GABA transport was abolished when the GAT-3-specific inhibitor SNAP5114 was applied to the cells. Together, these data highlight the superior transduction efficiency, and regulatory element-agnostic expression achieved by CRAAVR cells. Furthermore, we demonstrate the utility of CRAAVR cells as an AAV-transgene potency assay tool that could help streamline the development and validation of AAV-based therapeutics.

947. A Comprehensive Study of Truncated Inverted Terminal Repeats (ITRs) on the Productivity, Genome Packaging, and Potency of AAV Vectors

Yinxing Chen, Shiliang Hu, Lee William, Noel Walsh, Kayla Iozza, Neil Huang, Gregory Preston, Lauren Drouin, Nannan Jia, June Deng, Matthias Hebben, Jing Liao

Alexion Genomic Medicine, AstraZeneca Rare Disease, Lexington, MA

One of the challenges associated with working with Adeno-associated virus (AAV) lies in the inherent instability of its inverted terminal repeats (ITRs), which play a crucial role in AAV replication, encapsidation, and genome integration. The ITRs exhibit a high GC content and possess a palindromic structure, occasionally resulting in truncations and mutations during plasmid amplification in bacterial cells. Despite the pivotal role of ITRs, there exists a notable gap in comprehensive studies exploring how alterations in ITRs impact the essential characteristics of the final AAV vector. To address this knowledge gap, our study focused on designing ITRs with common variations, including single deletions in the B, C, or D regions at one end and dual deletions at both ends of the vector genome. These engineered ITR-carrying plasmids were employed to generate AAV vectors in HEK293 cells. Crude and purified AAV samples were collected for

the analysis of yield, capsid DNA-filled percentage, potency, and ITR integrity. The results of our investigation indicate that a single deletion had a negligible impact on AAV productivity, packaging efficiency, and potency. However, deletions at both ends, excluding the A region, exhibited notable negative effects on these characteristics. This valuable insight into the distinct roles of ITR regions—A, B, C, and D—sheds light on their significance in AAV production and DNA replication. Our work also proposes a novel strategy for quality control in both ITR-bearing plasmids and the ultimate AAV products, with the aim of ensuring the consistent production of high-quality AAV vectors.

948. A Multiplex Screening Strategy for Resolving Cell-Type Enhancer-AAV Expression Patterns in the Brain

Avery C. Hunker, John K. Mich, Naz Taskin, Amy Torkelson, Trangthanh Pham, Jeff Goldy, Christine Rimorin, Michael Tieu, Kimberly Smith, Ed S. Lein, Boaz P. Levi, Jonathan T. Ting

Allen Institute for Brain Science, Seattle, WA

Cis-acting regulatory enhancer elements are valuable tools for gaining cell-type specific genetic access. First identified as isolated peaks in chromatin accessibility datasets, putative enhancer sequences can be cloned and packaged into AAVs to drive custom gene expression in the mammalian brain. While recent advancements in peak detection have enabled large scale enhancer discovery, a significant bottleneck in enhancer-AAV tool generation is caused by the necessity for detailed expression pattern validation *in vivo*. Here we present a barcoded multiplex strategy for screening pools of enhancer-AAVs at cell-type resolution using a single cell isolation protocol optimized for the detection of AAV-derived transcripts. We executed a proof-of-concept study involving twenty enhancer-AAVs expressing in a variety of neuronal and non-neuronal cell types across the mouse brain and were able to successfully recapitulate previously reported enhancer specificities. We found this streamlined screening method rivals the granularity achieved by individual testing while only requiring a fraction of the time, mice, and resources essential for enhancer-AAV characterizations.

949. ITR Instability Challenge Solved: From Basic *E. coli* Genetics to Implications for rAAV Quantity and Quality

Marco T. Radukic[1], Dinh To Le[1,2], Timo Krassuski[1], Daniel Falk[1], Kristian M. Müller[1]

[1]Faculty of Technology, Cellular and Molecular Biotechnology, Bielefeld University, Bielefeld, Germany,[2]III. Department of Medicine, University Medical Center Hamburg- Eppendorf, Hamburg, Germany

Plasmids used for recombinant adeno-associated virus (rAAV) manufacturing typically encode truncated inverted terminal repeats (ITRs) of 130 bp or less, compared to the 143 - 145 bp wild-type ITRs. The small change may make a big difference to rAAV quantity and quality, but its effects remain understudied because of challenges in ITR sequencing and ITR genetic instability in *E. coli* that led to the utilization of truncated but stable ITRs in the first place. We developed a direct sequencing workflow to assess tens of thousands of ITRs on a single molecule level which allowed us to quantify ITR degradation in different E. coli strains and culture conditions. We found that wild-type ITRs were stabilized when consistently propagated at 42 °C compared to standard propagation at 37 °C or 30 °C. The effect was not due to typical heat stress responses, overexpression of single-strand binding protein, or induction of chaperone expression, while inhibition of the E. coli DNA gyrase exacerbated ITR degradation. We, thus, hypothesized that positive supercoiling preceding the plasmid replication fork leads to ITR extrusion from the double strand and subsequent cleavage and ITR loss by the 'structural maintenance of chromosome' proteins SbcCD. Indeed, only moderately significant levels of ITR degradation were observed in a single knockout ΔSbcC strain, JW0387-KC, cultivated at 37 °C, and no ITR degradation was detected in this strain at 42 °C, even after three days of continuous plasmid propagation. Importantly, producing rAAV in HEK293 cells with such wild-type ITR plasmids increased the genomic titer up to 7-fold, depending on the plasmid system used, while reducing reverse packaged backbone DNA. In conclusion, our work enables careful monitoring and maintaining "healthy" wild-type ITRs, which may be vital for manufacturing the highest quality rAAV products.

950. Short-Hairpin Sequences as Stoppers to Limit Replication-Competent AAV Formation and Propagation During Vector Packaging

Jisun Lee, Phillip WL Tai, Guangping Gao, Jun Xie

UMass Chan Medical School, Worcester, MA

Recombinant adeno-associated viruses (rAAVs) have emerged as one of the most advanced gene transfer systems for human gene therapy. Despite their great promise for treating a range of genetic diseases, rAAV manufacturing faces some challenges such as generating replication-competent (rc)AAV contaminants during vector packaging. It has been speculated that rcAAVs arise from non-homologous and random recombination between packaging plasmid DNAs and vector genomes during the replication and packaging of AAV vectors, producing pseudo-wild type genomes with intact *rep*/*cap* ORFs flanked by functional ITRs. However, attempts to reduce rcAAV by reducing recombination and, therefore rcAAV formation during AAV production by the 293 cell-triple transfection platform have not been effective. Thus, one potential strategy to diminish rcAAV formation and propagation is to compromise the replication of recombined rcAAV genomes. Our previous study revealed that nucleotide sequences that form short hairpins hinder normal replication of rAAV genomes by changing directionality of polymerase activity during rAAV manufacturing. We, therefore, hypothesized that flanking rep/cap ORFs with short-hairpin DNA sequences (shDNAs) as "stoppers" or inserting them into the intronic region of rep could reduce rcAAV by suppressing the rcAAV genome formation, replication, and propagation. We incorporated one shDNA with three different designs into the chimeric packaging plasmid expressing rep from AAV2 and cap from AAV1 (pRep2/Cap1): 1). flanking rep, 2). flanking cap, or 3). in the intron of rep. Furthermore, combination of two or three shDNAs at different positions was also introduced to pRep2/Cap1 plasmid. We then packaged the ssAAV vectors with these modified rep2/cap1 plasmids followed by CsCl gradient ultracentrifugation for purification and multiplex dd-PCR and silver stained SDS-Page analyses for rAAV and rcAAV genomes and rAAV virions respectively. Compared to the

original rep2/cap1 plasmid, all the rAAV1 preps produced by using modified rep2/cap1 plasmids showed 2- to 10-fold less rcAAV and no difference in VP1-3 ratio with rAAV1. Interestingly, modified rep2/cap1 plasmids harboring shDNA in the intronic region of rep were able to achieve vector yield comparable to that using original rep2/cap1 plasmid. In addition, such modifications on pRep2/Cap1 had no impact on the plasmid production. In summary, the incorporation of shDNAs in the rep/cap plasmid can reduce the frequency of rcAAV without compromising the vector yield and VP ratio. G.G. and J.X are co-corresponding authors.

951. AAV Full to Empty Ratio Study, Comparison of Analytical Techniques

Owen Leeser[1], Darren W. Begley[2], Dave Foster[1], Daniel Haq[1]

[1]Analytical Development, Resilience, Waltham, MA,[2]Resilience, Inc., Waltham, MA

After the production of Adeno Associated Virus (AAV) the final material is a mixture of full capsids (containing the gene of interest), empty capsids (without the transgene) and partial capsids (containing parts of the transgene). With the goal of limiting the number of empty and partial capsids. The effects of the empty and partial capsids are not well known and therefore can cause complications for the patients. It is important to determine the ratio of full to empty capsids but, there should be a greater emphasis on the full to total ratio as it factors in the partial capsids. Analytic Ultra Centrifugation is used as "the gold standard" to determine the ratio of full to empty capsids. Although there are other analytical methods that may be more feasible and have higher throughput. This study is a comparison between analytical techniques to determine full to empty ratio and the full to total ratio.

952. Evaluation of a Novel Strand-Specific Approach for AAV Genome Titer and Genome Integrity Quantification Using DNA Hybridization Biolayer Interferometry

David Dobnik[1], Nejc Košir[2], Rebecca Vollmeier Kovačić[2], Nejc Jakoš[2]

[1]National Institute of Biology, Ljubljana, Slovenia,[2]Niba Labs, Ljubljana, Slovenia

High specificity AAV genome titer is one of the most important assays, traditionally done using qPCR and digital PCR. Although both approaches have specific advantages and limitations, the availability of other target specific quantitative assays, which could fill some gaps, is limited. One of the promising developments in this field is a novel method using DNA hybridization biolayer interferometry (Figure 1) that shows promise of high accuracy, fast run time and high repeatability and precision. Additionally, the assay provides a strand specific quantitative information that could not be obtained by any other technique. The 30-minute assay involves single tube AAV capsid lysis plus a single hybridization step with DNA probes, followed by interferometry detection. We have evaluated the applicability of the assay for genome titer quantification of various AAV serotypes and have demonstrated appropriate repeatability, dynamic range, and robustness. Moreover, as evaluation of vector genome integrity is becoming more and more important, we have adapted the assay in a way to provide the strand specific results on quantity of full-length and fragmented genomes. To the best of our knowledge, this is the fastest approach to quantitatively evaluate vector genome integrity.

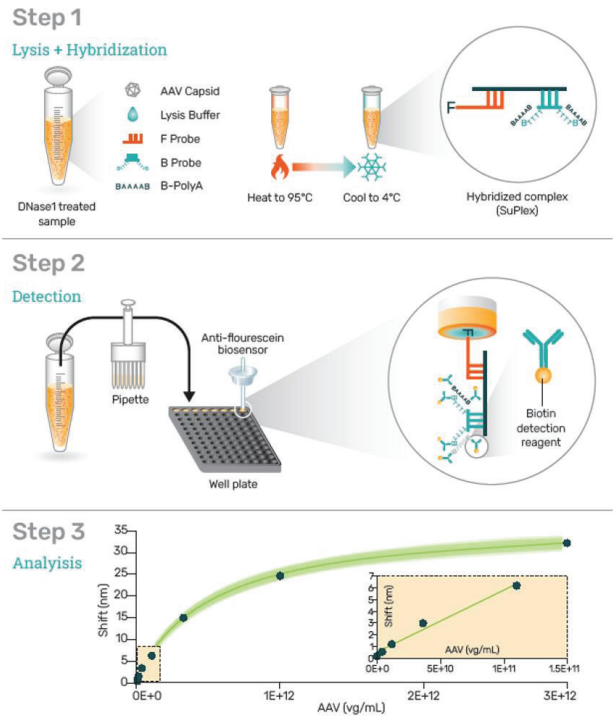


Figure 1: Schematic representation of Gator Bio BLI approach to vector genome titer quantification. First step includes single tube lysis plus hybridization of the two oligos, one labelled with fluorescein and other with biotin. In second step detection of hybridized targets is performed by Gator BLI system (in our case Gator Plus). After detection, analysis is performed in third step, where the wavelength shift can be analysed, and standard curve can later be used for unknown sample quantification.

953. Massively Parallel Screening of Barcoded AAV Capsid Variants for Neutralizing Antibody Reactivity

Kleopatra Rapti, Chiara Krämer, Ning Meng, Dirk Grimm

Department of Infectious Diseases/Virology, Section Viral Vector Technologies, Heidelberg University Hospital, Heidelberg, Germany

Recombinant adeno-associated viruses (rAAVs) stand out as highly promising vectors for delivering therapeutic genes to address genetic diseases. Their potential is evidenced by the approval of seven gene therapies over the past decade, as well as the approval of hundreds of clinical trials spanning various diseases. However, the presence of pre-existing neutralizing antibodies (NAbs), resulting from natural exposure to wild-type AAVs usually early in a patient's life, can impede the efficient transduction of target tissues by rAAVs. The conventional approach involves routine screening for NAbs, primarily in large animal models and human subjects, serving as an exclusion criterion. NAb assays fall into two primary categories: (1) cell-based assays gauging NAb inhibition of rAAV cell transduction, and (2) total anti-capsid antibody assays measuring total antibodies, irrespective of neutralizing activity. While cell-based assays, typically using luciferase as a reporter gene, are formally approved and utilized

for determining clinical enrollment cutoffs, they may not always be practical for pre-clinical studies due to time or sample constraints. To address this gap, we have devised a technology that allows swift and comprehensive screening of multiple serotypes against various samples by combining two methods: traditional cell-based NAb assays and next-generation high-throughput screening of barcoded AAV libraries. In a proof-of-concept approach, similar to cell-based assays, a library of barcoded natural AAV capsid variants carrying luciferase were mixed with samples and subsequently used to transduce cells. Transduction inhibition was evaluated at both DNA and RNA levels through next-generation sequencing (NGS) and bioinformatics analysis, outlining a serological profile for the library. In parallel, the same rAAVs were used individually to perform the classical cell-based NAb assay, enabling a head-to-head comparison of our high-throughput NAb NGS assay with the traditional NAb luciferase assay. We have expanded on this work by optimizing transduction efficiency and establishing the limit of detection. We have also performed the same assay using scAAVs and another transgene, enhanced green fluorescent protein, in order to determine the correlation of neutralization titers between the classical NAb assay and different types of libraries. In summary, our assay facilitates the simultaneous assessment of NAbs against AAVs on a large scale and offers an efficient and cost-effective alternative to conventional methods.

954. The Sixth Element: Identification and Evaluation of the Adenovirus L4 22/33K Region as a Requirement for Adeno-Associated Virus Production

Angela Adsero, Brendan Chestnut, Sara Shahnejat-Bushehri, Lalita Sasnoor, Travis McMurphy, Michael J. Swenor, Ryan Pasquino, Arun Pradhan, Victor Hernandez, Linas Padegimas, David J. Dismuke

Forge Biologics, Grove City, OH

Viral vectors based on adeno-associated virus (AAV) continue to be a leading modality in gene therapy. The most common method used for generating recombinant AAV (rAAV) vectors is by transient triple plasmid transfection of HEK293 cells. In this method the AAV replication (rep) and capsid (cap) genes are supplied via a RepCap plasmid, the ITR-flanked recombinant genome is supplied via the transgene plasmid, and the requisite Adenoviral (Ad) helper genes are supplied via an Ad helper plasmid. To improve rAAV manufacturability and safety we reasoned that excessive Adenovirus sequences could be removed or functionally replaced to make the Ad helper plasmid smaller and safer. The prevailing knowledge in the field has been that five Ad elements are necessary and sufficient to provide the Ad helper functions for rAAV production in HEK293 cells: E1a, E1b, E2a, E4ORF6, and VA RNA. Our data demonstrates that a sixth Ad element, known as the 22K protein within the L4 region is also required for rAAV production in the HEK293 transient transfection platform. Additionally, the L4 region 33K protein appears to synergistically boost titer and may possibly increase production titer in a dosage dependent manner. The L4 region, which contains the 22K and 33K coding sequences, resides in a region upstream of the E2a open reading frame (ORF). This upstream region also contains the E2a promoter. Thorough analysis of the E2a promoter region and substitution with alternative promoters showed that the upstream region is needed not only due to its E2a promoter activity, but because it also contains the L4 region expressing 33K and 22K proteins. E2a promoter activity was decoupled from the L4 region by testing multiple experimental and design changes, including providing the L4 region on a separate plasmid, reducing sequence homology to the natural E2a promoters, and relocating the L4 region within the helper plasmid to prevent *in cis* E2a regulation. We have utilized this knowledge of L4 22K/33K and the minimal promoter requirements for E2a to design smaller pEMBR™ Ad helper plasmids. These new Ad helper plasmids were tested in the production of a number of reporter and therapeutic vectors. The minimized pEMBR versions of the Ad helper plasmid led to substantial increases AAV yield, despite their smaller size. Commonly used Ad helper plasmids were developed as research tools and often contain up to 3500 bp upstream of the E2a ORF, with much of this sequence being dispensable. Our findings support that Ad helper plasmids can be optimized for high yields of rAAV production in HEK293 cells through the reduction and modification of the supplied Ad helper elements. This work also highlights the potential for improving the manufacturability of plasmids and AAV through better understanding and systematic design of the molecular elements needed for rAAV production.

955. Bridging the Gap in AAV Production Between Transient Transfection and Engineered Packaging Cell Lines Using Synthetic Biology and Advanced Analytics

Brandon Razooky[1], Michael Gapinske[1], Ed Aguilar[1], Andy Tran[1], Kailey Myers[1], Min Zong[1], Alexandra Cole[1], Kenny Shimada[1], Raquel Vega[1], Christos Galanis[1], Gilad Doron[2], Bryan St. Germain[2], Jason Carte[1], John F. Kerwin[2]

[1]National Resilience, Inc., San Diego, CA,[2]National Resilience, Inc., Waltham, MA

Adeno-associated virus (AAV) gene therapy has emerged as a groundbreaking approach for a wide range of genetic disorders. However, current manufacturing processes are costly due to the widespread use of transient transfection systems, which necessitate substantial amounts of high-quality DNA. To overcome this challenge, extensive efforts have been directed towards establishing packaging and producer AAV cell lines, eliminating the need for DNA synthesis. However, to date, many commercial and academic packaging cell lines do not produce the quality or quantity of AAV produced by transient systems, highlighting a gap in knowledge between transient settings and engineered cell lines. Here, we utilize a combination of high-throughput plasmid screening, advanced single-cell analytics, and synthetic biology to pinpoint bottlenecks in producer cell lines compared to transient transfection systems. Strikingly, we identify a novel set of previously undefined Adenoviral helper components essential for high titer AAV production. We then build on these findings to inform synthetic biology strategies to overcome challenges in establishing engineered packaging cell lines, including expressing AAV components at levels and ratios matching those observed in transient transfection settings, thus yielding producer cell lines with high titer AAV production. In summary, our research significantly advances AAV gene therapy by

identifying a pivotal helper protein, unraveling single-cell dynamics, and implementing synthetic biology principles for improved and cheaper vector manufacturing.

956. In-Trans Activation of the p40 Promoter by AAV Inverted Terminal Repeats

Anusha Sairavi[1], Pratheppa Rajagopal[1], Hiroyuki Nakai[1,2]

[1]Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR,[2]Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR

Meeting the high vector yields while keeping the desired properties of AAV vectors represents a major rate-limiting step in current AAV gene therapy approaches. Understanding AAV biology would help us better harness AAV's natural design, allowing us to create novel constructs that can enhance vector yields. Previous studies on AAV biology have shown that the AAV inverted terminal repeats (ITRs) activate the p40 promoter and indirectly drive AAV capsid protein expression through p5 promoter activation. They have also shown that the Rep binding sites (RBSs), the functional elements present in both ITRs and the p5 promoter, and their upstream placement are essential for the ITR and p5-mediated p40 activation. These prior observations have underscored the crucial roles of the in-cis placement of ITRs and p5 within the AAV viral genome as p40 activators and highlighted the importance of the positional relationship, whether positioned upstream or downstream, relative to the p40 promoter. Here, we show that AAV-ITR contained in recombinant AAV vector plasmids functions as an in-trans p40 promoter activator when AAV vectors are produced in HEK293 cells using a Rep-Cap and pHelper (i.e., an adenoviral helper) plasmids. In this study, we used a Rep2-AAV9Cap construct with the p5 promoter positioned downstream of the Cap ORF. A prominent feature of this construct is the presence of the 3' terminal potion of the p5 promoter (i.e., p5 remnant) upstream of the Rep ORF. As in-trans test constructs, we used an AAV vector plasmid containing a pair of AAV2-ITRs (ITR+ plasmid) and a respective control plasmid devoid of the ITRs (ITR- plasmid). Using these constructs and HEK293-based transient plasmid transfection, we investigated how the presence or absence of AAV-ITRs in trans might influence the level of AAV9 capsid protein expression from the Rep2-AAV9Cap plasmid, which is driven by the p40 promoter upstream of the AAV9Cap ORF. Consequently, we found that ITR+ plasmid significantly increased capsid protein expression from Rep2-AAV9Cap compared to ITR- plasmid in the presence of pHelper. Interestingly, when the p5 remnant was removed from Rep2-AAV9Cap, there was no increase in capsid protein expression with ITR+ plasmid, indicating a requirement of the p5 remnant for ITR-mediated in-trans activation of the p40 promoter. When ITRs are placed at both ends of Rep2-AAV9Cap, making ITR-Rep2-AAV9Cap-ITR plasmid providing ITRs in cis relative to the p40 promoter, capsid protein expression was comparable in the presence or absence of the p5 remnant. Comparison of capsid protein expression between ITRs in trans to that of ITRs in cis showed a significant difference, with the former yielding a higher expression. Based on these observations together with the insights obtained from prior studies, we propose a model for how ITRs activate the p40 promoter. In this model, when ITRs are provided in trans, the p5 remnant on its own activates the p40 promoter via DNA loops as previously reported, while the full-length p5 promoter is required for activation when ITRs are provided in cis. Further studies are warranted to understand the importance of the position of the p5 remnant relative to the position of the p40 promoter and how it would impact p40 promoter activation. Additionally, since the p5 remnant does not include the RBS, it would be interesting to see if the inclusion of the RBS on the p5 remnant would further enhance p40 activation. Studying the interplay of AAV viral genome elements in depth would help us better develop a system that can augment vector yields.

957. Massively Parallel In-Silico Protein Screening with AlphaFold and Molecular Docking for AAV and Therapeutic Payloads

Mark N. Miller, Andrey Smelter, Asher Baltzell, Jennifer Israel

Kriya Therapeutics Inc., Durham, NC

Identifying top candidates from a large library of protein variants is an essential task for the development of therapeutics, but the cost of synthesizing large libraries and experimentally screening their activity imposes a limit on practical library size and variant search-space. Generative deep-learning protein models and other in-silico approaches create libraries that far exceed biological library synthesis and in-vitro screening capacity, and these libraries can include variants with both potentially high activity and unusual sequence or structural features. These unusual features could bias traditional screens toward excluding variants despite the possibility of high activity. To rapidly identify promising protein variants while minimizing the cost of experimental screening, we built a computational framework to screen large protein sequence libraries in-silico based on modeled structure and binding to a ligand. Our system implements a massively parallel deep-folding (e.g. AlphaFold, ECMFold) deployment to rapidly generate protein structures from sequences, and subsequently uses this structure library as input to a parallel molecular docking system that models each variant's binding interactions with a ligand. The system's output is a per-variant score of predicted ligand-binding and protein stability, which can be easily used to identify a small number of candidates for experimental validation. Combining protein structure with predicted binding in a parallel compute environment enables rapid and practical screening of very large protein libraries as a tool to augment traditional library creation and screening approaches.

958. Stable Propagation of AAV2 Inverted Terminal Repeat (ITR) Elements in Plasmid Vectors

Vinod Jaskula-Ranga[1], Sheri Li[2], Fred Bunz[2]

[1]Hunterian Medicine, LLC, Cambridge, MA,[2]Johns Hopkins University School of Medicine, Baltimore, MD

Manufactured stocks of recombinant Adeno-Associated Virus (rAAV) typically contain a mixture of functional virions and defective particles, including empty capsids and virions that contain single-strand DNA genomes harboring deletions or contaminating sequences. The relative proportion of non-functional virions is typically significant. Moreover, the functional titer of viral preparations can vary among independently manufactured stocks. Homogeneity and consistency are particularly

important considerations when rAAV stocks are intended for clinical use. One probable source of rAAV heterogeneity is plasmid instability. Infectious rAAV particles are commonly rescued from plasmids that contain transgenes flanked by inverted terminal repeats (ITRs), which are the only genetic *cis* element required for packaging. The 145 base ITRs form stable, partial duplex hairpin structures that are critical for efficient genome encapsidation. In wild type AAVs, ITRs functionally contribute to virtually every stage of the viral life cycle. ITR instability was first observed in the original AAV2 isolates, which are ancestral to virtually all rAAV vectors currently in use. Propagation of these plasmids in standard bacterial strains results in the expansion of molecular subpopulations that contain ITR mutations, including deletions that are readily detectable by restriction digest. To minimize ITR instability, rAAV manufacturers frequently employ modified bacterial culture conditions that reduce saturation, such as growth under reduced temperature, and the use of specialized strains designed for the stable propagation of repetitive elements. We have employed advanced DNA sequencing methodology to rigorously investigate the basis for ITR instability. Unexpectedly, we observed that ITR instability in the founding AAV2 plasmids was asymmetric. While deletions could arise in either ITR, intermolecular heterogeneity was most prominent in the ITR positioned in closest proximity to the origin of replication (*ori*). By systematically positioning one or two ITRs in a variety of plasmid configurations, we were able to demonstrate that: 1) ori proximity was indeed the primary cause of ITR instability, and 2) instability was dependent on the presence of two ITRs. The latter finding suggests that recombination between ITR elements is a driver of plasmid instability rather than a consequence of instability. Most importantly, we have demonstrated that AAV2 ITR elements, when deliberately positioned in plasmid vectors, are inherently stable, even in standard bacterial strains grown under standard bacterial culture conditions. Many current rAAV vectors, which were ultimately derived from the original AAV2 isolates, harbor damaged ITRs. Among the most prevalent alterations is a recurrent 11-bp deletion. Indeed, a survey of rAAV2 plasmids submitted to Addgene revealed that a majority of vectors harbor this deletion. Our functional analysis of this mutant ITR suggests that its presence adversely affects critical aspects of vector manufacturing and function. Overall, our study highlights the importance of ITR stability for the safe application of rAAV therapeutics.

959. Increase AAV Productivity and Reduce Encapsuled Plasmid Backbone by Optimization of Plasmid DNA Usage During Upstream Process Development in AAVone Production System

Rongze Yang, Yuyan Wang, Zhen Shi, Yu Liu, Leming Wang, Cuiping Chen, Jiarui Liang, Daozhan Yu, Qizhao Wang

AAVnerGene Inc., Rockville, MD

Currently, the most used approach for manufacturing adeno-associated viral (AAV) vectors involves transiently transfecting mammalian cells with three plasmids. This method is versatile and relatively fast, but scaling up to meet the demands of clinical applications continues to be challenging. Total plasmid DNA (pDNA) amount and the ratio of the three plasmids are considered as important factors for achieving high productivity during upstream process. Although it may be different for different transgenes, a total pDNA amount of 0.75 to 1.0 μg is usually used for transfection of one million of cells. We previously reported a robust and highly efficient AAV production system, namely AAVone, in which all necessary components needed for AAV production in mammalian cells were consolidated into one plasmid with a backbone size around 13kb. Here we further optimized the plasmid dosage for transfection and found the AAVone system needs significantly lower amount of total pDNA to achieve higher productivity, which also results in significantly lower reverse packaging of plasmid backbone sequences into AAV capsids. The optimal total plasmid DNA amount needed for transfection is reduced to 0.25-0.30 μg for one million of HEK293T and 0.3-0.5 μg for HEK293 cells, about one half to one fourth of the amount used for triple-plasmid transfection system. The AAV productivity is increased up to 5-fold across different AAV serotypes compared to triple-plasmid transfection system for the same transgene. The reduced input plasmid in the AAVone system has also increased full-to-empty capsid ratio by ~10% (AAV2, from 30.02% to 38.48%) in the crude cell lysate, which is desirable for obtaining higher-quality AAV vectors with a higher proportion of functional, transgene-carrying capsids. Moreover, reduced input plasmid amount for transfection also reduces reverse packaged plasmid backbone sequences. In summary, reducing the input plasmid not only increases packaging efficiency, but also leads to increased full-to-empty ratios and reduced plasmid related impurities. AAVone represents a simple, cost-effective, and highly consistent AAV production system, particularly suitable for GMP-grade AAV production.

960. GMP (Good Manufacturing Practice) Testing Potential for Replication Competent Adeno-Associated Virus across Multiple Serotypes

Kaitlyn J. Knapp, Alexander Chiappini, Stacie Fichthorn, Marian L. McKee

Eurofins BioPharma Product Testing, Lancaster, PA

Replication competent virus testing is required by regulations for viral vector manufacturing to ensure the safety in resulting therapies. The regulations require that replication competent (rc) virus is monitored in virus seeds, unprocessed bulk (harvest), and drug substance (purified bulk). Adeno-associated virus (AAV) is a commonly utilized vector in many gene therapies. AAV vectors are designed to infect human cells and deliver the therapeutic genome in a specific area for the target condition without viral replication. Serotypes are key to the tissue selectivity. Vectors are modified to prevent replication, therapies must be monitored for the presence of replication-competent adeno-associated viruses (rcAAV), which could result in unintended side effects. Safety testing can be conducted to establish the presence of rcAAV in an intended sample, but many current methods are specified to AAV serotype 2 (AAV2) vectors. Serotype specificity is a challenge for methods which include a cell-based assay to amplify any replication competent virus present. We sought to explore methods for other AAV serotypes in order to offer an rcAAV method across multiple serotypes. For successful platform method development, understanding the infectivity of individual serotypes with the target cells used in the replication competent methods is required. To expand the scope of the

established rcAAV assays, AAV serotype 5 (AAV5), serotype 8 (AAV8), and serotype 9 (AAV9) were tested on cells from various tissue types. The standard for GMP testing with AAV2 is performed on HEK293 cells. Other cell lines were tested to expand testing alternatives. AAV serotypes expressing GFP (Green Florescent Protein) were used to infect HT1080, HeLa, and HEK293 cells. HT1080 cells acted as a control due to prior success across AAV5, AAV8, and AAV9. A range of Multiplicity of Infections (MOIs) for each serotype was tested to identify their individual infection sensitivity. Our results indicate that serotypes beyond AAV2 can infect cells that are suitable for rcAAV testing. These findings will expand rcAAV assay capabilities and the diversity of AAV serotypes that can be tested.

961. Scalable Cell-Based Assay of Tissue-Specific Regulatory Elements in Primary Human Tissues

Chris Stach, Ian Hill, Arturo Casini, Katelin Pratt, Jackie Thompson, Ben Gordon, Lila Wroblewska, Raja Srinivas, Alec Nielsen

Asimov, Boston, MA

The promise of adeno-associated viral (AAV) vector gene therapies relies on minimizing off-target effects of therapeutic payloads. One emerging approach is to steer gene expression toward specific tissues and cell types, using strategies such as engineering AAV capsids for targeted delivery, tissue-specific codon optimization of the payload, degradation of transcripts in off-target tissues with miRNAs, or by employing regulatory elements for tissue-specific expression. Previously, we reported a computational method to design tissue-specific promoters by applying artificial intelligence to multi-omics data, and validated its predictions using a reporter assay in mice, demonstrating greater than 100-fold dynamic range in expression between heart and liver tissues. The success of this design approach enables the design of thousands of candidate promoters, but screening at this scale is impractical in animal models. To address these challenges, we have developed a scalable cell-based assay that evaluates the specificity and activity of candidate regulatory sequences by testing their ability to drive a luciferase reporter across a panel of primary cells derived from human tissues. We demonstrate that AAV packaging, transduction, and luminescence assays can be performed in plate-based formats amenable to increased throughput. More importantly, by measuring tissue-specific promoters that we have previously characterized in mice, we show high correlation between the cell-based assay and the animal model. We further demonstrate that the assay can be extended to tissue-specific detargeting of payloads that incorporate sequences matching endogenous miRNAs. Together, these results indicate that our approach is well suited to accelerate the discovery of novel tissue-specific regulatory sequence designs by enabling screening of candidate sequences in large numbers in vitro before characterization in animal models.

962. Adapting a Replication Competent Adeno Associated Virus Assay for Commercial Manufacturing

Oxana M. Tsygankova[1], Leann Walsh[1], Brian Tomkowicz[2], Lana Sweet[1]

[1]Virology, Testing and Analytical Services, SK pharmteco - US, King of Prussia, PA,[2]Viral Vector Research & Development, SK pharmteco - US, King of Prussia, PA

Recombinant Adeno-Associated viruses (rAAVs)-based gene therapies are widely used for clinical purposes due to their unique characteristics. Unlike lentiviruses, they do not integrate into the host genome. Most manufacturing platforms for rAAVs includes a transient, triple-transfection of plasmid DNA into cells, mainly HEK-293, to produce viruses capable of infecting, but not self-propagating, in humans. However, the ability of otherwise replication incompetent viruses to acquire replication competence AAV (rcAAVs) has been observed. The assumption is that a recombination event occurs which swaps the *rep* and *cap* genes on one plasmid between the ITRs on the cis-plasmid. Subsequent packaging of these newly formed ITR-RepCap-ITR sequence will support replication of AAV *in vivo*. A universally applicable assay to monitor the emergence and estimate the frequency of recombination events during commercial manufacturing is required. Herein, we report on the development and application of a cell-based qPCR assay to monitor rcAAVs. Cell-based assays with a PCR endpoint are efficient assays and routinely used for rcAAV detection. Because all recombinant AAVs are use the AAV2-derived *rep* gene, our assay is built around Rep-targeting primer/probe sets to analyze test articles derived from transduced cells. To validate the assay, we use *cap* gene-specific qPCR. When applying this method for rcAAV detection on cells transduced with AAV1 or AAV2 serotypes we are able to detect rcAAV events at a limit of detection (LOD) of 10 Infectious Units. The incorporation of this assay to monitor the emergence of rcAAV for product release is necessary to ensure patient safety and provides a rapid assay for timely execution during manufacturing.

963. Seroprevalence of Liver Tropic AAV Capsids and the Evaluation of FcRN Inhibitors to Reduce Circulating AAV Antibodies

Yash Shah[1], Layth Awartani[1], Rohini Sheth[1], Charles Krause[1], Audry Fernandez[1], Angela Mote[1], Anna Tretiakova[1], Sherif Gabriel[1], Shari N. Gordon[1], Xiao Xiao[2], R. Jude Samulski[1,3]

[1]Asklepios BioPharmaceutical, Inc., Durham, NC,[2]Belief Biomed, Inc., Shanghai, China,[3]University of North Carolina at Chapel Hill, Chapel Hill, NC

Pre-existing Adeno-associated virus (AAV) neutralizing antibodies prevent efficient AAV transduction and reduce therapeutic efficacy. The prevalence of serotype specific AAV neutralizing antibodies (NAb) varies due to testing methodologies, geographic location, and population of interest. Recently, an AAV8/ AAV6 chimeric capsid, referred herein as AAV8/6 chimera, has garnered interest due to its transduction efficiency, liver tropism and safety profile in the context of hemophilia B (Xue et al. 2022). We directly compared the seroprevalence of AAV8/6 chimera to its parental wild type vectors AAV8 and AAV6 and evaluated the ability of a neonatal receptor (FcRn) inhibitor to

remove infused human antibodies and facilitate AAV transduction in a humanized mouse model. A library of 100 human serum samples were sourced from various regions within the United States from individuals ages 18-71 of mixed gender and ethnicity. All 100 serum samples were first screened in a NAb assay at a dilution of 1:5 and 1:20. Any serum samples with more than 50% inhibition at a dilution of 1:5 was defined as NAb positive, and all positive samples were titered. We observed a similar seroprevalence for AAV8 (48%), AAV6 (43%), and AAV8/6 chimera (43%) however, there were significant regional differences in seroprevalence ranging from as low as 15% in the Northeastern regions to 58% in Southern states. These data suggest that AAV 8/6 chimera shares similar antibody recognition regions as parental capsids and confirms the need for strategies to remove pre-existing antibodies and increase patient eligibility in systemically administered gene therapy studies. Recently FcRn inhibitors have been gaining interest in the AAV field as they accelerate antibody catabolism reducing circulating immunoglobulin G (IgG) levels. The FcRn inhibitor is a monoclonal antibody that inhibits IgG binding to FcRn. We infused varying levels of human intravenous immune globulin (IVIG) into humanized FcRn transgenic mice to model high vs low titer pre-existing AAV NAbs. The ability of FcRn inhibitor to reduce circulating AAV antibodies and facilitate transduction of liver tropic capsids was evaluated. Our findings confirm and extend the potential use of FcRn inhibitors as a feasible strategy to reduce pre-existing low level antibodies and expand the pool of patients eligible for gene therapy, given the wide variation in AAV seroprevalence in the general population.

964. Suppressing Prior AAV-Mediated Gene Expression Using AAV Delivered Artificial microRNAs

Miguel de Mulder Rougvie, Mara R. Kebret, Youjun Wu, Katie M. Stiles, Ronald G. Crystal

Genetic Medicine, Weill Cornell Medical College, New York, NY

When administered to a non-proliferating cell population, adeno-associated virus (AAV) gene transfer vectors mediate persistent expression. While persistent expression following a single administration is ideal for treating chronic disorders, current technology does not provide a means of turning off expression should there be adverse effects from the transgene product. To design a permanent "off switch", we hypothesized that incorporation of unique artificial microRNA (amiRNA) target sequences into the AAV expression cassette would, if necessary, enable suppression of transgene expression with subsequent delivery of a different serotype AAV vector coding for the amiRNA cognate to the target sequence in the previously administered expression cassette. To test this hypothesis, we introduced 22 nt unique sequences not found in the human or mouse transcriptome into the 3' untranslated region (UTR) of the transgene of a therapeutic AAV expression cassette. Two tandem copies of the amiRNAs targeting the unique sequences were inserted into the 3'UTR of the mCherry reporter gene in the "off" AAV expression cassette. In vitro assessment of six amiRNA/target pairs using a dual luciferase reporter assay identified 4 amiRNAs that efficiently suppressed transgene luciferase expression. Cells co-transfected with target site 3 (TS3) and amiRNA-T3B showed the highest reduction (80%, $p<0.0001$) in luciferase activity and was selected for further studies. The "off-switch" system was evaluated in vitro with an AAV5-based therapeutic vector coding for anti-immunoglobulin E (IgE) (AAV5-TBG-anti-IgE-TS3, human thyroxine binding globulin promoter driving a recombinant DNA-derived humanized IgG1κ monoclonal antibody that binds to human IgE, designed to provide long-term expression of anti-IgE to protect against allergen-induced reactions such as peanut allergy). This vector includes a microRNA target site (TS3) for amiRNA for silencing. Co-transfection of HEK293T cells with both anti-IgE with the target site and targeting amiRNA-T3B pAAV expression plasmids significantly reduced expression anti-IgE mRNA and protein compared with control amiRNA ($p<0.0001$). Assessment off the "off switch" in vivo in Balb/c mice (n=5) was carried out by intravenous administration of the therapeutic vector coding for IgE (AAV5-anti-IgE-TS3; dose $3.2x10^{10}$ gc) followed 4 weeks later by the AAVrh.10-based miRNA "off" vector (AAVrh.10-TBG-amiRNA-T3B, dose $1x10^{11}$ gc). Control mice administered only the therapeutic vector (AAV5-TBG-anti-IgE-TS3) expressed human anti-IgE in the serum (anti-IgE serum levels 18.4±13.8μg/ml at 10 weeks). Mice administered the "off" vector (AAVrh.10-TBG-amiRNA-T3B) 4 weeks later had marked suppression at 10 weeks of the therapeutic anti-IgE (serum anti-IgE levels 0.3±0.15μg/ml, $p<0.0001$). In summary, this data provides a proof-of-concept for the use of AAV vector expressing amiRNAs to switch off transgene expression, a strategy that may improve the safety of AAV-mediated gene therapies.

965. The Extracellular Matrix as a Barrier to Adeno-Associated Viral Gene Delivery

Yahya Cheema[1], Gregg Anthony Duncan[1], Devorah Cahn[1], Matthew Wolf[2]

[1]Fischell Department of Bioengineering, University of Maryland, College Park, MD,[2]National Cancer Institute, Frederick, MD

Introduction: Despite its wide use, AAV delivery efficiency and biodistribution can be greatly improved. The extracellular matrix (ECM) is a major component of the tissue microenvironment which poses a significant barrier to AAV distribution, preventing delivery of genetic cargo. In disease states, the ECM often changes in both composition and quantity, which can hinder AAV gene delivery. This proposed research aims to identify strategies to increase the efficiency of AAV gene therapy by furthering our understanding of its interactions with the ECM microenvironment. We hypothesize that both the AAV serotype and ECM composition will impact AAV transport and gene delivery. Materials and Methods: AAV2, AAV6, and AAV8 viral vectors were fluorescently labeled and filtered to remove free dye using Sephadex columns. We formulated decellularized ECM (dECM) hydrogels by digesting lyophilized and cryomilled ECM and combining the digest with ECM components. Lung, liver, and small intestinal submucosal (SIS) dECM hydrogels were constructed with the gelation time and ratio of core ECM components varying depending on source type. We then characterized AAV serotype diffusion via insertion of the labeled viral vectors of each serotype into wells containing decellularized ECM and conducted multiple particle tracking (MPT) via fluorescence video microscopy. Mean squared displacement (MSD) as a function of time was calculated and was used to compute and characterize the overall movement of the AAV viral particles through the ECM hydrogels. The effect of both AAV serotype and ECM type were assessed. Additionally, we evaluated the effect of AAV serotype on cellular uptake in 2D and 3D in vitro models via GFP gene expression.

Results and Discussion: We characterized the diffusion of the serotypes when loaded into liver, lung, and SIS dECM hydrogels via MPT, and found that the serotypes displayed lower MSD values in each hydrogel when compared to a similarly sized 20 nanometer poly-ethylene glycol coated nanoparticle control (Fig. 1, Fig. 2). The lower MSD values seen in the serotypes when compared to the similarly sized 20 nm control indicate a greater level of hindrance of AAV diffusion due to surface interactions. While there was no clear tropism preference for AAV2, AAV6, and AAV8 that could be quantified or observed, there were trends observed in AAV serotype behavior. (Fig. 2) We found that AAV2 diffusion was least affected by the presence of ECM regardless of tissue type, and that AAV8 diffusion was most affected by the presence of ECM. Conclusion: This work demonstrates that dECM hydrogels can accurately model the increased surface interactions in the ECM microenvironment that hinder or slow the AAV capsids used in gene therapy delivery. These results suggest a decrease in biodistribution of AAV2, AAV6, and AAV8 in these microenvironments. Current studies are examining this effect in 3D dECM-incorporated in vitro models.

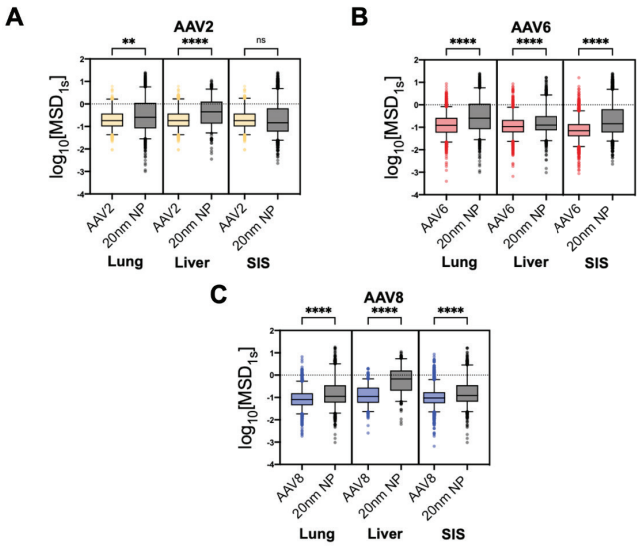


Figure 1. Diffusion of AAV Viral Vectors through a Decellularized ECM Model.
Box and whisker plots of log-based 1- values of the mean squared displacement values obtained via MPT at t=1s of AAV Viral Vectors in Lung, Liver and Small Intestinal Submucosal (SIS) dECM hydrogels. **A)** AAV2 viral vectors (yellow) MSD values at t=1s compared to the PEGylated 20 nanometer nanoparticle control. **B)** AAV6 viral vectors (red) MSD values at t=1s compared to the PEGylated 20 nanometer nanoparticle control. **C)** AAV8 viral vectors (blue) MSD values at t=1s compared to the PEGylated 20 nanometer nanoparticle control. Horizontal bars represent median values. Data sets statistically analyzed with One-Way ANOVA Kruskal-Wallis test. **p<.01, ****p<.0001.

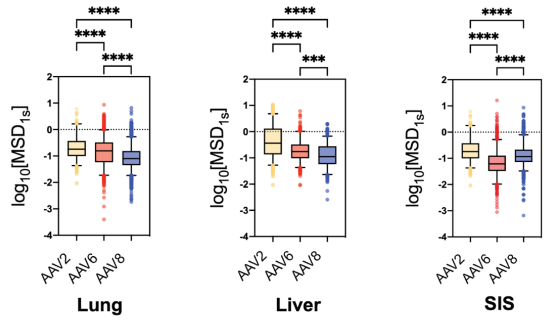


Figure 2. Differences in Diffusion Profiles of AAV2, AAV6, and AAV8 viral vector serotypes in a decellularized ECM hydrogel model from varying tissue sources.
Box and whisker plots of log-based 10 values of mean squared displacement values obtained via MPT at t-1s of AAV2 (yellow), AAV6 (red), and AAV8 (blue) viral vector serotypes in decellularized ECM hydrogels derived from lung, liver, and small intestinal submucosal tissue. Data sets statistically analyzed with One-Way ANOVA Kruskal-Wallis test *** p<.001, **** p<.0001.

966. Develop Next Generation All-in-One AAV Vectors for CRISPR/Cas Gene Editing with Paired Guide RNAs

Wenwen Huo[1], Nadia Amrani[1], Qin Xiang[1], Panos Argiras[1], Martina Donadoni[2], Anna Bellizzi[2], Ilker K. Sariyer[2], Franz Gerner[1], Jennifer Gordon[1], Guoxiang Ruan[1]

[1]Excision BioTherapeutics, Inc., Watertown, MA,[2]Temple University School of Medicine, Philadelphia, PA

Adeno-associated virus (AAV) vectors have the potential to deliver the CRISPR-Cas system as a therapeutic drug. However, the size of AAV cargo is typically limited to less than 4.7 kb, which makes it challenging to deliver CRISPR/Cas gene editing machinery, especially with more than one guide RNA expression cassette. Administering multiple AAV vectors at once can reduce dose and effectiveness, while increasing safety risks. This study describes developing an improved all-in-one AAV-Cas vector configuration with high gene editing activity and AAV productivity. We designed 14 dual-guide, all-in-one AAV-SaCas9 configurations by modifying the composite, location, and orientation of the SaCas9 expression cassette and the expression cassettes of the two guide RNAs that target the viral long terminal repeats (LTR) and the Gag gene of HIV-1. We introduced a human beta-actin gene-derived stuffer sequence in the backbone of the AAV plasmids to minimize AAV backbone packaging. We prepared AAV crude lysates from the 14 all-in-one AAV-SaCas9 plasmids to transduce an HIV-1 knock-in reporter cell line and examined the HIV-1 excision efficiency induced by the paired HIV-1 guide RNAs. The seven most efficient AAV-SaCas9 plasmids were further co-transfected in Hela cells along with the HIV-1 plasmid pNL4-3. Two AAV-SaCas9 configurations showed the highest SaCas9 and guide RNA expression levels and the highest HIV-1 excision efficiency. Next, we conducted a manufacturability test using 2L bioreactors for one top AAV9-SaCas9 configuration (with an AAV vector size of 4.7 kb) and two top AAV9-PlmCasX configurations (both with an AAV vector size of 4.5 bp). Compared to our first-generation AAV construct, the three new AAV9 vectors showed a 3-10 fold increase in AAV yield and infectious titer. The AAV9 vectors also displayed high purity and high vector genome integrity. To profile the entire AAV vector genome as an intact molecule, we used

PacBio SMRT sequencing. For all three AAV9 vectors, over 90% of the PacBio sequencing reads were mapped to the vector genomes, and 70-80% of the sequencing reads contained the Rep binding element (RBE) sequence for both ITRs. In addition, the AAV plasmid backbone packaging for all three AAV9 vectors was below 1%. Overall, our results demonstrate that these newly engineered AAV configurations can effectively produce AAV vectors with high yield, purity, and AAV genome integrity. This optimization of the dual-guide, all-in-one AAV vector significantly enhances the potential of AAV vector as a delivery platform for CRISPR/Cas gene editing therapeutics.

Thursday Posters: AAV Vectors - Capsid Engineering

967. A Novel Platform of Chemically Modified AAV Vectors with Enhanced Functionality and Tropism

Paul Heppenstall[1], Daniela Hainberger[1], Elena Groppa[1], Irene Schiavo[1], Denise Ferrarini[1], Alessandro Barenghi[1], Sara Trifari[2], Niccolò Bacchi[2]

[1]SISSA, Trieste, Italy,[2]Borea Therapeutics, Milan, Italy

Enhancing AAV functionality through capsid engineering has become an area of enormous growth for gene therapy. The most common method to achieve this is to use genetic engineering, whereby sequences encoding short peptides are inserted into exposed loops of the capsid proteins. While many new AAV genetic variants have been described with potential for clinical applications, these approaches also have a number of shortcomings. First, genetic modification often reduces yield and potency of AAV vectors. Second, since only small peptides can be accommodated into the capsid structure, modified variants may have only weak or promiscuous binding to their cellular targets. Third, the cellular receptor(s) of genetically modified AAVs are generally not known a priori, making translatability across species difficult. Here we describe an alternative approach to capsid engineering based upon modification of the AAV capsid through biorthogonal chemistry. By implementing a post-manufacturing functionalization step, we can make virtually any AAV vector amenable to accept a ligand that will re-direct, modify or enhance its tropism. We show that our approach can accommodate multiple classes of ligands, from active peptides to full-length protein domains, allowing pre-determined human validated targets to be used as binding receptors, and resulting in enhanced AAV vector potency with no impact on yield. To illustrate the flexibility of our approach, we describe how, depending on the ligand we choose, we can add new functionalities to AAV, such as improving transduction across a variety of cell types, achieving cell-specific targeting, or increasing transport across the CNS. Furthermore, we provide functional evidence of the in vivo efficacy of our targeted vectors using an experimental pain model. Collectively, these data demonstrate that chemical modification of the AAV capsid can produce vectors with novel properties, circumventing limitations associated with genetic engineering. By applying optimized chemistry to clinically relevant AAV capsids, performance and specificity of AAV mediated gene therapy can be enhanced across highly coveted target tissues.

968. "Sortagged" AAV: A Novel Chemo-Enzymatic Approach for Site-Specific Ligand Conjugation on AAV Vectors

Irene Schiavo, Elena Groppa, Daniela Hainberger, Fernanda De Castro Reis, Paul Heppenstall

SISSA, Trieste, Italy

Adeno-associated virus (AAV) is a versatile viral vector that can be employed for gene delivery for the treatment of a variety of human diseases. AAV vectors have been extensively engineered at both the level of the transgene cassette, and at the capsid structural level to improve the efficiency of gene delivery. However, depending on the capsid engineering technique used, there are still disadvantages concerning, for example, the ability of the modified vector to assemble correctly, or the fact that even when variants with a more desirable tropism are identified in vivo, the target receptor is usually unknown, which carries the risk of poor translatability and off-target effects.In this study, a chemogenetic protein ligation technology, based on sortase-mediated ligation, was developed in order to couple AAV vectors with a ligand of interest. Sortase-mediated ligation relies on the enzymatic activity of the bacterial enzyme sortase (SrtA) that cleaves a specific amino acid motif (LPXTG) in a target protein, and then ligates a nucleophile to it. AAV variants were obtained by introducing the sortase motif in two surface-accessible variable regions of the AAV2 capsid. SrtA was then used to functionalize the "sortagged" AAV with a crosslinker-reactive moiety. This exposed moiety undergoes a click-chemistry reaction with an azide-modified protein of interest, thus covalently ligating such protein to the AAV surface. As result, the AAV is decorated with the ligand of interest specifically at SrtA-recognised sites. Different protein ligands can be chosen to either boost transduction efficiency or to redirect the viral vector to cells expressing the cognate receptor. Validation experiments have shown that chemogenetically-modified AAV2 vectors acquire new functional properties conferred by the conjugated ligand. This strategy is an innovative technique to engineer AAV capsid combining the accuracy of an enzymatic approach with the modularity of a click-chemistry reaction. Our approach allows to conjugate virtually any ligand of choice to an AAV vector, and therefore open the possibility to develop new therapies for currently untreatable disease.

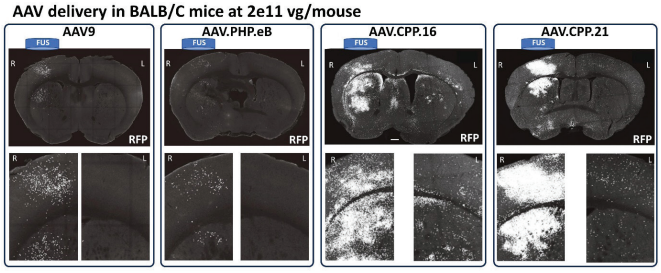
969. Engineered AAV Capsids Combined with Focused Ultrasound Blood Brain Barrier Opening for Improved CNS Transduction Efficiency

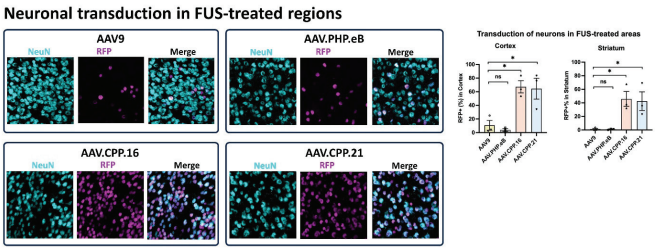
Paige Nowlin[1], Xinyu Nie[1], Zhenghui Li[1], Yongzhi Zhang[1], Lilyan Garrett[2], Bernie Simone Owusu-Yaw[1], Fengfeng Bei[1], Nick Todd[1]

[1]Brigham and Women's Hospital, Boston, MA,[2]Northeastern University, Boston, MA

Background Genetic engineering of adeno-associated viruses (AAVs) has led to impressive improvements in central nervous system (CNS) transduction efficiency. However, current engineered AAV vectors given systemically still require a large systemic dose to achieve high neuronal transduction, which can lead to peripheral organ toxicity and carries substantial production costs. For systemically administered

AAV vectors to achieve clinical utility and commercial viability, strong BBB penetrance and high neuronal transduction must be achieved at a substantially lower systemic dose. The technology of focused ultrasound-mediated disruption of the BBB (FUS-BBB opening) can play a complementary role in further enhancing the CNS delivery of engineered AAVs. FUS-BBB opening temporarily increases the permeability of the BBB through mechanical forces on vessel walls created by the interaction of the ultrasound pressure wave and systemically circulating microbubbles. This physical approach offers an orthogonal set of BBB crossing mechanisms, that can be optimized in combination with the BBB crossing capabilities of genetically engineered AAV capsids. Here we demonstrate CNS transduction gains by combining FUS-BBB opening with two engineered AAV capsids, AAV.CPP.16 and AAV.CPP.21. **Methods** Experimental conditions in BALB/C mice were designed in a 4 x 2 fashion: AAV9 vs AAV.PHP.eB vs AAV.CPP.16 vs AAV.CPP.21; No FUS vs FUS (N=3 mice per condition). FUS-BBB opening was targeted to the right cortex and striatum, leaving the left cortex and striatum as the No FUS condition. Microbubbles were given by tail vein injection (Definity, 20 µL/kg) and FUS sonications were applied at 0.34 MPa intensity, 10 ms bursts and 1 Hz repetition frequency over 120 seconds. AAVs packaging nuclear red fluorescent protein (RFP) were given by tail vein injection immediately after FUS treatment at a dose of 2.0e11 vg/mouse (~8.0e9 vg/g). Mice were sacrificed 3 weeks after treatment for immunohistochemistry to quantify neuronal transduction. **Results** Figure 1 shows representative images of RFP signal for each AAV capsid. Greater RFP signal is seen in the FUS-treated right hemisphere for all capsids. Figure 2 shows representative NeuN, RFP and merged images from regions of interest within the FUS-treated right hemisphere and results quantifying the percent of neurons in cortex and striatum transduced for each AAV capsid. One-way ANOVA indicates a significant main effect over the AAV capsid type ($F = 12.47$ and $p = 0.0022$ for cortex; $F = 7.99$, $p = 0.0086$ for striatum), with post-hoc comparison showing significantly greater neuronal transduction for AAV.CPP.16 and AAV.CPP.21 (* $p < 0.05$) but not for AAV.PHP.eB. FUS-BBB opening improved delivery to the mouse brain for all AAV capsids tested. However, particularly strong delivery was seen for the novel capsids AAV.CPP.16 and AAV.CPP.21. Our next steps will be to characterize transduction efficiency as a function of AAV dose and demonstrate large-volume FUS-BBB opening can improve delivery to the whole mouse brain.





970. Deep Learning-Guided and Structure-Informed Multifunctional AAV Capsid Design Pipeline for Manufacturability and Redirected Tropisms

Cui Zhang[1], Zhuoyang Lv[2], Zhiwei Lu[1], Yongjian Zhang[1], Pengfei Yuan[1]

[1]EdiGene Inc., Beijing, China,[2]Department of Computer Science, Brown University, Rhode Island, RI

The recombinant adeno-associated virus (AAV) is a highly promising vector that is transforming the field of gene therapies for human diseases. Capsid engineering, which involves modifying the interactions between the capsid and cell surface receptors, has been achieved through a combination of knowledge-based design and iterative directed evolution. This approach has successfully produced AAV variants with notably altered biodistribution and other beneficial properties, such as improved manufacturability. Despite the capability of screening millions of capsid variants in a single library, conventional methods fall short in exploring the vastly larger combination pool and efficiently identifying the small fraction of vectors with the desired characteristics for clinical use. The process is also marred by the tedious and costly nature of multiple selection and testing rounds across different tissues. In response to these challenges, we introduce a comprehensive method that integrates deep learning-based models for sequence-to-characteristic predictions and a structural approach for capsid classification and clustering. This strategy simplifies the screening process and expedites the discovery of new capsids. Our method utilizes random 7-mer NNK insertional capsid libraries, with packaging and enrichment screening conducted across various tissues including the liver, muscle, and central nervous system (CNS). A deep-learning framework has been developed, capable of predicting manufacturability and tissue-specific targeting based on peptide sequences. From an initial experimental library of approximately 360,000 variants, representing a fraction of the 1.28 billion possible sequences, each peptide sequence was transformed into a one-hot encoded 7x20 two-dimensional array. A deep learning model was then trained for binary classification on production efficiency, supplemented by regression models to assess the enrichment scores for each target tissue. The integration of an attention mechanism to capture global sequence relationships and 1-D convolution for local context analysis has resulted in a classification model with an impressive 98% accuracy. Our regression model also consistently achieved over 70% accuracy in its predictions. By combining these models, we constructed a condensed, multi-trait capsid library of 300 manufacturable candidates targeting the CNS and muscle. This library was experimentally validated, with over 50% of the variants showing

targeted tissue enrichment—a success rate significantly higher than that achieved through traditional screening methods. Moreover, we utilized AlphaFold2 for predicting oligopeptide structures, facilitating the clustering of variants into distinct clades based on structural similarities. This approach aided in the deduplication and efficient classification of top candidates showing robust tissue enrichment in mouse models. Preliminary data from a non-PHP.eB-clustered variant demonstrated high and consistent CNS transduction in both C57BL/6 and BALB/c mice, underscoring the effectiveness of structure-based capsid classification. In summary, our proposed pipeline represents a significant advancement in AAV capsid engineering. It drastically narrows the search space for potential variants, reduces the number of screening and testing cycles needed, and demonstrates the powerful synergy of deep learning and structural analysis in expanding therapeutic possibilities.

971. Novel AAV Variants for Huntington's Disease Gene Therapy

Brian Lewandowski[1], David E. Leib[2], Yonghong Chen[1], Paul T. Ranum[2], Luis Tecedor[1], Sakshi Arora[1], Ashley Robbins[3], Bryan P. Simpson[2], Elena Lysenko[1], Megan S. Keiser[4], Xueyuan Liu[5], Jang-Ho Cha[2], Beverly L. Davidson[1]

[1]Children's Hospital of Philadelphia, Philadelphia, PA,[2]Latus Bio, Philadelphia, PA,[3]University of Pennsylvania, Philadelphia, PA,[4]Ohio State University Wexner Medical Center, Columbus, OH,[5]The Children's Hospital of Philadelphia, Philadelphia, PA

Huntington's disease (HD) affects a distributed network of brain areas including the basal ganglia and cerebral cortex. However, current state-of-the-art AAV capsids do not sufficiently target areas critical for HD therapy without large doses using prolonged surgery times. To overcome this challenge, we performed a screen of tens of millions of peptide-modified (PM) AAV capsid variants in adult rhesus macaques to identify novel variants that transduce relevant areas following a single focused low dose infusion. Our screen identified multiple variants with robust transduction across basal ganglia and cortical regions in rhesus. To test the across-species translatability of our PM-AAV screening approach, we took the top ~150 capsid variants identified after two rounds of screening in rhesus macaque and tested their performance in rhesus, African green monkey (AGM) and in mice. Capsid performance measured from next generation sequencing (NGS) results in rhesus and AGM was highly correlated, with a Pearson's correlation of 0.85. Similarly, there was enrichment of top capsid variants in mouse medium spiny neurons (MSNs) when assessed by single cell sequencing methods. One PM-AAV variant, DB-3, was consistently highly enriched in each round of selection. Biodistribution of AAV-DB-3 was characterized by native fluorescence of AAV-DB3. mTFP following relatively low dose injections in rhesus. We observed remarkably efficient transduction of basal ganglia regions and layer V/VI projection neurons across multiple cortical areas, including primary motor cortex. A critical target for HD gene therapy are MSNs in the basal ganglia. We assessed cell-type tropism in the caudate and putamen using RNAscope™ fluorescence *in situ* hybridization with probes targeted to MSN marker genes. DB-3 demonstrated high selectivity for MSNs, transducing ~50% of MSNs in both regions and only 5-20% of non-MSN cell types. We also assessed DB-3 transduction outside the nervous system. In digital droplet PCR of liver samples, DB-3 transgene levels were below the threshold of detection. To test whether DB-3 would exhibit similarly high transduction efficiency in human cells, we quantified transgene expression in neurons derived from human iPSCs using qPCR. At a dose of 5.0E3 vg/cell, DB-3 showed a ~5700-fold increase in transgene expression compared to the wild-type parental serotype. In summary, DB-3 exhibits remarkable tropism for therapeutically relevant HD-related brain regions and improved transduction efficiency in both human and NHP neurons at doses orders of magnitude lower than those currently in clinical use. DB-3 will be a powerful tool for the delivery of therapeutic transgenes for HD and other disorders affecting the brain.

972. Engineering Human-Translatable AAV Vectors with Receptor-Targeting Moieties for CNS Gene Delivery

Richard Alexander Martino, Ka Ying Sharon Hung, Mark Julian, Nadia D'Lima, Mengying Zhang, Xiaodong Lu, Cailtin Meeks, Samantha Desmarais, Aditya Utturkar, Shawn Kyle Bourque, Misha Chittoda, Harini Dhamodaran Sampath, Pete Clarner, Ankur Thomas, Bin Wang, Muccini Arnold, Samneet Kashyap, Quan Jin, Levi Griffin, Tania Seabrook, Emily Rouse, Yuting Liu, Adem Koksal, Benjamin Smith, Tom Cameron, Paul Weinreb, Junghae Suh, Joseph Arndt, Yuan Yuan

Biogen, Cambridge, MA

Treatments for neurological diseases would be expanded with adeno-associated virus (AAV) vectors capable of efficiently crossing the blood-brain barrier (BBB) and transducing cells of the central nervous system (CNS). CNS-targeting AAV vectors have been identified from selections of large AAV libraries in animal models, but these methods can produce vectors with species-specific activity due to a lack of phylogenetic conservation in cognate cell surface receptors. An alternative approach involves engineering AAV vectors that directly target desired human receptors, a strategy that would augment human translatability. In this work, we sought to improve AAV CNS transduction by inserting protein domains targeted to a human receptor into the AAV capsid. We first developed targeting domains cross-reactive to murine, cyno, and human receptor homologs. We then designed a cohort of genetic fusions with distinct sets of peptide linkers. We built a barcoded AAV library of these variants and screened in cynomolgus macaques as well as in a knock-in (KI) mouse model. Several variants emerged as attractive hits showing enhanced CNS transduction over AAV9 in both models. We further validated several variants in the humanized KI mouse model. One such variant was superior with significant improvement in transgene mRNA expression over AAV9, and robust neuronal transduction throughout the brain. In summary, we successfully engineered AAV capsids with targeting domain insertions that efficiently transduce the CNS through engaging with a human receptor. With a robust transduction profile in a humanized mouse model, these vectors hold clinical promise for the treatment of CNS diseases.

973. Discovery of TRACER AAV Capsids Escaping Pre-Existing Neutralizing Antibodies

Damien Maura, Jennifer Yu, Xiao-Qin Ren, Matthew Child, Rupinderjit Kaur, Zachary Thorpe, Jiachen Liu, Netsanet Gebremedhin, Daniel Henry Cox, Weitong Chen, Brett Hoffman, Tyler Moyer, Ambreen Sayed-Zahid, Johnny Yao, Charlotte Chung, Todd A. Carter, Mathieu E. Nonnenmacher

Voyager Therapeutics, Cambridge, MA

Adeno-Associated Virus (AAV) gene therapy shows great promise to treat a wide array of human genetic diseases. However, patient eligibility to receive AAV gene therapy is limited by the presence of pre-existing anti-AAV antibodies resulting from prior exposure to natural AAVs. These antibodies restrict AAV biodistribution and target tissue transduction, especially for systemically administered AAVs. Recent capsid engineering efforts have successfully improved AAV tissue tropism, and paved the way for enhancing other AAV properties. In this study, we leveraged our proprietary RNA-driven TRACER evolution platform to detarget our 2nd generation AAV9-derived CNS tropic capsids from neutralizing antibodies (NAbs). We evolved immunogenic capsid surface regions, and screened the resulting libraries *in vitro* in presence of monoclonal NAbs as well as human intravenous immunoglobulin (IVIG) to maximize the clinical relevance. Capsid libraries were also screened in marmoset and in a mouse passive immunization model with human IVIG. We identified several capsid families with specific amino acids enriched by IVIG selection pressure at several mutation hotspots. In pooled capsid library assays, top candidates evaded IVIG up to 20-fold better than the parental capsid in mouse while retaining over 50% brain transduction of the parent capsid in mouse & marmoset. Individual validation of these capsids in mouse confirmed that top candidates evaded human IVIG 10 to 30-fold better than AAV9. Most importantly, these capsids were able to complete evade ~40% of neutralizing human serum samples. Remarkably, combining mutations from different capsid regions further improved NAb evasion, with seroprevalence decreasing from 50% down to 20% in a panel of 50 human serums. Overall, this study identified a 3rd generation of bioengineered AAV capsids with mutations conferring evasion from pre-existing neutralizing antibodies while retaining the improved CNS tropism of their 2nd generation parent. These novel capsids have the potential to significantly improve a key bottleneck in AAV treatments by increasing patient eligibility to receive AAV gene therapies.

974. AAV9 Production Fitness Modeling with Machine Learning

Daniel H. Cox, Richard Lu, Hassan Gheisari, Netsanet Gebremedhin, Jiachen Liu, Mathieu E. Nonnenmacher, Weitong Chen

Voyager Therapeutics, Cambridge, MA

In the search for good vectors for viral-mediated gene-therapy, often large combinatorial libraries of mutant capsids are screened. The objective is to find capsids that will form novel viruses that both produce well and exhibit enhanced tropism toward a particular tissue. Typically, the sequence space represented by such libraries is much larger than the number of capsids that can be effectively screened in a single experiment, and it is common for a high percentage of the mutant capsids to fail to form viable virus. Thus, it would be useful to screen out such low-production-fit capsids in silico, at the design stage, to prevent wasted space in the subsequent experimental stage. To do this we have developed machine learning models to predict production-fitness from capsid sequence. Indeed, for capsid sequences consisting of 7 or 9 amino-acid insertions in variable region 8 of AAV9, we have developed classifiers that can distinguish between high and low production fit capsids with AUC values as high as 0.98 and regression models whose predictions correlate with measured values with r values ≥ 0.95. Furthermore, we have found these machine learning models to effectively guide the design of high-production-fit libraries as tested experimentally, and we have used hamming-distance constraints to spread test sequences as evenly as possible over the relevant search space. The theoretical limits of this approach will also be discussed.

975. Identification and Characterization of a Highly Conserved Cell Surface Receptor Utilized by Engineered BBB-Penetrant AAV Capsids with Enhanced Brain Tropism in Non-Human Primates and Mice

Brett Hoffman, Tatiana Knox, Tyler Moyer, Ishan Shah, Xiao-Qin Ren, Weitong Chen, Emily Rouse, Damien Maura, Mathieu Nonnenmacher

Voyager Therapeutics, Lexington, MA

Novel engineered blood-brain barrier (BBB)-crossing AAV capsids have demonstrated significant improvements in CNS tropism and thus increased therapeutic efficiency. However, the unpredictability of cross-species activity by engineered capsids has hampered progress to the clinic. Therefore, the identification of the receptors utilized by such novel capsids is important to predicting their utility in treating human conditions. For instance, the recent identification of receptors for multiple CNS-tropic capsids have explained their lack of cross-species activity as either due to lack of a human receptor ortholog or poor conservation of critical residues. Here we report the identification of a highly conserved brain vascular receptor specifically bound by the cross-species VCAP-101/102 engineered capsid class. This capsid family was previously reported to exhibit a 50-fold increase in BBB penetrance in both macaques and mouse. Ectopic overexpression of the human receptor isoform in cultured cells led to a significant increase in capsid binding and transduction while no difference was observed with the parental capsid, AAV9. Importantly, receptor isoforms from macaque, mouse, and pig also facilitated transduction by VCAP-102, highlighting the cross-species functionality of this capsid class. Neutralization of capsid-receptor interaction with anti-receptor antibodies or small molecule inhibitors completely reverted the gain of transduction by VCAP-102, further supporting a direct role in transduction. Additional studies in a transwell cell culture model demonstrated that the VCAP-102 receptor dramatically increased the transcytosis of capsids across polarized cells, strongly supporting a mechanistic role in capsid translocation across the BBB *in vivo*. Previous studies have demonstrated that expression of the VCAP-102 receptor significantly increases with age in both humans and rodents, and we observed a corresponding 2-fold increase of CNS transduction in aged mice relative to their young counterparts. Conversely, mice

pre-treated with a small molecule inhibitor of VCAP-102 receptor showed significantly reduced brain transduction, while no measurable effect was observed on liver transduction. Importantly, the CNS transduction by other capsids with a different receptor usage was unaffected by the inhibitor, ruling out the possibility of non-specific effects on viral transduction. Employing in silico structural modeling, we have further characterized the molecular mechanism governing the interaction between capsid and receptor. In summary, our discovery of a conserved cross-species receptor facilitating BBB passage by a novel engineered AAV capsid class represents a significant step forward in the development of targeted CNS therapeutics. Understanding the molecular mechanisms underpinning this interaction provides a foundation for the rational design of next-generation AAV vectors.

976. Establishment of a Predictive Transcytosis Model to Recapitulate Capsid-Receptor Interaction and Phenotype of BBB-Penetrant AAV Variants

Xiaoqin Ren, Ishan Shah, Tyler Moyer, Brett Hoffman, Elisabeth R. Knoll, Brian C. Ezell, Mathieu E. Nonnenmacher

Voyager Therapeutics, Cambridge, MA

Delivery of genetic payloads across the blood-brain barrier (BBB) remains a critical challenge for treatment of neurological diseases. Recent years have seen rapid progress in the engineering of BBB-penetrant viral and non-viral gene transfer vehicles, but questions remain regarding the translatability of these discoveries to human patients. In the absence of true in-human observations, translation between animal models and humans can be inferred from phenotypic equivalence across various primate and/or rodent species, or from the identification of the molecular mechanisms of BBB penetration, for example by identification of a vascular receptor. We have previously identified VCAP-102, an Adeno-Associated Virus (AAV) capsid variant showing improved BBB penetration in both primates (old world and new world) and rodents, and we were able to determine that VCAP-102 binds to a BBB surface receptor showing a high degree of conservation across species, including humans. In order to further characterize the mechanism of action of VCAP-102, we generated a transwell model using Madin-Darby Canine Kidney (MDCK) cells overexpressing VCAP-102 receptor and showed that this cell line allowed very efficient transcytosis of VCAP-102 capsids, but not the parental AAV9. Conversely, parental MDCK cells, which do not naturally express the receptor, were non-permissive to either capsids. We confirmed the receptor-mediated transcytosis of generation 2 capsids in our transwell model, consistent with their in vivo potency.

977. One Amino Acid Substitution Makes the AAV2 Capsid Mutant BR1 Permeable to the Murine Blood-Brain Barrier

Ayumu Konno, Hayato Kawabata, Hirokazu Hirai

Neurophysiology & Neural Repair, Gunma University, Maebashi, Japan

Adeno-associated viruses (AAVs) are small, non-enveloped DNA viruses that infect dividing and non-dividing cells. AAV vectors are promising for gene therapy because of their broad tissue and cell affinity, efficient gene expression, and minimal immune response. AAV serotypes, AAV9 and AAV2, are known to interact differently with brain microvascular endothelial cells (BMVECs). Namely, AAV9 is effectively transcytosed through BMVEC and leads to the transduction of brain parenchymal cells, whereas AAV2 barely crosses the endothelial barrier and transduces BMVECs. Because of these properties, the development of blood-brain barrier (BBB)-permeable AAV vectors such as PHP.B (Deverman et al., 2016) and AAV-F (Hanlon et al., 2019) has generally been based on AAV9. In this presentation, we report that AAV2-BR1, a capsid mutant derived from AAV2, has acquired BBB permeability by a single residue amino acid substitution.AAV2-BR1 is an AAV vector previously reported to enable specific and efficient transduction into BMVECs (Körbelin et al., 2016). This mutant has heptapeptide (NRGTEWD) insertions with a "stuffer" sequence (G and A) at both sides between amino acid positions R588 and Q589 of the AAV2 capsid protein. Importantly, BR1 has one more mutation from N to Q at position 587 (Figure). To produce the BR1 capsid for brain microvasculature transduction, we inserted 9 amino acids (7 insertions plus 2 stuffer amino acids) between R588 and Q589 of the AAV2 capsid, where an amino acid at position 587 was still asparagine (termed BR1N). Because we assumed that BR1N might be sufficient for BMVEC transduction, we produced BR1N capsid vectors and intravenously injected them into C57BL/6 mice. However, BR1N transduced cells of the central nervous system rather than BMVECs. Although BR1 and BR1N are presumed to use the same receptor for entry into BMVECs, the substitution of one amino acid (Q to N) may have resulted in a profound change in intracellular trafficking. This suggests that receptor binding alone does not determine the final outcome *in vivo* and that further improvements of capsids within predetermined receptor usage may open new avenues for capsid engineering.

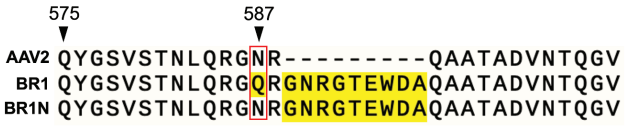


Figure. Amino acid sequence alignment of AAV capsids (AAV2, BR1, BR1N). BR1 and BR1N, AAV2 capsid mutants, have 7 amino acid (NRGTEWD) insertion with 'stuffer' sequence (G and A) at both sides in AAV2 capsid between positions 588 and 589. BR1 also has an asparagine (N) to glutamine (Q) substitution at position 587. Amino acids distinct from those of AAV2 are highlighted by a yellow background.

978. Characterization of Brain Targeting CereAAV™ Gene Transduction in Cynomolgus Macaque and Further Improvement of Gene Delivery Capability by Amino Acid Substitutions

Yoshinori Tanaka, Sayuri Ueda, Nozomi Okuda, Hiroaki Nariki, Sachiko Okamoto, Tatsuji Enoki

Takara Bio Inc., Kusatsu, Shiga, Japan

Recombinant adeno-associated virus vectors (AAVs) have been widely used as vehicles to deliver gene of interest for both research and therapeutic purposes. Zolgensma a treatment for spinal muscular atrophy, was approved by the US Food and Drug Administration (FDA) and numerous pre-clinical and clinical studies have been conducted using AAV2 and AAV9 serotypes to targeting brain diseases via local and systemic administration. In particular, AAV9 have been frequently used for gene delivery into the brain including central nervous system

(CNS), and several AAV9 derivatives such as AAV-PHP.eB, have been identified to transduce CNS efficiently. On the other hand, it has been reported that an AAV2 variant, designated AAV-BR1 efficiently transduced the Blood Brain Barrier (BBB) -associated endothelial cells by systemic administration. However, there is still no AAV2 variant is reported to transduce CNS across the BBB efficiently. To overcome the limitations of AAV2, we addressed to develop a novel brain-targeting AAV vectors based on AAV2 serotype. To date, we have demonstrated that CereAAV, which was obtained by directed evolution using random peptide-inserted AAV2 library, can efficiently transduce in mouse and marmoset brain by systemic injection. In this study, we aimed to assess the gene transduction efficiency of CereAAV in cynomolgus macaque and improve the transduction efficiency through capsid optimization of CereAAV in rodent model. We evaluated the gene transduction efficiency of CereAAV in a cynomolgus macaque using the AAV barcode library method and found that CereAAV also exhibited higher gene transfer efficiency to the brain compared to AAV9 in the non-human primate model. To elucidate the transduction mechanism of CereAAV, we performed alanine scanning of the insertion peptide of CereAAV capsid to identify the important amino acids for brain transduction. As a result, we found that the sixth amino acid residue in the inserted peptide of CereAAV plays a crucial role in gene delivery to the mouse brain by intravenous administration. Furthermore, we identified a novel CereAAVmt-6Y mutant with a specific and higher transduction efficiency in the mouse brain compared to CereAAV through a single amino acid substitution. The gene transduction in the brain of CereAAVmt-6Y was approximately 90-fold higher than that of AAV9 in luciferase reporter assay in rodent models. Recently, Kawabata et al. demonstrated that a single amino acid substitution, changing glutamine to asparagine at position 587 (AAV-BR1N) in the AAV-BR1 capsid, could increase the BBB permeability and redirect gene delivery form vesicular endothelial cells to neurons in the mouse brain. We have confirmed, the Q587N substitution also increased the gene transduction of CereAAVmt-6Y. Currently, further studies are underway to evaluate the transduction efficiency and characterize the transduction targets of CereAAV variants including Q587N substation, in rodent and non-human primate models.

979. Characterization of Engineered AAV Capsids for Improved Transduction Efficiency through Rational Design

Jingjie Hu[1], Panos Theofilas[1], Travis Bainbridge[2], Yoon Min Kim[2], Beyza Bulutoglu[2], Oded Foreman[3], Honglin Chen[1], Annalisa Lattanzi[1]

[1]Molecular Biology, Genentech, Inc., South San Francisco, CA,[2]Protein Chemistry, Genentech, Inc., South San Francisco, CA,[3]Research Pathology, Genentech, Inc., South San Francisco, CA

The engineering of adeno-associated virus (AAV) capsids for enhanced transduction and tissue-specific targeting is crucial for gene delivery and therapies. This would be beneficial for reducing vector dose, resulting in less immunogenicity and toxic effects. AAV9 serotype has become the preferred vector for systemic administration due to its ability to transduce a variety of tissues and organs. In this study, we tested AAV9-derived capsid mutants, engineered with a rational design approach for increased binding affinity to the universal AAV receptor (AAVR). The goal was to augment cell entry and intracellular trafficking, ultimately aiming to improve transduction efficiency. All AAV9 capsid mutants were packaged with an ITR-CAG-GFP-ITR payload for simplicity of imaging and flow cytometry analysis. We initially evaluated the *in vitro* transduction efficiency of the AAV9 capsid mutants in HEK293T and SH-SY5Y (neuroblastoma) cell lines. We observed 2- to 4-fold increases in transduction efficiency for two mutations and a synergistic enhancement up to 6-fold when they were combined. Their AAVR dependency was further confirmed in AAVR+/− and AAVR−/− HEK293T cell lines generated by CRISPR/Cas9 editing. Subsequently, we analyzed the transduction capability of the AAV9-derived capsid mutants in comparison to the wild-type control in C57BL/6J adult male mice via retro-orbital systemic injection or local brain injection. The systemic injection suggested slightly increased transduction in liver tissue and significantly detargeting from the heart for one of the two mutants that showed enhancement *in vitro*. We chose intracerebroventricular injection to test the differences in expression levels and biodistribution of these AAV9-derived capsid mutants compared to a wild-type control. Four weeks post-injection, the left hemisphere was harvested for DNA analysis of the AAV vector copy number (VCN). Meanwhile, the right hemisphere was processed in serial sections to evaluate the vector biodistribution using immunofluorescence for GFP reporter positivity. Our results revealed robust localized expression adjacent to the injection site for most mutants. Furthermore, the two capsid mutations with the best *in vitro* transduction ability also demonstrated an increased GFP signal intensity and a wider biodistribution area, which was consistent with the increased VCN quantification in the area of interest. Notably, in both *in vitro* and *in vivo* studies we found that mutants with the highest binding affinity to AAVR (30- to 50-fold) dramatically reduced their transduction ability. This suggests that an excessively high AAV-receptor binding affinity eventually results in an impaired vector's ability to enter the nucleus. Overall, this study demonstrates that the AAV9 capsid protein can be engineered to enhance its efficiency in transducing cells both *in vitro* and *in vivo*. Our findings suggest that these mutations can be utilized in future gene therapy research and be combined with other engineered AAV9 capsids.

980. Optimizing Cell-Type Specificity of AAV Gene Therapy Vectors for the Human Brain

JP McGinnis[1], Josh Ortiz-Guzman[2], Maria Camila Guevara[3], Sai Mallannagari[3], Daniel Curry[4], Ben Arenkiel[3]

[1]Department of Neurosurgery, Baylor College of Medicine, Houston, TX,[2]Rio Grande Urology, El Paso, TX,[3]Department of Human and Molecular Genetics, Baylor College of Medicine, Houston, TX,[4]Department of Neurosurgery, Texas Children's Hospital, Houston, TX

Molecular-based therapies promise to improve outcomes for our most frustrating pathologies. To date, however, we have no method that can deliver such sequence-based therapies selectively to the cell types involved. Virtually all prior work on adeno-associated viruses, the most common vector for neurological gene therapies, has studied capsid tropism in model organisms, primarily mice, despite abundant data that demonstrates a lack of translation across species. For this reason, we have developed an ex vivo organotypic system that uses surgically resected human brain tissue to screen libraries of AAV variants.We consent patients undergoing resective neurosurgical

procedures for any tissue not needed for clinical use. After sectioning the tissue, transducing with virus libraries, and allowing time in culture, we use both imaging and single-nuclei sequencing to characterize each variant's tropism.Resected human brain tissue is viable for up to four weeks, and is unaffected by addition of AAVs up to MOIs of 60,000. Appreciable transgene expression takes approximately 14 days. We find that transduction with 14 known AAV variants, including AAV2, the most common vector used in clinical trials, show quite low transduction of human neurons. Astrocyte transduction is better, in some cases as high as 50%. Ongoing work involves dissecting promoter behavior within human tissue in various cell types, along with large-scale library transduction.There is no prior work characterizing the cellular AAV tropism in human brain tissues, even those variants currently FDA-approved. This work shows the limited ability of animal models to predict the efficiency and specificity of AAV transduction, which relies on cell surface molecules that are known to be divergent between species and even strains of model organisms. These results provide the foundation for large-scale AAV gene therapy vector screening using human brain tissue, especially from diseases for which no durable therapy is currently available.

981. AAV Capsid Engineering for Improved AAVR Binding to Enhance Transduction Efficiency

Travis W. Bainbridge[1], Yoon Min Kim[1], Jingjie Hu[2], Panos Theofilas[2], Maciej T. Paluch[1], Oded Foreman[3], Honglin Chen[2], Annalisa Lattanzi[2], Beyza Bulutoglu[1]

[1]Department of Protein Chemistry, Genentech, South San Francisco, CA,[2]Department of Molecular Biology, Genentech, South San Francisco, CA,[3]Department of Research Pathology, Genentech, South San Francisco, CA

Immunogenicity and the consequent toxic side effects present major obstacles to broader AAV usage1, and this can become especially problematic with the highest doses used in the clinic (E13-E14 vg/kg). One way to overcome this is to improve the transduction efficiency of AAVs, which could allow for the use of lower doses, hence less immunogenic material. AAV transduction involves two key mechanistic events: i) AAVs binding to cell surface receptors followed by internalization, ii) intracellular trafficking of AAVs. The universal AAV receptor, AAVR, was identified as a key receptor for AAV infection, playing an important role in intracellular AAV trafficking.2 Recently, the protein-protein interactions between AAVR and AAV1, AAV2, AAV5, AAV9 were mapped, offering an opportunity to manipulate the affinity between the receptor and the AAV capsid.3,4. In this study, we engineered the AAV capsid to increase the affinity between the capsid proteins and AAVR for improved trafficking and cargo delivery to nucleus. We generated AAV9 virus-like particles and mutated the residues at the receptor-binding interface to all 20 amino acids. We then screened the variants bearing single point mutations as well as combinations of mutations via surface plasmon resonance (SPR), and identified AAV9 variants with improved AAVR affinity (Figure 1A). A handful of promising AAV9 variants carrying a GFP reporter gene were tested in vitro in HEK293T and SH-SY5Y neuroblastoma cells, where we observed up to 6-fold improvement in transduction efficiency. The transduced cells also showed a higher level of GFP expression, compared to the cells transduced with WT AAV9 (Figure 1B).We observed an optimal range for AAVR affinity improvement as AAV variants with highest AAVR affinity resulted in decreased transduction, compared to WT AAV9.

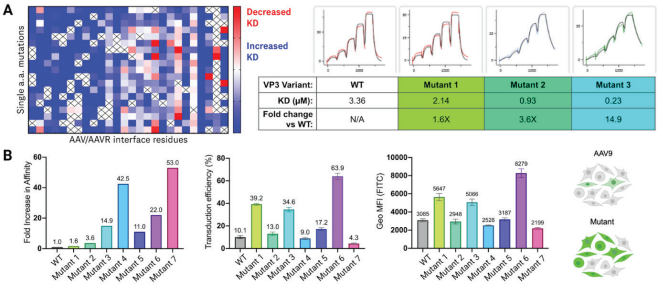


Figure 1. (A) Left: Heatmap demonstrating the AAVR fold-affinity change (relative to WT AAV9). Red denotes an increase in affinity (decreased KD). Blue denotes a decrease in affinity (increased KD). X denotes no capsid formation or WT residue. Right: Exemplary SPR sensograms. Point mutation-based engineering efforts resulted in variants with a wide range of fold increase in AAVR affinity. (B) When tested in vitro in SH-SY5Y neuroblastoma cell line, several AAV9 mutants performed better, compared to WT, both in terms of number of transduced cells as well as the intensity of the transgene (GFP) expression. In addition, we tested some of the AAV9 variants in vivo in C57BL/6J adult mice. Two variants with improved in vitro transduction ability, also demonstrated higher vg copy numbers in the brain tissue and wider biodistribution, compared to WT AAV9, when administered locally via intracerebroventricular injection (2E10 vg/mouse). These promising results demonstrated that engineering viral capsid proteins to modulate their binding affinity to the AAV-trafficking receptor AAVR can be advantageous. This approach can also be complementary to other capsid engineering efforts focusing on enhanced tropism, and can be used in a broad range of gene therapy applications. References: 1. Verdera et al., Mol Ther 2020; 2. Pillay et al., Nature 2016; 3. Zhang et al., Nat Microbiol 2019; 4. Xu et al., Mol Ther Methods Clin Dev 2022.

982. Identification of Novel CNS-Tropic AAVs Using In Vitro Binding to Candidate Receptors Combined with *In Vivo* Screening, Generative AI, and Structural Modeling

John Reece-Hoyes[1], Jackson Lee[2], Jie Tan[3], Estrella Lopez-Gordo[3], Caroline Curll[3], Fion Chan[3], Sri Siripurapu[3], Stephanie Malyszka[3], Kimberly Le[3], Allegra Fieldsend[4], Haley Knight[3], Paul Freeman[3], Matt Bennett[3], Esther Aribilola[3], Ramin Kamran Sami[3], Shahrzad Parker[3], Jordan Shufro[3], Graham Lilley[3], Bryan Mastis[3], Roberto Calcedo[3], Matt Edwards[3], Sherry Cao[3], Charles F. Albright[5]

[1]Vector Biology, Affinia Therapeutics, Waltham, MA,[2]Computational Science, Affinia Therapeutics, Waltham, MA,[3]Affinia Therapeutics, Waltham, MA,[4]Affinia Therapeutics, Inc., Waltham, MA,[5]Research, Affinia Therapeutics, Waltham, MA

Adeno-associated virus (AAV) capsids with improved central nervous system (CNS) tropism are needed to unlock the potential of AAV vectors for CNS diseases. In vivo screening of random peptide insertion

capsid libraries has identified novel AAV capsids with superior neural tropism compared to naturally occurring capsids. However, deconvoluting the transduction mechanism can be challenging, and engineered capsids can exhibit species-specific tropism, which potentially complicates their therapeutic utility. To overcome these limitations, we used an in vitro binding platform to select novel AAVs that bind to target human receptors with the potential to enable AAV trancytosis into the CNS parenchyma. We first selected 10-15 potential receptors based on either their ability to transcytose other cargo or other relevant factors. For instance, the transferrin receptor was included in our list of high-priority receptors based on its ability to transcytose antibodies and AAVs in animal models. The extracellular region of these receptors was then immobilized on beads as a fusion protein and used to affinity purify AAV capsids from diverse AAV9-based libraries with peptide insertions. A variety of capsid libraries were constructed, including those with random peptide insertions or designed to uniformly sample the vast sequence space. Through these screens, we identified a diverse collection of AAV variants that were both reproducible and unique to a given receptor. Importantly, peptide sequence motifs emerged from these binding hits for a given receptor, indicating specific receptor binding. Binding hits were then used to construct candidate libraries. To further diversify the experimental hits, we designed additional capsids using a deep learning-based modeling and sequence generation framework. The combined libraries were then tested in cell-based assays and animal models, including human protein knock-in mice and non-human primates. These studies resulted in the successful identification of novel AAV capsids with improved CNS tropism compared to AAV9 in animals, for more than one receptor. Structural modeling of the CNS-tropic capsids revealed similarities and differences in the putative binding conformation of the capsids with their cognate receptor. Based on the performance ranking of individual capsids in cell-based assay and animal models, several candidate AAV variants were selected for clonal studies in animals and for mechanistic studies. The results from these studies will be reviewed.

983. Optimization of a Generative Algorithm for Designing Novel rAAV Capsids with Enhanced Yields

Lijun Liu[1], Jiali Yang[2], Lele Niu[2], Jianfei Song[1], Zeqi Cai[2], Jia Xu[2], Hua Gao[2], Wei Mao[2], Hui Shi[1], Guodong Jia[2], Xinglin Yang[2], Yafeng Deng[1], Weiran Shen[2]

[1]Hangzhou Carbosilicon AI Technology Development Corp., Ltd., Hangzhou, China,[2]OBiO Technology (Shanghai) Corp., Ltd., Shanghai, China

Although AAV vectors are widely utilized in gene therapy, existing vectors lack optimization in terms of yield, specificity, and potency. In the pursuit of improving vector performance and exploring uncharted possibilities beyond the current library strategy, we have refined a generative algorithm for the exploration and testing of novel capsid sequences. This study utilizes publicly available data from AAV2 to train a generative algorithm employing a diffusion model to predict capsid assembly. We generated 8000 diverse AAV2 VP sequences and assessed their viability through DNase-resistant capsid assembly assays. Furthermore, we extended the algorithm's application to predict sequence migration and generation within the AAV9 domain, leveraging both similarities and differences. Envisioned sequences were synthesized, constructed, and compiled into a vector library to assess their actual survival rates. Results revealed that generated sequences exhibited a viability proportion exceeding 90% within a mutation range of 7 to 20. Importantly, selected sequences not only demonstrated enhanced yield but also exhibited a capacity for functional transduction. The mutation of additional sequences displaying high viability holds promise for generating innovative capsids tailored to specific targets and increased specificity.

984. Directed Evolution of AAV1, AAV5 and AAV9 Variants for Efficient Gene Delivery to Eye

Nachiket Pendse, Eduardo Magdaleno, Fabio Luna, Tan Ge, Steph Hintzen, Jake Hill, Cameron Mcelroy, Jayson Chen, Lawrence Tam, Eloise Hudry, Helen Booler, Kirsten Stoner, Kelley Penraat, Mark Hedgeland, Shraddha Babaria, John Walker, Scott Ellis, Fatih Ozsolak

Novartis Biomedical Research, Novartis Pharma AG, San Diego, CA

In recent years, adeno-associated viral (AAV) vectors have demonstrated efficacy and safety for ocular, neurological, musculoskeletal, and metabolic liver diseases in clinic. Naturally occurring AAV serotypes along with engineered capsids variants have been shown to enhance tropism to the neural retina and retinal pigment epithelium (RPE). The current FDA approved ocular gene therapy is for the treatment of RPE65-associated Leber congenital amaurosis (LCA) - an inherited retinal disorder (IRDs), utilizing sub-retinal delivery of AAV2 carrying the therapeutic cargo to the RPE. Despite its effectiveness, sub-retinal gene delivery is surgically complex and could only be accessible to a limited number of patients. Therefore, the development of platform capabilities including novel capsids and delivery methods may enable 'in-office' ocular gene therapies through broadening ocular tissue tropisms and expanding patient access. In the present investigation, we utilized our 'state of the art' Tangential Suprachoroidal (TS) Microinjector delivery device as well as previously described intra-vitreal (IVT) route for administration of AAV capsid libraries into non-human primates. To identify retinal specific capsids, we selected serotypes AAV1, AAV5 and AAV9 as the parental capsid. We utilized directed evolution approach by targeting the *CAP* gene of serotypes AAV1, AAV5 and AAV9 in variable loop VIII region and inserted randomized 7mer NNK library. Then, we administered such AAV libraries separately to non-human primate eyes via the suprachoroidal and IVT delivery routes. After one round of in-vivo selection and deep sequencing analysis, based on whole retina RNA enrichment scores, we identified key capsid candidate profiles of AAV1 and AAV5 with almost no virtual off-targeting in peripheral organs such as liver in NHPs. The results from our study demonstrate for the first time that TS Micro device mediated discovery of novel AAV1 and AAV 5 variants displayed high retina tropism and will enable the selection of ocular tropic variants with improved safety and efficacy profile for human ocular gene therapies.

985. Development of Blood-Brain Barrier Penetrant AAVs through Receptor-Targeted Capsid Engineering

Ankitha Nanjaraj, Stephanye Frias, Satria Sajuthi, Lori Andrews, Russell Darst, Stephen Wist, Yuri Bendaña, Hung Tran, Sarah Mueller, Bryan Zeitler, Amy Pooler, Matthew R. Tiffany, David Ojala

Sangamo Therapeutics, Inc., Richmond, CA

The development of AAV capsids that cross the blood-brain barrier (BBB) has the potential to enable transformative genomic medicines for the treatment of neurological disorders. Previously we described SIFTER, a method for in vivo selection of AAV capsids that mediate improved central nervous system (CNS) delivery after administration into the vasculature or cerebrospinal fluid. This method has yielded highly efficient capsids for CNS delivery in non-human primates and can be coupled with receptor identification efforts to elucidate mechanisms by which AAV capsids cross the blood-brain barrier. Here we describe a complimentary engineering approach to develop AAV capsids targeting human receptors that are highly expressed in endothelial cells comprising the blood-brain barrier. AAV capsid libraries based on different parental serotypes, peptide insertion sites, and peptide sizes were first screened in vitro for enhanced binding to purified human BBB receptors. Library members with enhanced binding to the purified receptor were additionally screened for binding and transduction in cells engineered to overexpress these human BBB receptors. Two of the human BBB receptors we targeted for capsid evolution yielded especially notable results. The first receptor is highly conserved across species and abundantly expressed at the blood-brain barrier. Receptor-targeted library screening identified two capsid families with a differentially enriched amino acid motif that mediates enhanced receptor binding and transduction. This receptor interaction is preserved for both human and cynomolgus macaque orthologs, increasing the likelihood of translation between pre-clinical models and clinical applications. The second receptor is less well conserved across species but is likewise highly expressed in the human blood-brain barrier. We identified capsids with markedly improved binding and transduction in cells that overexpress this human BBB receptor. Based on these results we are evaluating these receptor-targeted capsids in vivo to assess CNS transduction efficiency and therapeutic potential for the treatment of neurological disorders.

986. Directed Evolution of Rationally-Engineered AAV Capsid Variants with Enhanced Central Nervous System (CNS) Tropism Following Systematic Administration

Hongxing Wang, Sha Zhu, Weiqun Zhang, Gabriela C. Toro, Na Liu, Lingzhi Zhao, Joan Shen

NeuShen Therapeutics, Lexington, MA

Clinicalsuccess in gene therapy has proven the potential of adeno-associated virus(AAV) as the leading viral delivery tool for in vivo therapeutics across manydisorders. Despite this, the inherent limitations of natural AAV capsids to crossthe blood-brain barrier (BBB) pose a significant clinical challenge,particularly in the context of neurological disorders. Recent efforts in AAVcapsid engineering have yielded some BBB penetrating AAV variants with enhancedCNS tropism and transduction in rodent and non-human primate (NHP), butnone of these new AAV variants have advanced to clinical stage, primarily dueto poor translatability across animal species or manufacturing/stability issueof novel AAV variants. Thus, there is an urgent need to develop novel AAVvariants with enhanced BBB penetrability, improved vector manufacturability anddesirable CNS tropism. At NeuShen Therapeutics, we employed structure-guidedpeptide insertion/substitution and amino acid mutagenesis approaches to rationallyengineer a total of 176 novel capsid variants, incorporating modifications tovariable regions (VR) 4, 5 and 8 of AAV9, AAV5 and AAVrh10. Additionally, weutilized 11 CNS benchmark capsids as reference controls. Each capsid variant wasindividually cloned and labelled with two specific barcodes. To assess the manufacturabilityof novel capsid variants, all variants were individually produced and vectortiter in cell lysate was assessed by quantitative PCR (qPCR). About 40% ofAAV9-derived and AAVrh10-derived variants exhibited better vector titer comparedto parental capsids. All capsid variants were pooled at equal vector genome andintravenously administered into rodent (C57BL/6 and BALB/c mice) and cynomolgusmacaque for 3-4 weeks of directed evolution. Specific barcoded DNA and RNA ofnovel capsid variants were recovered from multiple brain tissues and primaryperiphery tissues and analyzed by next generation sequencing (NGS). Based onNGS data from rodent evolution, a dozen of novel AAV9-derived variants andseveral AAV5-derived and AAVrh10-derived variants exhibited improved (up to70-fold) brain transduction and lower liver tropism in C57BL/6 or BALB/c micecompared to parental AAV9, AAV5 or AAVrh10. Some variants exhibited improvedCNS tropism in both rodent strains. Ongoing efforts involve furthercharacterization and validation of the most promising capsid variants. The NGSdata from NHP evolution will be expected in early March 2024, following whichthe data of CNS-tropic AAV variants from rodent and NHP will be aligned andintegrated. Taken together, our findings demonstrate the successfulestablishment of a capsid discovery platform for the development of CNS-tropiccapsid variants.

987. Structural Characterization of Engineered AAV Capsids and Their Interactors by Cryo-ET

Tyler Brittain[1], Seongmin Jang[1], Erin E. Sullivan[1], Timothy Shay[2], Ting-Yu Wang[1], Tsui-fen Chou[1], Viviana Gradinaru[3]

[1]Caltech, Pasadena, CA,[2]CLOVER Center, Caltech, Pasadena, CA,[3]California Institute of Technology, Pasadena, CA

Recombinant adeno-associated virus (AAV) vectors are the current optimum platform for therapeutic gene delivery. Despite this, clinical reports continue to call attention to the need for improved safety, potency, and cellular specificity. To address this, AAV capsids have been engineered on two fronts: (1) to evade antibody detection, thereby decreasing the immune response, and (2) to specifically target desired tissues and cell types. Understanding the interaction mechanisms of natural and engineered AAV capsids, enables structure-guided engineering of vectors with enhanced immunological and targeting properties. Structural analysis of AAVs has largely been restricted to single-particle Cryo-EM with few examples of Cryo-ET being utilized. Here we show across two different ligand-AAV interactions the limitations of single-particle can be overcome through sub-tomogram

averaging. Our lab identified murine carbonic anhydrase IV (Car4) as a receptor for two capsids, 9P36 and 9P31, enabling blood-brain barrier (BBB) crossing. Car4 is promising due to its conservation across rodent and primate species. Here we present the single-particle Cryo-EM structures of the two engineered capsids with and without their receptor. Inherent flexibility within their interaction prevents resolving Car4 through single-particle methods, which can be overcome and visualized using sub-tomogram averaging. Structural analysis of the 9P36/9P31-mCar4 interaction provides a framework from which to engineer future BBB-crossing vectors. Separately, a cell micro-array screen found a novel interaction between the cytokine interleukin-3(IL3) and all AAV9-based capsids. XL-MS/MS and site-directed mutagenesis was used in tandem with cryo-ET to reveal the binding location on the surface of AAV9. This structural analysis hints at a larger role in destabilizing core AAV interactions. The methodology herein shows how cryo-ET and sub-volume averaging can outshine traditional single-particle methods for small complexes bound to large symmetric particles. Additionally, the information learned has far-reaching implications for engineered BBB crossing vectors in primates & all AAV9-based capsids.

988. Expression-Based Selection Identifies a Microglia-Tropic AAV Capsid for Direct CSF Routes of Administration in Mice

Miguel Santoscoy[1], Paula Espinoza[1], Killian S. Hanlon[2], Carrie Ng[1], Suzanne Hickman[1], Rachel E. Bennett[1], Casey A. Maguire[3]

[1]MGH, Charlestown, MA,[2]Harvard Medical School, London, United Kingdom,[3]The Massachusetts General Hospital, Charlestown, MA

Microglia, resident myeloid-lineage cells of the central nervous system, exhibit a range of heterogeneous phenotypes due to their context-dependent functionalities in brain parenchyma. *In vivo* targeting of microglia using gene delivery systems is crucial for studying brain physiology and developing gene therapies for dementia and other brain disorders. However, they have been extremely resistant to transduction by viral vectors, including adeno-associated virus (AAV) vectors. This may be due to several factors, including the phagocytic nature of these immune cells and the ability to degrade pathogens such as viruses. Another challenge of microglia transduction may relate to the natural neuronal tropism of AAV and the relatively low percentage of microglia (~10%) compared to other cell types in the brain. The combination of this tropism "sponge" towards neurons and unfavorable stoichiometry may impact whether a given AAV particle is able to interact with microglia. Fortunately, in the past two years, there has been some progress demonstrating the feasibility and potential of using AAV to transduce microglia after direct intraparenchymal vector injection. Data suggests that combining specific AAV capsids with microglia-specific gene expression cassettes to reduce neuron off-targeting will be key. However, no groups have developed AAV capsids for microglia transduction after intracerebroventricular (ICV) injection. The ICV route of administration has advantages such as increased brain biodistribution while avoiding issues related to systemic injection. Here, we developed and validated a variant of the AAV transgene-expression selection system, iTransduce, combined with an AAV9 peptide display library for microglia transduction in mice after ICV injection. For the first round of selection, we injected CX3CR1-GFP mice ICV with the AAV9 library and rescued capsid DNA from flow-sorted GFP+ microglia. To select capsids that could transduce microglia over other cell types in the brain, we engineered an AAV-CD68-Cre-p41-Cap-7mer cassette to allow Cre expression in microglia. In the second round, we injected Ai9$^{CAG\text{-}FS\text{-}tdt}$ x CX3CR1-GFP mice. Expression of Cre in microglia resulted in tdTomato expression, and we flow-sorted GFP+/tdTomato+ microglia and GFP+ only microglia and performed sequencing to assess unique peptide inserts. We chose one variant, MC5, which was in the top 5 reads for the highest presence in the GFP+/tdT+ fraction. Interestingly MC5's peptide sequence has high identity with a domain within Syndecan-4, which may provide insight into its microglia-tropic properties in the future. The ability of MC5 for specific and efficient microglia transduction was validated with a head-to-head comparison with reported microglia-specific capsid MG.1.2 and parental capsid, AAV9, using a previously reported microglia-selective AAV transgene expression cassette, AAV-Iba1-GFP-WPRE-mir9.T4x-mir129.T4x. In preliminary stereological quantitation in the cortex and immediately surrounding the ventricles, MC5 showed a trend towards improved transduction efficiency of microglia when compared to the reported microglia-selective MG1.2 capsid as well as AAV9. MC5 and MG1.2 showed similar transduction specificity towards microglia over other transduced cells. AAV9 showed the poorest specificity. In conclusion, our *in vivo* selection strategy has identified a new AAV capsid capable of improved and more specific transduction of microglia after ICV injection. MC5 can serve as a valuable research tool, and a potential therapeutic candidate.

989. Engineering Human-Translatable CNS-Targeting AAV Capsids through Differential Directed Evolution in a Humanized Mouse Model

Mengying Zhang, Nadia D'Lima, Quan Jin, Samneet Kashyap, Margaret Kelemen, Emily Rouse, Pete Clarner, Maria Bloom, Ming Liu, Fu-Chia Yang, Xiaodong Lu, Ankur Thomas, Bin Wang, Kate Henry, Aissatou Diallo, Merilla Michael, Darryna Guy-Williams, Joe Arndt, Thomas O. Cameron, Caitlin Meeks, Benjamin Smith, Mark Julian, Taras Tuczkewycz, Daniel Ferretti, Junghae Suh, Yuan Yuan

Biogen, Cambridge, MA

Safe and efficient gene delivery remains a major challenge for central nervous system (CNS)-targeted gene therapy. Recombinant adeno-associated viruses (rAAVs) have emerged as promising engineering scaffolds for CNS-targeting vectors, as demonstrated by a myriad of blood-brain barrier (BBB)-crossing and neurotropic vectors engineered through directed evolution in mouse and non-human primates (NHPs). While enabling selection of novel capsid variants capable of transducing target CNS cells through diverse mechanisms, most CNS-targeting directed evolution approaches produce capsids with uncertain human translatability due to genetic differences between human and model organisms. To overcome this limitation, we developed Differential Directed Evolution, a species-specific receptor-targeted approach to engineer CNS-targeting capsids with human translatability. We evolved in parallel a peptide insertion AAV library in WT mice and a humanized knock-in (KI) mouse

model, and bioinformatically identified human receptor-dependent CNS-targeting capsid candidates with significantly and reproducibly higher CNS transduction in humanized KI mice compared to WT controls. A number of peptide motifs emerged in the candidate pool, indicating a robust selection pipeline. To validate top candidates, we confirmed human receptor-dependent transduction in CHO cells as well as enhanced CNS transduction in humanized KI mice for a subset of variants. We selected several top hits and further evaluated them in singleton format in the humanized KI mouse model, and observed that all top capsids led to superior and widespread CNS transduction in KI mouse brains. In summary, we established Differential Directed Evolution as a novel approach combining directed evolution and humanized mouse models to engineer human-translatable AAV vectors. Through this method, we identified potent human receptor-targeted CNS capsids, which may facilitate therapeutic gene delivery for a variety of neurological disorders.

990. Identification of a CNS-Targeting AAV Capsid Family with NHP and Human Cross-Reactivity through Directed Evolution

Jin Quan[1], Ka Ying Hung[1], Samneet Kashyap[1], Emily Rouse[1], Margaret Kelemen[1], Xiaodong Lu[1], Misha Chittoda[1], Harini Dhamodaran Sampath[1], Samantha Desmarais[1], Shawn Kyle Bourque[1], Aditya Utturkar[1], Davide Gianni[1], Gregory Dillon[2], Sueon Sim[2], Nam-Kyung Yu[2], Kurt Cox[3], Bekim Bajrami[3], Anh Nhu[2], Pete Clarner[1], Richard Alexander Martino[1], Caitlin Meeks[4], Jimmy Tran[5], Amit Kumar[5], Tania Seabrook[1], Kate Henry[1], Jinkuk Choi[1], Shuyun Deng[1], Joseph Cabral[1], Junghae Suh[1], Yuan Yuan[1]

[1]Gene Therapy Accelerator Unit, Biogen, Cambridge, MA,[2]Genetic and Neurodevelopmental Disorders Research Unit, Biogen, Cambridge, MA,[3]Chemical Biology & Proteomics, Biogen, Cambridge, MA,[4]Biologics Drug Discovery, Biogen, Cambridge, MA,[5]Nonclinical Safety, Biogen, Cambridge, MA

The most promising route to achieve widespread therapeutic gene delivery to central nervous system (CNS) is by overcoming the blood-brain barrier (BBB). Directed evolution of peptide insertion AAV libraries has yielded a myriad of novel capsids capable of crossing the BBB and transducing CNS cells in mouse and non-human primates (NHPs). However, it remains to be demonstrated whether capsids evolved in model organisms are suitable for clinical applications, as cross-species genetic differences may impact engagement between capsids and cognate BBB receptor homologs. The key to unraveling the uncertainty around human-translatability, therefore, lies in identifying cognate BBB receptors followed by affinity comparison between capsids and receptor homologs. We have identified a CNS-targeting AAV capsid through three rounds of directed evolution in cynomolgus macaques. In adult cynomolgus macaques treated with immunosuppressants (n=3), this novel AAV capsid led to widespread, significantly higher CNS transduction compared to AAV9, with deep brain nuclei and brain stem showing highest overall transgene expression. In addition, the AAV capsid was well tolerated with minimal safety or pathology findings. To further improve the potency of this novel capsid, we screened in NHP a saturation mutagenesis AAV library carrying a conserved motif, and identified additional variants with further enhanced CNS and neuronal tropism.

991. Human CA4-Targeting AAV Capsids Efficiently Transduce the CNS in Humanized Mice

Nuria Roxana Botticello-Romero, Jason Wu, Qin Huang, Thomas Beddow, Ken Y. Chan, Pamela Brauer, Fatma Elzahraa Eid, Alina Chan, Benjamin E. Deverman

Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA

The development of adeno-associated viruses (AAVs) that efficiently target the human central nervous system (CNS) promises to boost the impact of gene therapies for neurological disorders. We engineered a suite of peptide-modified AAV9 capsids to cross the blood-brain barrier (BBB) by directly binding to the human GPI-linked enzyme Carbonic anhydrase IV (hCA4). Using our recently described receptor-targeting approach, we identified three capsids with distinct sequences, BI-hCA4-1, BI-hCA4-2, and BI-hCA4-3, that more efficiently transduce hCA4-expressing cells compared to wild type AAV9. When systemically injected into mice that express hCA4 on the brain microvasculature, the BI-hCA4 capsids transduced cells throughout the CNS more efficiently than AAV9 (Fig. 1). This enhanced tropism was not observed in wild type mice. We are now using site-saturation mutagenesis and a generative machine learning model paired with an AAV production fitness model to further improve the ability of the capsids to bind hCA4 and cross the BBB *in vivo*. In summary, we demonstrate that human CA4 is a promising new target for engineering CNS-directed gene delivery vectors.

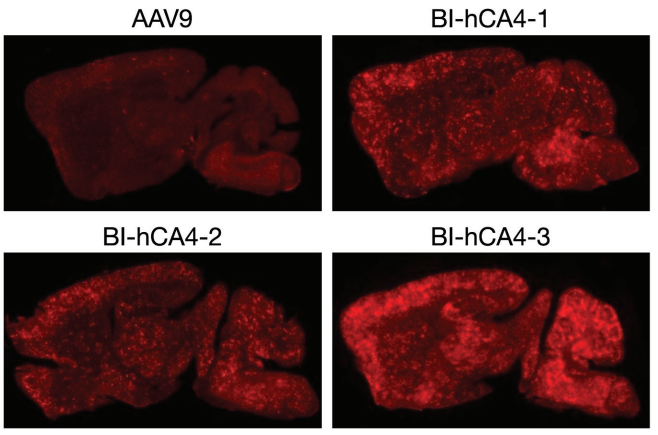


Figure 1. Human CA4-targeted capsids achieve efficient gene delivery to the CNS. The hCA4 gene was first delivered to the CNS endothelium by intravenous administration of AAV-BI30:CAG-hCA4-miR122-WPRE. Next, AAV9 or BI-hCA4:CAG-mScarlet-P2A-Luciferase-SV40pA were administered intravenously. Representative images show AAV transduction in sagittal brain sections of mice three weeks after the delivery of the hCA4-binding AAV.

992. Directed Evolution of AAV2 Libraries Yields Capsids with Improved Performance in the Central Nervous System and Cross-Species Translatability

Xiaojing Shi, Sean Gross, Michelle Ling, Reem Elteriefi, Zach Mason, Caden Holloway, Yixi Wang, Mark Chin, Christopher Octeau, Kimberly McDowell, Brandon Wheeler, Nicholas Flytzanis, Nick Goeden, Susan Catalano

Capsida Biotherapeutics, Inc., Thousand Oaks, CA

Central nervous system (CNS) disorders represent an area of major unmet medical need. The delivery of genetic therapies to the CNS is a significant challenge, particularly in the context of broad delivery throughout the brain and/or spinal cord. Intravenous delivery of engineered Adeno-Associated Virus (AAV) capsids capable of crossing the blood-brain barrier (BBB) is an attractive strategy for achieving broad and uniform transduction of the CNS. Current efforts to engineer AAV capsids for improved transduction of the CNS have largely focused on AAV9, primarily due to its ability to cross the BBB more efficiently than other serotypes. However, the use of AAV9 is limited by its high transduction of the liver and the resulting toxicity. Alternative AAV serotypes may offer distinct advantages regarding tropism, manufacturability, and safety profile. In light of this, we aimed to investigate other AAV serotypes for CNS tropism engineering, with a particular focus on AAV2, which has been extensively studied and utilized in clinical trials, providing a wealth of structural information that can aid in engineering efforts. We modified surface-exposed loops of AAV2 that are known to be important for brain and liver tropisms and employed our high-throughput, non-human primate (NHP)-based AAV engineering platform to evolve for capsids with CNS enrichment. Following two rounds of screening in NHPs, we identified three capsid families, defined by distinct consensus sequence motifs, that showed notable BBB-penetrance and liver de-targeting capabilities. Subsequent pool studies of the top capsids from each family in both NHPs and rodents highlighted one family with remarkably improved brain performance across species. Further characterization of a specific novel capsid from this family revealed a 50-fold increase in vector biodistribution in the brain and a 20-fold decrease in the liver compared to wild-type AAV9 in cynomolgus monkeys, along with extensive brain transduction in rodents. Continued engineering of this novel capsid yielded next-generation variants with up to 80-fold enhancement in CNS transduction relative to the parent novel capsid in NHPs. Taken together, our work identified AAV2-derived capsids with a combination of desirable characteristics, including robust CNS penetrance, cross-species translatability, and an improved safety profile due to reduced liver viral load. The identified variants have the potential to be utilized in future gene therapy applications for CNS disorders.

993. Unveiling Novel AAV Variants for Blood-Brain Barrier Crossing through Computational-Guided Directed Evolution

Eunhyun Ji, Eunji Lee, Kye Il Joo

Ewha Womans University, Seoul, Korea, Republic of

Adeno-associated viruses (AAVs) have emerged as common gene delivery vehicles for treating numerous human diseases. Gene therapy using AAV in neurological disorders is a leading platform because they are considered safe, low immunogenic, and long-term persistency. However, enabling the noninvasive transportation of AAVs across the blood-brain barrier (BBB) poses a substantial challenge in achieving optimal clinical applicability. In this study, we employed an integrated approach that combines experimental selection strategies and computational modeling to synthesize chimeric AAV capsid variants and predict their ability to traverse the BBB through AAV capsid-receptor binding. To discover the novel AAV capsid candidates with improved tissue- or cell-specific tropism for brain targeting, we generated a library of engineered AAV capsids using directed evolution techniques, including AAV capsid DNA shuffling and peptide display. A Next Generation Sequencing (NGS) analysis was conducted on the tissues and cells infected by the generated AAV library to isolate AAV candidates capable of penetrating the BBB. Concurrently, computational modeling was utilized to evaluate the binding affinity of the newly discovered AAV capsids to BBB-related receptors, facilitating a comparative analysis with experimental data. Taken together, these results enable the discovery of AAV capsid variants capable of crossing the BBB, primarily by quantifying their receptor binding affinities, through a comprehensive approach that integrates AI-enhanced structural prediction and molecular modeling. The outcomes of this investigation have the potential to guide the application of noninvasive, customized gene therapy for brain disorders.

994. AAV(BBB): A Novel AAV Variant Targeting the Central Nervous System with Super-High Efficiency and Specificity

Bing Wang, Yu Liu, Yuyan Wang, Leming Wang, Qizhao Wang, Daozhan Yu

AAVnerGene Inc., Rockville, MD

Treating diseases of the central nervous system (CNS) is notably challenging, largely because the blood-brain barrier (BBB) restricts the entry of many therapeutic drugs. Utilizing AAV vectors in gene therapy offers a potential solution to overcome this barrier. AAV9 is recognized for its ability to breach the BBB and is commonly employed for CNS delivery. Numerous studies have utilized AAV9 as a template to develop new AAV vectors with improved BBB-crossing capabilities, examples of which include AAV-Php.eB and AAV-Mac. Using our ATHENA I platform, we verified that AAV9 outperformed other AAV serotypes in brain tissue of C57B6 mice. However, AAV9 also shows significant transduction in heart, kidney, lung and liver. AAV-Php.eb, which is derived from AAV9, has also been reported to express highly in various tissues. Furthermore, the ability to cross the blood-brain barrier (BBB) varies among species. AAV-Php.eb, developed using the C57B6 mouse model, is effective only in this specific strain and not in others like B6C3, 129/SV or non-human primates (NHPs). Consequently, there is a significant demand for new AAV variants

with enhanced targeting to the central nervous system (CNS). In this context, we have engineered a new AAV capsid, named as AAV(BBB), based on the data of our ATHENA I evolution studies. AAV(BBB) shows a remarkable increase at RNA level by approximately 20-fold and at DNA level by 26-fold in mouse brain, Compared to AAV9. More importantly, AAV(BBB) demonstrates a specific affinity for the mouse brain, with ultra-low DNA accumulation and RNA expression in other organs including liver. Thus, AAV(BBB) has the potential to drastically increase the drug efficacy, and decrease AAV dose, side effects and cost for gene therapy of CNS disorders.

Thursday Posters: AAV Vectors - Preclinical and Proof-of-Concept In Vivo Studies

995. Optimizing AAV Dosing for *In Vivo* Screens in the Mouse Brain

Alex Lenail
MIT, Cambridge, MA

In vivo, AAV-driven CRISPR and ORF perturb-seq screens are emerging as powerful techniques to assay gene function in organismal contexts. In order to perform an in vivo perturb-seq screen, AAVs carrying CRISPR constructs or transgene open reading frames are delivered to an organism, with some method of targeting the organ system under study. The ideal dose of AAV yields as many study-relevant cells expressing a single AAV payload as possible per animal - too low a dose requires more animals, and too high a dose results in too many multiplets from which it is very challenging to garner useful data. The relationship between AAV dose and infected cells is relatively straightforward in vitro, where "poisson loading" results in a characteristic relationship between AAV dose and infection. However, in the case of an organism, cells may be differentially vascularized, differentially or susceptible to infection, resulting in the need for empirical measurement of cargo expression at different AAV dosages in the in vivo cells under study. We performed a dose-escalation experiment to ascertain the optimal dose of AAVs to perform efficient in vivo perturb-seq screens in the murine brain by systemic injection of AAV capsid PHP.eB. We found that infectivity in different brain regions differed substantially, in agreement with known vascularization patterns. We found strikingly diminishing returns with increasing AAV dosage. Finally, we found no evidence for cooperativity among AAV infections, providing a green light for perturb-seq screens in the murine CNS.

996. Gene Therapy Development for DDX3X Syndrome

Andrea Boitnott, Anjala Jiji, Yuhui Hu, Xin Chen, Steven J. Gray
UT Southwestern Medical Center, Dallas, TX

DDX3X syndrome is a neurodevelopmental disorder first described in 2015, and today it is thought to be one of the leading known genetic causes of intellectual disability in females. The syndrome is caused by mutations in the Dead-Box Helicase 3 X-Linked gene (*DDX3X*), an abundant and ubiquitously expressed RNA helicase. Despite the clinical burden of DDX3X syndrome and the known genetic cause, no study to date has determined whether gene-based therapies have the potential to treat this disorder. Our hypothesis is that DDX3X gene reinstatement, after altered DDX3X function during prenatal development, will restore proper protein function and, therefore, ameliorate disease symptoms preclinically in a timepoint dependent manner that is sensitive to the level of DDX3X. To determine preclinically if gene replacement therapy for DDX3X syndrome is beneficial, we developed a first generation AAV9 gene therapy to test in mice. In this vector, a functional copy of *DDX3X* is expressed from a relatively weak ubiquitous promoter. Mice modeling DDX3X syndrome, as well as littermate controls, were dosed at postnatal day 1 (P1) via two routes of administration—bilateral intracerebroventricular or lumbar intrathecal—and behavior phenotypes were assessed across development from P2-20 and as adults, at 3 and 12 months. The treated DDX3X syndrome mice showed limited evidence for benefit in a subset of all tests conducted, including time in center of an open field and gait analysis. Conversely, some behaviors became worse, such as decreased rotarod performance. Our results suggest that the dosage of DDX3X is important for behavior, where not enough or too much expression could each lead to altered behavior. To further test the dose-sensitivity of DDX3X, we treated mice with an AAV9 vector that overexpressed DDX3X from a strong ubiquitous promoter, and this resulted in rapid death of mice as early as 1-week post-injection when dosed at P1. Our results clearly demonstrate that overexpression of DDX3X is detrimental, even relatively subtle overexpression that can occur with a deliberately weak promoter. These studies suggest that there could be beneficial outcomes with a DDX3X gene therapy approach, but only if expression can be carefully regulated to avoid transgene overexpression.

997. A Novel Approach to Treat Stuttering: Preclinical Gene Therapy in Animal Studies

Camryn Bragg, Ruli Zhang, Shahriar SheikhBahaei
Neuron-Glia Signaling and Circuits Unit, National Institutes of Neurological Disorders and Stroke, Bethesda, MD

Stuttering is a neurodevelopmental disorder that affects nearly 70 million adults worldwide. People who stutter exhibit repetition, prolongation, pauses, or hesitations in their speech. Recently, it has been shown that specific mutations in the gene that codes for N-acetylglucosamine-1-phosphate-transferase subunits α,β, *GNPTAB*, cause stuttering symptoms in humans and rodents. Yet, the cellular and physiological consequences of the causal mutation are still unclear. Previously, our efforts to characterize the disorder in genetically engineered, *Gnptab*-mutant mice have shown effects on rhythmic motor behaviors, including breathing, grooming, and vocalization. In the present study, we evaluate the effectiveness of a gene therapy method to improve these motor behaviors in the mutant mice. Here, we used viral vectors to introduce a functional copy of the gene in P0 mice via intracranial injections. Our preliminary analysis suggests that pre-clinical gene therapy has improved vocal behaviors in the mutant animals. This study can be used as proof of principle that gene and cell therapies may be a possible avenue for the treatment of neurodevelopmental motor control disorders.

999. Allele-Specific Silencing of Mutant ATXN3 Mitigates Motor Deficits and Neuropathology in a Severely Impaired Mouse Model of Spinocerebellar Ataxia Type 3 (SCA3)

Ana Carolina Silva, Carina Henriques, Diana Lobo, Ana Rita Fernandes, Miguel Lopes, Dina Pereira, Sonia Duarte, Rafael Baganha, Sara Lopes, Magda Santana, Rui Jorge Nobre, Luis Pereira de Almeida

Center for Neuroscience and Cell Biology, Coimbra, Portugal

Spinocerebellar Ataxia Type 3 (SCA3), also recognized as Machado-Joseph Disease (MJD), stands as a dominantly inherited and fatal neurodegenerative disorder characterized by the aberrant repetition of CAG trinucleotides within the ATXN3 gene. This anomalous expansion leads to a toxic gain-of-function in the ATXN3 protein, inducing neuropathology and neurodegeneration, particularly in the cerebellum. Presently, there exists no therapy capable of modifying disease progression.Consequently, our investigation delved into the therapeutic potential of an AAV encoding artificial microRNAs (miATXN3) precisely targeting the mutant ataxin-3 allele. We administered this therapy via intra-cisterna magna (ICM) in severely impaired transgenic SCA3 mice.Motor performance analysis unveiled significant and robust enhancements in coordination, balance, and gait at 5-, 8-, and 11-weeks post-injection, as assessed through rotarod performance, beam-walking testing, and CatWalk XT analysis. Importantly, histological analysis indicated an improvement in neuropathology, evident through a reduction in the number of ATXN3 inclusions and the prevention of layer thickness shrinkage within the cerebellar lobules of AAV9-miATXN3-injected mice when compared with vehicle-treated counterparts. These outcomes were reinforced by a dose-dependent reduction in mutant ATXN3 mRNA levels in the cerebellum. Furthermore, the end-of-experiment assessment (13 weeks post-ICM injection) revealed widespread detection of AAV genome copies and miATXN3 levels in disease-relevant brain regions. In summary, our findings endorse an AAV-based therapy for SCA3, marking a significant stride toward imminent clinical translation for SCA3 patients.

1000. Developmental Age Effects for AAV9/SLC6A1 Gene Therapy in Heterozygous and Homozygous SLC6A1 KO Mice

Weirui Guo, Matthew Colby Rioux, Frances Shaffo, Yuhui Hu, Steven J. Gray

University of Texas Southwestern Medical Center, Dallas, TX

The human SLC6A1 gene encodes the highly conserved gamma-aminobutyric acid (GABA) transporter GAT-1, a protein crucial for GABAergic signaling in the central nervous system (CNS), and thus crucial for maintaining the excitatory/inhibitory signaling balance in the brain. A subset of patients presenting with rare forms of infantile encephalopathy with intellectual disability (ID) have been shown to be heterozygous for loss-of-function mutations in SLC6A1.To test our hypothesis that broad delivery of the human SLC6A1 gene across the CNS using adeno-associated virus type 9 (AAV9) to restore normal GABAergic signaling can provide an effective treatment and long-term therapeutic benefit for SLC6A1-Related Disorder, we designed two AAV9 vectors with different promoters (one is a weak universal promoter - JeT; the other is a neuronal-specific promoter - MeP229) and conducted preclinical gene therapy studies using heterozygous and homozygous SLC6A1 KO mice at different developmental ages. Both vectors were well-tolerated when administered to WT mice intrathecally (IT), at either post-natal day (PND)7-10 or PND28, out to 6 months and 1-year post-injection, respectively. The two AAV9/SLC6A1 vectors were tested separately via intracerebroventricular (ICV) administration in neonatal mice, lumbar intrathecal (IT) administration at the age of postnatal day (PND) 5, 10 or 28, and intravenous (IV) administration of AAV-PHP.eB at PND23. Neonatal ICV administration of both vectors ($3x10^{11}$ vg per mouse) resulted in significantly reduced seizures assessed by EEG in heterozygous and homozygous SLC6A1 KO mice, as well as improvement in several behavioral phenotypes of homozygous SLC6A1 KO mice. IT administration of both vectors ($5.6x10^{11}$ or $1.12x10^{12}$ vg per mouse) at PND5 showed near-complete normalization of EEG patterns in $SLC6A1^{+/-}$ heterozygous mice at either dose. Interestingly, the abnormal EEG patterns were not rescued in the homozygous $SLC6A1^{-/-}$ KO mice, suggesting that complete absence of GAT1 through early postnatal development makes them refractory to treatment rescue. Likewise, IT administration to PND10 $SLC6A1^{-/-}$ KO mice showed modest and statistically non-significant improvements in nest building and rotarod, but no improvement in EEG. The PHP.eB/SLC6A1 (IV) administration at PND23 ($2x10^{10}$, $2x10^{11}$ or $1x10^{12}$ vg per mouse) failed to reduce EEG seizures in heterozygous or $SLC6A1^{-/-}$ KO mice, and the $2x10^{10}$ or $2x10^{11}$ vg doses only showed a moderate nest building behavioral improvement (the 1 $x10^{12}$ vg group wasn't assessed). Treatment at PND 28 in $SLC6A1^{-/-}$ KO had no apparent treatment benefit. When administered ICV to PND1 mice, the JeT vector resulted in approximately 50% early mortality and some sporadic adverse effects while the MeP229 vector resulted in 17% early morality also with some sporadic adverse effects. No adverse effects were observed across all the IT or IV treated animals, except for some possible sporadic events in the JeT PND5 injection group (<5% of animals). Overall, our data demonstrate compelling treatment activity when mice are treated at an early development age (neonatal and PND5). We also identified that outside of the neonatal treatment window, the severe homozygous KO model is refractory to treatment, whereas the heterozygous mice that genotypically match human patients have displayed remarkable treatment activity. Treatment of heterozygous mice older than PND5 is ongoing, but the lack of rescue at PND23 with PHP.eB suggests that early intervention is critical for this disorder.

1001. Dorsal Root Ganglion Pathologies and Responses to Gene Therapy in Mice with Sialidosis

Wuh-Liang Hwu[1,2], Yu-Han Liu[1], Karine Chang[1], Hao-Chun Wang[1], Ni-Chung Lee[3], Yin-Hsiu Chien[3]

[1]Nation Taiwan University Hospital, Taipei, Taiwan,[2]Center for Precision Medicine, China Medical University Hospital, Taichung, Taiwan,[3]National Taiwan University Hospital, Taipei, Taiwan

Patients with sialidosis (mucolipidosis type I) type I typically present with myoclonus, seizures, ataxia, cherry-red spots, and blindness because of mutations in the neuraminidase 1 (*NEU1*) gene. Although ataxia is one of the main symptoms of the disease, there is no loss

of cerebellar Purkinje neurons in the disease. We created a *Neu1* knockout (*Neu1*$^{-/-}$) mouse model of sialidosis, and the homozygous *Neu1*$^{-/-}$ mice exhibited impair rotarod performance. We examined the dorsal root ganglia (DRG) of *Neu1*$^{-/-}$ mice. Grossly, DRG of *Neu1*$^{-/-}$ mice were larger than those of wild-type mice. H&E staining of the affected DRG revealed vacuolation of small cells (microglia) and pale cytoplasm of large cells (neurons). Immunofluorescence staining was performed for the storage material LAMP1. DRG neurons of *Neu1*$^{-/-}$ mice exhibited a variable, homogeneous increase in LAMP1 staining intensity, while microglia exhibited strong and clumping LAMP1 stain. However, the shape and number of peripherin (nociceptor) or neurofilament (mechanoreceptor) positive neurons looked similar between the wild-type and *Neu1*$^{-/-}$ mice. Ultrastructure of DRG was further elucidated by electron microscopy. Microglia were found to contain large vacuoles. On the contrary, neurons were filled with small vacuoles more homogeneous in size with depletion of rough endoplasmic reticulum, but the morphology of mitochondria still looked normal and there was no degeneration of nuclei. We have developed an adeno-associated viral (AAV) vector AAV9-P3-NP which included the human *NEU1* promoter, *NEU1* cDNA, IRES, and *CTSA* cDNA. In *Neu1*$^{-/-}$ mice received neonatal injections of AAV9-P3-NP, LAMP1 accumulation decreased in both the neurons and microglia of DRG. Therefore, DRG-related symptoms of sialidosis may be treatable by AAV-mediated gene therapy.

1002. Gene Editing Strategies to Treat Spinocerebellar Ataxia Type 1

Kelly Fagan[1], Guillem Chillon[1], Ellie Carrell[2], Beverly L. Davidson[2]

[1]University of Pennsylvania, Philadelphia, PA,[2]Children's Hospital of Philadelphia, Philadelphia, PA

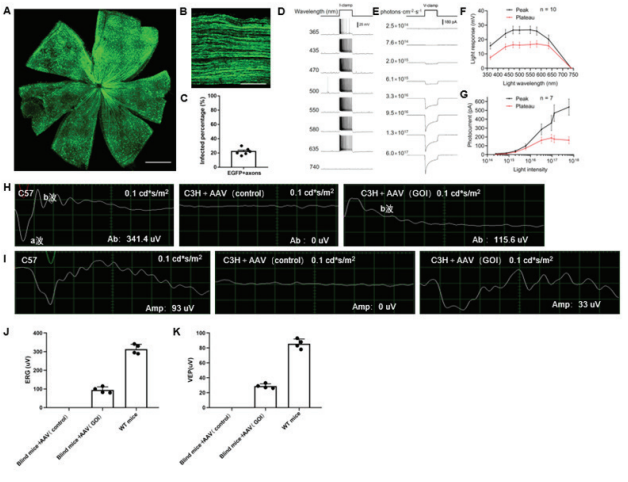
Spinocerebellar ataxia type 1 (SCA1) is a dominantly inherited neurogenetic disease caused by expansion of the polyglutamine repeat region in *ATXN1*. Normal *ATXN1* alleles contain 6-42 CAG repeats with interspersed CAT nucleotides, while disease alleles have 39-100+ uninterrupted CAG repeats. SCA1 patients present with cerebellar Purkinje cell (PC) loss, progressive loss of motor coordination, respiratory issues and eventual death. Currently there are no disease modifying treatments; however, previous work from our laboratory has demonstrated the potential of gene therapy as a treatment modality, specifically RNAi. Here, we tested two CRISPR-Cas9 strategies aimed to reduce *ATXN1*; a single guide RNA (gRNA) that targets near the exon-exon junction to induce nonsense mediated decay and a second approach which employs a dual guide system to excise the CAG repeat region. Both single and dual gRNA strategies significantly reduced *ATXN1* RNA by 56% and 57%, respectively when screened in HEK-Cas9 cells. Next, we tested for editing in human cells and in mice models. SCA1 patient iPSCs and unaffected matched sibling donors were differentiated into neuronal progenitor cells and transgenes expressing *Sp*Cas9 and dual gRNAs were delivered via AAV1. Treated experimental cells showed full CAG deletion. In B05 mice, a transgenic model of SCA1 that expresses human *ATXN1* (*hATXN1*) with 82 uninterrupted CAG repeats under a PC-specific promoter, co-delivery of Cas9 and gRNA reduced ATXN1 mRNA levels by 36% (dual gRNAs) and 54% (single gRNA), respectively. Additionally, rotarod performance by B05 mice treated with the single gRNA strategy was significantly better than vehicle-treated B05 mice and not statistically different from wild-type animals. Importantly, there was no increase in inflammatory markers in treated animals. In combination, these data present CRISPR-Cas9 as a potential approach for SCA1 therapy.

1003. Optogenetic Therapy Partially Restores Vision in Blind Mice

Xiaohu Wei[1], Lingjie Cui[1], Shanzhong Zhang[1,2], Cheng Xie[1], Sijie Sun[1], Xiaohua Liu[1], Zhiping Cui[1], Shijun Weng[3]

[1]Ugenex Therapeutics Ltd.Co, Suzhou, China,[2]Otovia Therapeutics, Shanghai, China,[3]Institute of Brain Science of Fudan University, Shanghai, China

Retinal neurodegenerative diseases, such as retinitis pigmentosa (RP) and age-related macular degeneration (AMD), eventually lead to degeneration of photoreceptor cells, resulting in blindness. We are dedicated to restoring vision in patients through optogenetic therapy, utilizing the expression of photosensitive ion channel proteins in the remaining neurons of the retina. Our designed photosensitive ion channel products and their various variants, delivered via AAV2/7m8, induce the generation of electro-retinogram (F-ERG) in blind C3H mice under normal white light stimulation. Additionally, Visual evoked potentials (F-VEP) are recorded in the brain's visual cortex, indicating the restoration of the complete visual pathway. These products exhibit long-term expression for at least 14 months without adverse effects. Enhanced photosensitivity and photodynamics, significantly lower photosensitivity thresholds, increased light response speed, and maximum response frequency are observed in these products and their variants. Covering the visible light spectrum, with optimal absorption wavelength in the safe red-shifted range, the products demonstrate minimal phototoxicity, surpassing other photosensitive ion channel protein products. Given the favorable efficacy and advantages in light sensitivity, photodynamics, and wavelength characteristics observed in blind mice, these products hold significant potential in the treatment of retinal neurodegenerative diseases.



(A), In C3H mice with photoreceptor cells degenerative blindness at 12 weeks of age, intravitreal injection of AAV was performed, and retinal wholemount 7 weeks post-injection revealed efficient expression of the product in retinal ganglion cells (RGCs), with axons converging at the

optic nerve (B). Approximately 20% of RGCs were infected (C). (D), Whole-cell current-clamp recording RGCs in UGX-GOI-treated C3H retinal wholemount, the light-evoked spiking traces of GOI-expressed RGCs with 2 s light pulses at different light wavelengths under about 3×10^{17} photons/cm^{2}s. (E), Voltage-clamp recording RGC, the light-evoked current traces of GOI-expressed RGCs with 2 s light pulses at 580 nm under different light intensities measured as photons/cm^{2}s.(F), The light wavelength and voltage response relationship were measured at peak and plateau currents. n = 10. (G), The light intensity and current response relationship were measured at peak and plateau currents. n = 7. (H), F-ERG data were collected using a light intensity of 0.1 cd*s/m^2. Compared to the control group, the experimental group expressing our product exhibited recorded ERG signals. The a-wave, specific to photoreceptor cells, was minimal, while the b-wave, specific to inner layer cells, was relatively high, indicating restoration of the visual pathway in the eye region. Statistical analysis is represented in panel (J) n=4. (I), VEP data were also collected using the same light intensity, revealing recorded VEP signals in the experimental group expressing our product compared to the control group. This implies that the visual pathway in the eye region has been restored, and signals can be further transmitted to the visual cortex. Therefore, partial restoration of vision was observed in blind mice after expressing our product. Panel (K) presents the VEP statistical analysis, n=4.

1005. Validation of TDP-43 Splicing Repression as a Therapeutic Strategy for ALS and FTD

Tianyu Cao, Aswathy Mallika, Irika Sinha, Meghraj Baghel, Katie Irwin, Jonathan Ling, Philip C. Wong
Pathology, Johns Hopkins University School of Medicine, Baltimore, MD

Nuclear depletion and cytoplasm aggregation of Tar RNA-binding protein 43 (TDP-43) is a major pathology underlying amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD) and many neurodegenerative diseases. Previous findings showed that the major function of TDP-43 is splicing repression, which serves to protect the transcriptome by preventing aberrant splicing. Lossing TDP-43 in the nuclear leads to mis-splicing of cryptic exons, and especially depletion of a critical gene for synapse function, UNC13A. However, whether restoring splicing repression can be a therapeutic strategy for neurodegeneration is still under investigation. Here we show that a previously characterized chimeric repressor (termed CTR), which possesses only splicing repression of TDP-43, is effective for TDP-43 loss of function. We found that in vivo delivery of CTR by AAV to a mouse model lacking TDP-43 in forebrain neurons showed a rescue of CA2/3 neurons loss and hippocampus shrink, together with a significantly reduced expression of the cryptic exons than the AAV GFP injected group, including the cryptic UNC13A expression in the hippocampus. Our results demonstrate that TDP-43 loss of function mediated cryptic splicing, calcium channel disruption and neurodegeneration can be effectively restored by CTR. This finding may provide preclinical evidence for the treatment of ALS/FTD.

1006. scAAV9.hGAMT Results in a Durable and Dose-Dependent Correction of the Biochemical Phenotype in a Mouse Model of Guanidinoacetate Methyltransferase (GAMT) Creatine Deficiency

Robyn T. Binsfeld[1], Troy B. Webster[1], Ilona Tkachyova[2], Emahnee Cover[3], Tesla Peretti[1], Brianna M. Quinville[1], Melissa Mitchell[4], Andreas Schulze[2,5], Jagdeep S. Walia[4,6]
[1]Centre for Neuroscience Studies, Queen's University, Kingston, ON, Canada,[2]Peter Gilgan Center for Research and Learning, The Hospital for Sick Children, Toronto, ON, Canada,[3]Faculty of Arts and Science, Queen's University, Kingston, ON, Canada,[4]School of Medicine, Department of Pediatrics, Queen's University, Kingston, ON, Canada,[5]Departments of Pediatrics and Biochemistry, University of Toronto, Toronto, ON, Canada,[6]Kingston Health Sciences Centre, Kingston, ON, Canada

Guanidinoacetate methyltransferase (GAMT) is an essential enzyme in the biosynthesis of creatine, a small molecule that plays an important role in energy metabolism. Creatine deficiency disorders are inborn errors of metabolism resulting in several neurological manifestations including developmental delay, intellectual disability, and epilepsy, emphasizing the importance of creatine's role in the brain. Endogenous synthesis of creatine takes place through the enzymatic function of arginine:glycine amidinotransferase (AGAT) to form guanidinoacetate (GAA) which is then converted to creatine by GAMT. GAMT-deficiency is an autosomal recessive disorder resulting in little to no available intracellular creatine accompanied by an accumulation of the potentially neurotoxic metabolite, GAA. Current treatments for GAMT-D focus on diet intervention to supplement creatine and reduce GAA production, however this does not restore creatine or GAA levels in the brain, leaving many of the symptoms unattended. Previously we presented a proof-of concept study showing that the *scAAV9.hGAMT* vector had the potential to restore expression and function of GAMT in murine models of GAMT-D following intrathecal administration at 7 weeks post-treatment. Here, for the first time we now show the dose-response following treatment with *scAAV9.hGAMT* resulting in durable expression detected at 5-months post-treatment. *scAAV9.hGAMT* was administered intrathecally at three doses ranging from $6.25e^{10}$ to $2.5e^{11}$vg/mouse. A dose-dependent increase in creatine and decrease in GAA accumulation was detected in several tissues and the serum of treated mice including the central nervous system. Monthly blood collections showed these metabolites stabilized at 6 weeks post-treatment and were maintained until the long-term endpoint of 20 weeks post-injection in the serum. GAMT protein was restored and detectable by western blotting in the liver at both short and long-term endpoints in a dose-dependent manner. Overall, we have shown that *scAAV9.hGAMT* represents a promising therapeutic for the treatment of GAMT creatine deficiency and is under further investigation for safety and dose optimization for better translation for clinical use.

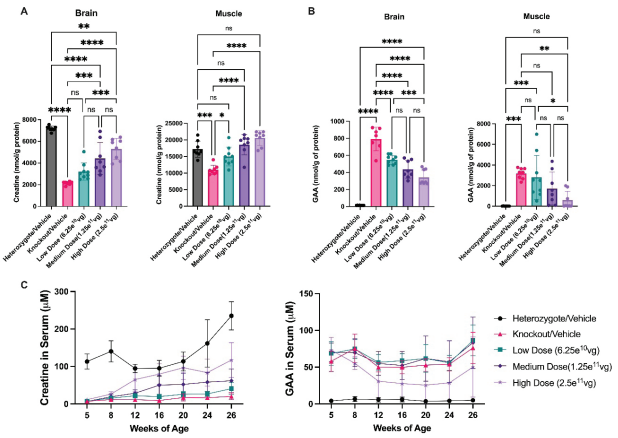


Figure 1. Creatine and GAA levels are corrected in a dose-dependent manner in treated animals compared to untreated controls. A. Creatine is significantly increased in animals treated with the medium and high doses compared to untreated knockouts in both the brain and muscle tissues. **B.** GAA levels are significantly decreased in the brain at all three doses however this is only evident at the high dose in the muscle tissues. **C.** Creatine increased over time in the serum with the greatest increase observed at the highest dose and no differences seen with the lowest dose. This increase stabilized at 6 weeks post injecton and remained for the duration of the study until the endpoint at 20-weeks post-treatment. GAA accumulation decreased over time following the same trend as the increase in creatine, however there was no observable differences in the medium or low doses compared to untreated knockouts. (* p<0.05, **p<0.01, ***p<0.001, **** p<0.0001)

1007. Considerations for Species Differences in Pre-Clinical Gene Therapy Development

Meghan Eller, Krishanna Knight, Rachel M. Bailey

UT Southwestern Medical Center, Dallas, TX

For gene therapy proof-of-concept studies, cell and animal models are used to demonstrate safety and therapeutic benefit, which typically involves expressing human genes in a non-human species. For many genes, this cross-species approach is well tolerated due to high homology, however species compatibility can still be an issue even in well-conserved genes. In this study, we aimed to determine the species compatibility between human and mouse *ECHS1*, a gene that encodes short-chain enoyl-CoA hydratase (ECHS1) with 88% species homology. ECHS1 functions within mitochondria to catabolize branched chain amino acids and fatty acids, supporting energy production within cells. Loss-of-function variants in ECHS1 cause a Leigh-like syndrome, and the feasibility of developing a gene replacement therapy for this disorder has yet to be explored. Towards this, 2 self-complementary AAV9 constructs were developed, which both contained codon optimized human *ECHS1* cDNA driven by a weak ubiquitous promoter. The second vector transgene was also CpG depleted in an effort to reduce immunogenicity. Prior to virus production, both plasmids were confirmed to express ECHS1 at similar levels following transfection of cells. To assess toxicity in vivo, wildtype (WT) mice were treated with high and low doses of each virus via intrathecal lumbar puncture at post-natal day 10. There were no differences in weight or survival across treatments or compared to vehicle, however, 70% of mice that received a high dose of the CpG-depleted transgene developed progressive hindlimb clasping that did not resolve up to 1-year post-injection. This behavior was not associated with any histopathological abnormalities across tissues at 2 months post-injection, but animals that developed clasping had significantly higher ECHS1 expression than animals without clasping. This suggests that overexpression of human ECHS1 is not well-tolerated in mice, although it is unknown whether this is due to species differences or to ECHS1 itself. To further investigate potential species incompatibility with ECHS1 overexpression, human (HEK293T) and mouse (N2A) cell lines were used. Cell viability and ATP levels were used as indicators of overall cell health and ECHS1 function. When ECHS1 is reduced by genetic knock-out or siRNA, ATP levels are significantly decreased in both mouse and human cells. Interestingly, when human ECHS1 was overexpressed in mouse cells, viability was not affected but ATP levels were significantly reduced. In contrast, ATP levels were not impacted by mouse Echs1 overexpression in mouse cells. Further, overexpression of human ECHS1 in human cells led to a two-fold increase in ATP levels, suggesting improved cell health and/or increased ECHS1 function. Taken together, these results support that overexpression of ECHS1 may not be inherently toxic, and rather there is species incompatibility. As ECHS1 functions as a homohexamer, co-expression of human and mouse ECHS1 may have a dominant negative effect and reduce functionable ECHS1 complexes. Experiments to overexpress mouse ECHS1 in human cells are ongoing and will be reported. Future studies will develop AAV9 constructs to overexpress mouse Echs1 in WT mice, which we hypothesize will be well tolerated. These findings emphasize the importance of considering species compatibility in pre-clinical studies, and caution scrutiny of the impact of human transgene expression in different model systems.

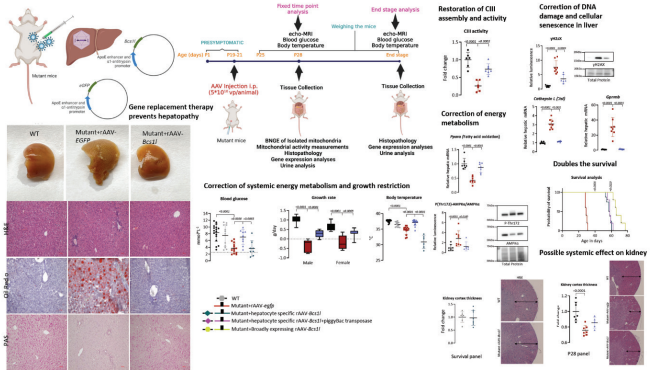
1008. Effective Treatment of Complex III Deficient Mice with Hepatocyte-Targeted Gene Therapy

Rishi Banerjee, Janne Purhonen, Vineta Fellman, Jukka Kallijärvi

Faculty of Medicine, University of Helsinki, Helsinki, Finland

Mitochondrial disorders comprise a large group of monogenic inborn errors of metabolism, typically with compromised respiratory chain (RC) function, leading to a wide spectrum of manifestations from myopathies to encephalopathies and multi-organ disease. Mutations in the RC complex III (CIII) assembly factor gene *Bcs1l* are the most common cause of CIII deficiency (1). The most severe of them is GRACILE (Growth Restriction, Aminoaciduria, Cholestasis, liver Iron overload, Lactic acidosis, and Early death) syndrome, described in Finland (2). Our group generated a GRACILE mutation knock-in (Ser78Gly mutation in Bcs1l) mouse model that recapitulate most of the clinical manifestations (3, 4). The *Bcs1l*$^{p.S78G}$ mice show a severe juvenile-onset disease with growth restriction, kyphosis, and decreased bone mineral density and fat mass. Early histopathology and CIII deficiency are mainly seen in the liver and kidney. It is not known to which degree the concomitant systemic and metabolic phenotypes growth restriction, loss of adipose tissue and hypoglycemia dependent on the liver. Therefore, to interrogate the contribution of the metabolically compromised hepatocytes, we used recombinant adeno-associated viruses (rAAVs), serotype 9 and a hepatocyte-specific human ApoE enhancer and α1-antitrypsin promoter, to express wild-type BCS1L in the *Bcs1l*$^{p.S78G}$ liver starting at presymptomatic age (P21). . Along with the expression cassette, the vector also has flanking PiggyBac transposon recognition sequences, which ensures efficient integration. To ensure persistent expression, we used this novel bi-virion AAV system, which consists of the vectors carrying *Bcs1l* and PiggyBac transposase, and the survival was analyzed. In the juvenile mice, a single intraperitoneal injection of rAAVs improves CIII assembly and activity and normalizes mitochondrial alterations in mutant liver. Moreover, the gene replacement therapy prevented lethal hepatopathy, like lipid accumulation, glycogen depletion in juvenile mice. It also prevented cell proliferation, DNA damage and cellular senescence in liver.

Moreover, it also rescued all the metabolic error, critical for systemic effect by liver. Strikingly, the liver-targeted gene therapy was sufficient to correct systemic phenotypes like hypoglycemia, hypothermia and decreased growth. Lastly, it dramatically increases the survival of these animals by two fold. Thus, we propose that liver-targeted gene therapy is an effective strategy to treat mitochondrial disease phenotypes with mainly visceral manifestations, such as GRACILE syndrome. Reference 1. Banerjee, R., Purhonen, J. and Kallijärvi, J. (2021), The mitochondrial coenzyme Q junction and complex III: biochemistry and pathophysiology. FEBS J. https://doi.org/10.1111/febs.16164 2. Fellman, V., Rapola, J., Pihko, H., Varilo, T. & Raivio, K. O. Iron-overload disease in infants involving fetal growth retardation, lactic acidosis, liver haemosiderosis, and aminoaciduria. Lancet Lond. Engl. 351, 490-493 (1998) 3. Levéen P, Kotarsky H, Mörgelin M, Karikoski R, Elmér E, Fellman V. The GRACILE mutation introduced into Bcs1l causes postnatal complex III deficiency: a viable mouse model for mitochondrial hepatopathy. Hepatology. 2011;53(2):437-447 4. Purhonen, J., Grigorjev, V., Ekiert, R. et al. A spontaneous mitonuclear epistasis converging on Rieske Fe-S protein exacerbates complex III deficiency in mice. Nat Commun 11, 322 (2020)



1009. NGGT006, an Advanced Gene Therapy Vector for Treating Familial Hypercholesterolemia

Shengmei Wen[1], Hai Zhang[1], Yufeng Guo[1], Qingzhou Wang[1], Yiting Liu[2], Guang Qu[1,2], Lixin Jiang[1,2]

[1]NGGT (Suzhou) Biotechnology Co., Ltd., Suzhou, Jiangsu, China,[2]NGGT, Inc, Walnut Creek, CA

Introduction: Homozygous Familial Hypercholesterolemia (HoFH) is a rare and devastating genetic metabolic disorder characterized by absent or severely reduced capacity to catabolize circulating Low density lipoprotein (LDL) particles by the hepatic LDL receptor (LDLR). HoFH patients present abnormally high total plasma LDL-cholesterol (LDL-C) levels, resulting in severe atherosclerosis and often leading to early onset of cardiovascular disease. Treatment aimed to reduce plasma LDL-C level for these patients is therefore essential. Unfortunately, most HoFH patients are unresponsive or respond very poorly to the existing therapies which cannot overcome the deficiency of LDLR. Thus, functional replacement of the defective LDLR via AAV-based liver-directed gene therapy could be a valuable approach to treat HoFH. **Methods/Results:** Using an optimized expression cassette combined with a high liver-tropism AAV vector, we have developed NGGT006, an rAAV8-based vector expressing human LDLR (rAAV8-hLDLR). In the hepatocyte line Huh7 cells infected with different multiplicities of infection (MOIs) of NGGT-006, dose-dependent increases of the expression of hLDLR protein as well as the activity of LDL-C particle uptake were observed, indicative of an effective in vitro biopotency of hLDLR delivered by NGGT006. In the LDLR-deficient mice on Western Diet containing 0.21 % cholesterol and 40% fat, a single intravenous (IV) injection of NGGT006 significantly corrected hypercholesterolemia at the dose as low as of 8.0E10 vector genome (vg)/kg. At the dose of 4.0 E11 vg/kg, 12 weeks after injection, NGGT006 effectively reduce the area of atherosclerotic deposition with substantially reduced LDL-C and total cholesterol (TC) in the peripheral blood. Furthermore, NGGT006 demonstrated therapeutic effects in LDLR gene depleted golden syrian hamsters, another rodent model of FH. It has been reported that coronary atherosclerosis in the aged LDLR null hamsters exhibit similar lipid metabolic profiles as those in humans. In these hamsters on normal chow diet, NGGT006 at 1.5E13 vg/kg significantly and sustainedly reduced serum LDL-C during a 32-week period after a single jugular vein injection. In LDLR null hamster fed on a high fat chow diet (containing 0.5 % cholesterol and 33% fat) with abnormally high serum LDL-C induced, NGGT006 at 1.25E13 vg/kg also achieved a robust 50% reduction of serum LDL-C and TC from 2-week post injection and maintained the effect for at least 27 weeks. In addition, when injected into LDLR +/- hamsters on normal chow diet, NGGT006 at 1.5E13 vg/kg significantly and stably reduced the level of serum LDL-C for 20 weeks. **Conclusions:** These studies demonstrate the therapeutic efficacy of liver-directed gene therapy approach of hLDLR gene replacement and support the further development of NGGT-006 in treating FH.

1010. NGGT003, an Advanced Gene Therapy Vector Carrying a New FVIII Variant for Treating Hemophilia A

Qing Cao[1], Qingzhou Wang[1], Yufeng Guo[1], Shengmei Wen[1], Yiting Liu[2], Guang Qu[1,2], Lixin Jiang[1,2]

[1]NGGT (Suzhou) Biotechnology Co., Ltd., Suzhou, Jiangsu, China,[2]NGGT, Inc, Walnut Creek, CA

Introduction: Hemophilia A (HA) is an X-linked bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII), resulting in extensive bleeding tendencies, hematomas and joint deformities. A common treatment for hemophilia A is protein replacement with recombinant or plasma-derived coagulation FVIII. However, this therapy requires lifelong medication. AAV-based gene therapy for hemophilia A could achieve a lifetime cure by a single dose. Roctavian was recently approved for treating HA at a dose as high as 6E13 vg/kg. In order to improve the treatment efficiency and reduce the potential risks caused by high doses, we developed NGGT003, an rAAV8 based vector carrying a new human FVIII (hFVIII) variant, hFVIII-L1, aiming to increase plasma hFVIII level compared to the commonly used hFVIII-SQ and to reduce the treatment dose. **Methods/Results:** In HepG2 cells overexpressing the AAV receptor (HepG2-AAVR), 5- to 24-fold higher activity of hFVIII was detected in the supernatant of cells infected with NGGT003 (AAV8-hFVIII-L1) than AAV8-hFVIII-SQ at different multiplicities of infection (MOIs). In the FVIII -/- mouse model, 6 weeks after tail vein injection of vectors at a dose of 4E12 vg/kg, plasma hFVIII activity mediated by NGGT003 was 7.1-fold higher than that by AAV8-hFVIII-SQ, as well as a shorter bleeding

time. NGGT003 significantly reduced the bleeding time at the dose as low as 8E11 vg/kg. **Conclusions:** These in vitro and in vivo results demonstrated that NGGT003 achieved higher efficacy at a low dose, suggesting that it has a great potential to improve the treatment for HA patients at a much lower dose to ensure the safety.

1011. *In Situ* Gene Editing of Hematopoietic Stem Cells via AAV-Delivered CRISPR Guide RNAs

Alborz Karimzadeh[1], Rebekah Kim[1,2], Vivian Garcia[2], Michael Florea[2], Bryan Peacker[2], Shio Kobayashi[1], Drake Watkins[1], Kathleen Messemer[2], Jing Zeng[3], Daniel E. Bauer[3], Thomas F. Serwold[1], Amy Wagers[1,2]

[1]Joslin Diabetes Center, Boston, MA,[2]Harvard University, Cambridge, MA,[3]Boston Children's Hospital/Harvard Medical School, Boston, MA

Hematopoietic stem cells (HSCs) are the self-renewing, multipotent, and engraftable source of all cells in the blood system. Accordingly, hematopoietic stem cell transplantation (HSCT) has been utilized as a definitive cure for hematological disorders. Although HSCT has been improved upon for decades, complications associated with HSCT still relegate it to a last-resort option in many cases. Efficient gene editing of HSCs while they remain in the body has the potential to eliminate the requirement for HSCT while still leveraging the potency of HSCs for blood cell repopulation in treating monogenic blood disorders. Towards this goal, in this study, we aimed to optimize adeno-associated virus (AAV) vector design and delivery to achieve CRISPR guide RNA (gRNA) mediated *in situ* HSC gene editing in *Sp*Cas9-expressing mice. Using Ai9;*Sp*Cas9 reporter mice, which carry a broadly expressed *Sp*Cas9 transgene together with a Lox-STOP-Lox (LSL)-TdTomato reporter cassette, we first tested whether systemic administration of AAVs carrying *Sp*Cas9-compatible gRNAs designed to target sequences 5' and 3' of the LSL cassette would result in excision of the STOP codons and expression of TdTomato in HSCs. In adult mice, administration of single-stranded AAVs (ssAAVs) carrying one copy of the 5' gRNA and one copy of the 3' gRNA ("1x gRNAs") yielded rare, but readily-detectable, TdTomato+ HSCs, demonstrating the sensitivity of this system to reveal gene-editing events at the single-cell level, even in rare HSCs. We then leveraged this platform to identify modifications that might improve HSC editing rates. Substitution of a self-complementary AAV (scAAV) format improved editing rates by ~6-fold compared to ssAAV-1xgRNAs. Inclusion of an additional copy of each gRNA also boosted HSC editing rates by ~25%. Vectors incorporating both these modifications achieved editing rates of more than 5% TdTomato+ HSCs in adult animals injected with scAAV-2xgRNAs. Notably, CFU and transplantation assays confirmed that such *in situ* AAV-CRSIPR edited HSCs sustain intact multi-lineage engraftment potential. We also evaluated the adaptability of our platform to earlier intervention strategies by systemic delivery to neonatal Ai9;*Sp*Cas9 animals. Excitingly, we observed HSC editing rates exceeding 20% of the total HSC pool in mice injected within the first 3 days of life with scAAV-2xgRNAs and, for the first time, documented multi-vector transduction of HSCs *in situ* upon dual AAV delivery of separated CRISPR machinery. Finally, we tested the potential therapeutic utility of *in situ* CRISPR editing using gene-modified mice carrying the constitutive *Sp*Cas9 transgene in which the mouse hemoglobin A (HBA) and HBB genes had been replaced with human HBA, HBG, and HBB or HBS (sickle HBB). Systemic delivery of scAAV carrying gRNAs targeting the BCL11A binding motif, located within the HBG promoter, effectively targeted this motif in HSCs, resulting in ~40% modified reads and modest induction of fetal hemoglobin (HbF). Taken together, this study introduces a new and effective platform for AAV-mediated *in situ* modification of HSC genomes in mice, delineates multiple design features that promote robust gene editing in these cells, and offers initial proof-of-concept for the therapeutic application of this approach to hemoglobinopathies and potentially other congenital blood disorders.

1012. AAV-hTREM2 Reduces Pathological Amyloid-Beta in 5xFAD Mouse Model

Jessica Willen[1], Carter Daniels[2], Jeremiah Hendricks[2], Milankumar Kothiya[2], Priyam Raut[2], Lindsay Carter[3], Neda Masoudi[4], LiChin Wong[2], Sitharthan Kamalakaran[2], Ben Shykind[5], Franz Hefti[2], Mansuo Shannon[2], Anindya Sen[4]

[1]Preclinical Research and Development, Eli Lilly, New York, NY,[2]Eli Lilly, New York, NY,[3]Eli Lilly, Indianapolis, IN,[4]Prevail Therapeutics, New York, NY,[5]Prevail Therapeutics/Eli Lilly, New York, NY

TREM2 (Triggering receptor expressed on myeloid cells-2) is a cell surface transmembrane glycoprotein expressed primarily in cells of myeloid lineage, including microglia. TREM2 directly binds to amyloid-beta, facilitating microglial congregation around plaques, limiting plaque accumulation and promoting plaque removal *TREM2* mutations associated with increased risk of AD decrease microglial response to amyloid plaques. In mouse models of AD, increasing TREM2 levels enhances microglia activation, promoting their clustering around plaques, amyloid-beta uptake and plaque compaction. AAV-hTREM2 is a gene therapy candidate designed to reduce deposition and toxicity of amyloid-beta in AD by increasing TREM2 levels. The hTREM2 vector was packaged in two different capsids, AAV9 and AAV-PRV, a novel capsid which shows broader CNS biodistribution and an increased microglial transduction compared to AAV9. In the 5xFAD mouse model of AD, we evaluated the effects of AAV9-hTREM2 and AAV-PRV-hTREM2, administered by intracerebroventricular (ICV) injection. Post-treatment, animals were assessed for biodistribution, TREM2 protein levels, and amyloid-beta levels. hTREM2 dosing in this model resulted in broad biodistribution and dose-dependent increases in TREM2 protein levels for both capsids, but significant reduction of amyloid-beta isoforms and aggregates only in mice treated with the AAV-PRV capsid. This study demonstrates the potential of a TREM2-based therapy for AD and highlights the critical importance of the optimal capsid for targeting disease-associated cell types.

1013. AAV-hTREM2 Gene Therapy, a Disease Modifying Treatment for ALSP Associated with CSF1R Mutations (ALSP-CSF1R)

Neda Masoudi[1], Jessie Willen[1], Carter Daniels[1], Beverly Ann Jenkins[1], Ellen Conceicao Furber[1], Milankumar Kothiya[1], Michael Banjoko[1], Raghu Gowda[1], Jeremiah Hendricks[1], Yi-Ya Fang[1], Priyam Raut[1], Adnan Arnaout[2], Fares Bassil[1], LiChin Wong[1], Garrett Daniels[1], Jorge Francisco Haller[1], Sitharthan Kamalakaran[3], Travis Lewis[3], Theresa Day[1], Ben Shykind[4], Mansuo Shannon[1], Franz Hefti[1], Anindya Sen[5]

[1]Preclinical Research and Development, Eli Lilly, New York, NY,[2]Eli Lilly and Company, New York, NY,[3]Eli Lilly, Indianapolis, IN,[4]Prevail Therapeutics/Eli Lilly, New York, NY,[5]Eli Lilly, New York, NY

Adult-onset Leukoencephalopathy with axonal Spheroids and Pigmented glia (ALSP) is a rare neurological disorder characterized by demyelination of white matter, spheroidal axons, and pigmented glia. Mutations in the colony stimulating factor 1 receptor (CSF1R) gene, a transmembrane tyrosine kinase receptor that expresses in brain microglia have been linked to the majority of ALSP cases. CSF1R function is necessary for the development and maintenance of microglia. Similar to CSF1R, TREM2 (Triggering receptor expressed on myeloid cells-2) is a receptor expressed in brain microglia, sharing an overlapping signaling pathway with CSF1R to activate microglia. Thus, we have developed PR009, a rAAV gene therapy to deliver a functional h*TREM2* gene, as a disease-modifying, one-time treatment for ALSP-CSF1R. PR009 is designed to enhance microglial function by increasing cellular TREM2 levels to compensate for CSF1R loss-of-function and ameliorate ALSP-CSF1R-associated pathophysiology. To test the efficacy of PR009, we developed *in vitro* and *in vivo* CSF1R-inhibition models to mimic CSF1R loss observed in patients. In a CSF1R-inhibition mouse model, treatment of PLX3397, a CSF1R inhibitor, resulted in depletion of the microglial pool. PR009 ICV dosing resulted in broad biodistribution throughout the CNS, dose-dependent increase in hTREM2 expression, effective attenuation of microglial depletion and maintenance of microglial health. Ongoing safety and tolerability studies in NHPs and rodents will provide valuable safety profile of this drug candidate. Currently, there is no disease-modifying therapy for ALSP-CSF1R and the use of available drugs is limited to managing disease associated symptoms. Our findings suggest that PR009 has the potential to treat the underlying cause of ALSP-CSF1R by expressing hTREM2 in the microglia of patients, resulting in restoration of microglial function and survival.

1014. The Postnatal Injection of AAV9-FOXG1 Rescues Corpus Callosum Agenesis and Other Brain Deficits in the Mouse Model of FOXG1 Syndrome

Shin Jeon[1,2], Jaein Park[2,3], Shibi B. Likhite[4], Ji Hwan Moon[2,5], Dongjun Shin[2,3], Liwen Li[2,3], Kathrin Meyer[4], Jae W Lee[2,3], Soo-Kyung Lee[2,3]

[1]Systems Pharmacology & Translational Therapeutics, Institute of Immunology, University of Pennsylvania, Philadelphia, PA,[2]FoxG1 Research Center, University at Buffalo, Amherst, NY,[3]Biological Science, University at Buffalo, Amherst, NY,[4]Nationwide Children's Hospital, Columbus, OH,[5]Samsung Genome Institute, Seoul, Korea, Republic of

Heterozygous mutations in the *FOXG1* gene manifest as FOXG1 syndrome, a severe neurodevelopmental disorder characterized by structural brain anomalies, including agenesis of the corpus callosum, hippocampal reduction, and myelination delays. Despite the well-defined genetic basis of FOXG1 syndrome, therapeutic interventions targeting the underlying cause of the disorder are nonexistent. In this study, we explore the therapeutic potential of adeno-associated virus 9 (AAV9) mediated delivery of the *FOXG1* gene. Remarkably, intracerebroventricular injection of AAV9-FOXG1 to *Foxg1* heterozygous mouse model at the postnatal stage rescues a wide range of brain pathologies. This includes the amelioration of corpus callosum deficiencies, the restoration of dentate gyrus morphology in the hippocampus, the normalization of oligodendrocyte lineage cell numbers, and the rectification of myelination anomalies. Our findings highlight the efficacy of AAV9-based gene therapy as a viable treatment strategy for FOXG1 syndrome and potentially other neurodevelopmental disorders with similar brain malformations, asserting its therapeutic relevance in postnatal stages.

1015. OCU410ST (AAV5-h*RORA*) Modifier Gene Therapy Rescues Retinal Degeneration in a Mouse Model of Stargardt Disease

Monica Akula[1], Pushpendra Singh[2], Neena Haider[3], Arun Upadhyay[4]

[1]Research and Development, Schepens Eye Research Institute, Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, MA,[2]Research and Development, Ocugen, Malvern, PA,[3]Research and Development, Schepens Eye Research Institute, Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts, USA, Boston, MA,[4]Ocugen, Inc., Malvern, PA

Background: Stargardt disease (STGD) affects ~ 1 million individuals globally. This rare disease is clinically characterized by slow loss of central vision in both eyes from early childhood. Underlying common cause involves >1,200 variants in gene mutations in the ABCA4 gene, which follows an autosomal recessive pattern of inheritance. Mutations of the ABCA4 gene lead to defective processing and transportation of all-trans retinaldehyde, from the photoreceptors to retinal pigment epithelium (RPE) cells. Our preclinical studies revealed that Retinoic acid related orphan receptor alpha (RORA), a nuclear hormone receptor, regulates the inflammatory response pathways that are impacted in Stargardt's disease demonstrating its therapeutic potential.
Methodology: We evaluated AAV5-*hRORA* as a modifier gene therapy

for STGD using the *Abca4*$^{-/-}$ mouse, an established model for STGD. A Low (1×10^8 vg/eye), Medium (1×10^9 vg/eye) or high (4×10^9 vg/eye) dose of AAV5-*hRORA* was administered subretinally in the *Abca4*$^{-/-}$ mice at one month of age. Fundus Blue Light Autofluorescence (BAF) imaging was used to measure the presence of lipofuscin deposits, Electroretinogram (ERG) analysis was performed to measure the retinal function. Immunohistofluorescence (IHF) was done to detect the presence of several molecular biomarkers. Follow -up assessments were performed at 1-, 4- and 7-months post-treatment. **Results:** Three different doses of *RORA* transgene delivered through AAV5-*hRORA* at 1 month (1M) showed reduced sub-retinal and RPE lipofuscin deposits in *Abca4*$^{-/-}$ treated eyes compared with untreated eyes correlating with improved photoreceptor function as assessed with electroretinography (ERG) analysis. Furthermore, *RORA*-treated *Abca4*$^{-/-}$ mice showed restored *CD59* expression at 4M and 7M and attenuated expression of amyloid precursor protein (APP) that gives rise to β-amyloid a drusen component, compared to untreated eyes. **Conclusions:** AAV5-*hRORA*-treated retinas demonstrate improvement in multiple clinical, functional, and molecular markers in *Abca4*$^{-/-}$ mice. This is the first study evaluating the impact of AAV5-*hRORA* in rescuing retinal degeneration associated with Stargardt disease. Ocugen, Inc. is currently conducting phase 1/ 2 safety and efficacy study for OCU410 in Stargardt patient (NCT05956626).

1016. Two Years Study of Safety and Efficacy of AAV Vaccine in a Preclinical Spontaneous Canine Model of Oral Melanoma

Ester Molina[1], Caitlin Yung[2], Colin Caine[1], Hisae Yoshitomi Kuoch[3], Karina Krotova[1], Antonella Borgatti[2], George Aslanidi[4]

[1]The Hormel Institute, Austin, MN,[2]University of Minnesota, Minneapolis, MN,[3]Hormel Institute University of Minnesota, Austin, MN,[4]University of Minnesota, Austin, MN

We previously demonstrated the possibility of using capsid and expression cassette optimized AAV vectors as cancer vaccine. Multivalent AAV vaccine which carry three melanoma antigens, glycoprotein 100 (Gp100), tyrosinase (Tyr) and tyrosinase related protein 1 (TRP1) proved its effectiveness in mice model. Oral melanoma (OM) is an aggressive disease that often courses with metastasis in distant organs, such as lungs and lymph nodes which eventually cause the death of the patients. The mainstream therapy is surgical removal of the primary tumor followed by intense chemotherapy or radiotherapy. The therapy is rarely effective and associated with serious adverse effects for the patients. Dog's immune system is much similar to humans, and they develop spontaneous OM with higher frequency than humans, which make them an ideal pre-clinical model to test our vaccine. 8 companion dogs with confirmed early stages of OM were vaccinated with equal amount of the 3 different AAV expressing human tumor antigens (Gp100, Tyr and TRP1). Some dogs have undergone surgery and radiotherapy prior injection of AAV vaccine. 5 males and 3 females were enrolled in the study with ages range between 6.5 and 14.3 years. 5 dogs received dose of $3x10^{12}$ vg/ dog and 3 animals received $9x10^{12}$ vg/ dog. Animals were followed for up to 9 months. On each visit animals were evaluated for blood biochemistry and hematological analysis, and chest radiography to track metastatic spared in the lungs. Additionally, blood samples were collected to analyze in our lab to determine the immune reaction against both the viral capsid and the encoded antigen(s). Globulins and white blood cell (WBC) count were tracked for a sign of inflammatory responses and 3 dogs presented high values 2 weeks after AAV vaccine administration. 4 of the dogs displayed high values for liver enzymes (alanine transaminase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (ALS)). However, baseline values for these parameters were on the high end before vaccination, suggesting that their increased cannot be a consequences of the treatment. The results showed that none of the animals had measurable an adverse reaction against AAV vaccine. The selected inflammatory markers (IFN-γ, TNF-α, interleukin 6 (IL-6), MYD88, and TLR9) showed that at least 2 dogs presented a significant increase in all markers, suggesting acute immune system activation. The IFN-γ ELISPOT analysis demonstrated that at least 1 out of 4 dogs presented a significant increase in antigen-specific $CD8^+$ T cells against all three tumor antigens (Gp100, Tyr and TRP1). All dogs enrolled into study were tested for presence of neutralizing antibody (NAb) against AAV6 and met eligibility requirement of titer 1:2-1:8. Post vaccination monitoring showed that all animals presented elevated NAb titers with peak between 1 and 4 weeks after AAV administration. 4 animals presented a slight increase in NAb titer 1:14- 1:21, while three animals presented a significant reaction with NAb titer 1:88-1:182. However, NAb titer declined at later time points for most of the dogs suggesting the possibility of vector re-administration. The variable survival time making impossible to definitely evaluate efficacy of AAV vaccine. However, significantly, 3 dogs are currently disease-free and 2 dogs survived over 560 days after vaccination. Our study presents for the first time the attempt of using AAV cancer vaccine in large spontaneous animal model of OM closely recapitulating human disease development. Our results indicate that AAV vaccine based on tumor antigens is safe in dogs, which open possibility to expand repertoire of diseases beneficial from AAV vectors based therapy.

Thursday Posters: AAV Vectors - Product Development Manufacturing and Approval Considerations

1018. Avoiding Host and Viral (Non-Viral Genomic) DNA/RNA Contamination in Viral Particles Produced for Clinical Applications Under GLP/GMP and Profiling of RNA/DNA Packaged Inside Viral Particles

Praveensingh B. Hajeri, Masato Yamamoto, Adam Herman

Department of Surgery, University of Minnesota, Minneapolis, MN

Background: Viruses are increasingly being used as vectors for delivering therapeutic cargo. Often, viruses themselves are used as therapeutic agents for, e.g., oncolytic viruses. In all such cases, efficient amplification and purification of viral particles is an essential step. It includes designing, producing, testing, and validating viral particles that meet the standards of GLP, GMP, and standards of impurities. Viral particle assembly and packaging in cells is an organized process.

The process differs between different viruses based on whether they are enveloped, the type of genome, the structure of the protein capsids, etc. There are several reports that mention host and viral RNAs (non-genomic) get packaged in mature and empty virus particles, which may have functional importance to the virus infection cycle in general. It is not completely clear if all the host and viral non-genomic RNAs found in viruses are of functional importance or if they get packaged randomly. Most importantly, the experiments used in several such reports were not stringent enough to rule out the possibility of such RNAs bound outside the viral particles rather than getting packaged inside. So, at least in some viruses, it still remains to be investigated if host and viral RNAs get packaged inside viral particles at all using very stringent and efficient methods. For clinical applications, such impurities may affect the quality of the viruses (prepared under GLP and GMP) and may lead to many unwanted and untargeted effects on patients. Several reports suggest that some host/viral RNA contamination is unavoidable in adenoviruses and AAVs. The standards for testing such contaminants and how much contamination is acceptable are not very clear and are still being debated. ***Materials and methods:*** We tested viruses (AdV and AAVs) prepared with current state-of-the-art methods and standards and observed significant host and viral DNA/RNA contamination. Viruses were prepared using ultracentrifugation. We deployed robust DNA/RNA extraction methods and next-gen sequencing methods to identify if there are RNAs and to determine the sequences, if any, are present. We used current state-of-the-art methods available through our genomics core facilities and also our novel NGS library prep methods, which are more sensitive than others. ***Results:*** We could identify most of such contamination is only from outside of the viral particles rather than inside. We could not detect any of the transcripts (host / viral) reported earlier to be packaged inside viral particles, and also, no new transcripts were detected. This is contradictory to several earlier reports. We also devised novel methods to remove DNA/RNA contaminants from AdV particles, which seem to be mostly from outside. We hope our study and recommendations can be implemented to prepare better-quality viral particles with no contamination of nucleic acids from hosts. This will help in meeting zero contamination levels for clinical applications and manufacturing.

1019. Automated Alkaline Lysis System for cGMP Production

Baoyu Zhu

OBiO Technology (Shanghai) Corp., Ltd., Shanghai, China

Background: Supercoiled plasmid is a key raw material for gene therapy, cell therapy, nucleic acid vaccine and other fields. It is usually obtained by high-density fermentation of Escherichia coli. How to improve the lysis efficiency of supercoiled plasmid in large-scale plasmid extraction process to obtain gram scale high-purity supercoiled plasmid is a key technical bottleneck in the industry. Methods: In order to break through the technical bottleneck of the industry, OBiO independently designed a large-scale plasmid Lysis system suitable for the platform process. It consists of four parts: front/back weighing and mixing unit, group pumps, unit mixer and program console. The speed ratio of the power group pump is accurately adjusted by the program algorithm, so that the weight ratio of the material and liquid can be quickly and accurately controlled, so that the two-phase material and liquid can be accurately mixed in the mixer to ensure uniform lysis effect. During the lysis process, the contact time of alkaline solution, acid neutralization time, stirring speed and stirring time in the process of plasmid lysis can be accurately controlled to ensure consistent production between batches; The lysis production adopts single-used consumables under the laminar flow hood to minimize the risk of contamination to the environment. By this optimized process of the platform, 50-100L fermentation broth could be processed per batch in large-scale production, and 4-6g of supercoiled plasmid stock solution could be produced in the following purification process. The yield of supercoiled plasmid from SEC pool to fermentation pool could be more than 40% after purification, and the HCD residue Could be < 0.2%, and the plasmid lysis efficiency Could be stable above 90%. Keywords: Cell and gene therapy, Plasmid lysis, Automated alkaline lysis system, Supercoiled plasmids

1020. Improving rAAV Functionality through Chromatographic Enrichment of Full Capsids

Hannah Mosheim[1], Douglas Steinhauff[2]

[1]Gene Delivery Process and Analytical Development, Bristol Myers Squibb, Seattle, WA,[2]Bristol Myers Squibb, Seattle, WA

Recombinant adeno-associated virus (rAAV) is one of the most commonly used delivery vectors in gene and cell therapy development. Significant research has been done towards understanding AAV biology and the development of scalable and robust manufacturing processes and technologies. Despite advances in vector production, vector supply and potency remain obstacles for commercial viability. One of the main challenges of rAAV production is the formation of empty rAAV particles, which do not contain the gene of interest and reduce vector quality. To resolve this, anion exchange chromatography (AEX) can be used to selectively enrich full rAAV particles. The initial study used fractionation of AEX elution to generate rAAV pools with a range of full capsid concentrations and measured transduction frequency (TF) using a primary T cell assay. The results showed that increasing the fraction of full capsids led to higher transduction frequency and required a lower volume of vector to reach saturation for gene expression. A subsequent study investigated AEX process parameters with the aim of increasing selectivity for full rAAV particles. The ionic strength of the wash step was identified as a key process lever for increasing selectivity. Enriched rAAV pools recapitulated the trends in higher TF at reduced vector input. This data illustrates the value of AEX chromatography for reducing product-related impurities and improving vector functionality.

1021. Scalable Purification of Adeno-Associated Virus Produced in the Gibco™ CTS™ AAV-Production System

Nils Williston[1], Natalie Glover[1], Collin Rasmussen[1], Natalie Miller[1], Emily Jackson-Holmes[2], Chao Yan Liu[1], Kenneth W. Thompson[1], Jonathan Zmuda[1]

[1]Thermo Fisher Scientific, Frederick, MD,[2]Thermo Fisher Scientific, Carlsbad, CA

Given the tremendous interest in the use of viral vectors for gene therapy applications, there is a critical need to scale AAV manufacturing processes to deliver products able to fulfill clinical and commercial needs. As the AAV gene therapy space continues

to grow the speed with which sponsors look to move their therapies into clinical testing has accelerated. Also, large quantities of AAV are needed per dose with a maximum dosage of 1.5x 10^{17} vector genomes per kilogram of body weight for systemic treatment. To provide a scalable upstream solution for commercial-grade AAV production using the triple transfection method, we previously developed the Gibco™ CTS™ AAV-MAX Production System. Here, we present data on the downstream purification of an anti-CD19CAR-AAV6 produced using the AAV-MAX system at the 20L scale in a single-use Wave bioreactor system including detailed procedures for each step of the downstream process: 1) cell lysis and nuclease treatment, 2) clarification by depth filter train, 3) concentration and buffer exchange by hollowfiber tangential flow filtration, 4) affinity chromatography using the POROS™ CaptureSelect™ AAVX Affinity Resin, 5) full capsid enrichment by anion exchange chromatography, 6) final concentration and buffer exchange by hollowfiber tangential flow filtration, and 7) sterile filtration. Using droplet digital PCR with primers and probes targeting the anti-CD19CAR we determined viral genome titers and achieved 40% overall recovery throughout the entire purification process. Analysis of the purified AAV by mass photometry determined that 75% of the capsids were full and ex vivo transduction of T cells demonstrated the biological activity of the resultant AAV. We also provide data on vector quality including host cell DNA and protein clearance as well as residual plasmid DNA levels in the final product. To further demonstrate scalability of the AAV-MAX System in stirred tank reactors, we produced the same anti-CD19CAR-AAV6 at the 50L scale in Thermo Scientific DynaDrive single use bioreactors with associated downstream purification. This work demonstrates the scalable, end-to-end production and purification of AAV vectors using the AAV-MAX Production system and provides guidance to those looking to manufacture highly purified AAV at clinical and commercial scales.

1022. Evaluating Three Genes of Interest in an Adeno-Associated Viral Vector Platform Process

Catherine Ludolph, Hannah Seo

Gene Delivery Process Development, Bristol Myers Squibb, Seattle, WA

Recombinant adeno-associated virus (AAV) vectors for cell and gene therapies are commonly produced via triple transient transfection in suspension HEK293 culture, utilizing replication and helper plasmids, as well as a plasmid containing the gene of interest to be expressed (pGOI). With a robust AAV platform, the GOI plasmid can be changed with minimal upstream process optimization, allowing for accelerated development. In this work, we aimed to demonstrate the robustness of our internal upstream AAV platform. With the same upstream process, we can produce AAV with high vector genome titers and a high fraction of full capsids, despite changing the GOI. Of the many key parameters that influence AAV vector production, we focused on the total concentration of plasmid DNA introduced into the system, the viable cell density at the time of transfection (VCD), and the ratio of DNA to transfection reagent (N:P). The maximum achievable vector genomes and percent full capsids produced vary with GOI; however, there is consistency in the optimal values for the process parameters investigated, suggesting a robust platform. As more GOI are developed in this upstream platform, the targets identified provide a reliable starting point for optimization and the opportunity to continue outlining a robust design space for transfection.

1023. Digital PCR-Based Assays for AAV and VCN Quantification

Dirk Vollenbroich[1], Christoph Mueller[2], Ali Kaan Aldere[2], Nicole Paland[2]

[1]Minerva Biolabs Inc., Skillman, NJ,[2]Minerva Biolabs GmbH, Berlin, Germany

Gene and cell therapies for treating patients with uncurable diseases or genetic disorders have been rapidly evolving over the last decade. Manufacturing of gene therapy products involves process development to ensure more efficient therapeutics and analytical development to ensure the therapeutics' quality, efficacy, and safety. Gene and cell therapies include in vivo and ex vivo approaches. In vivo approaches locally or systemically deliver gene editing reagents or genes directly to the target site. In contrast, ex vivo approaches involve altering the patient's cells outside the organism and re-applying the modified cells to the patient. To ensure efficacy and to avoid toxicity effects of the final product, the therapeutic dose before administration must be determined. We have developed two quantification assays for the determination of the titer of adeno-associated virus (AAV) vectors, commercially available as MiQuant® AAV Kit, as well as for the determination of the vector-copy number (VCN) for CAR T-cell therapy, available as MiQuant® CAR T Cell Kit. Quantification is based on the novel PCR quantification technology, digital PCR (dPCR). dPCR is based on the distribution of the bulk reaction into thousands of partitions, so an individual end-point PCR amplification is undergone in each partition. This makes dPCR less vulnerable to PCR inhibitors, less dependent on the efficiency of the primers, and more precise, especially for lower concentrations of the target. Both quantification kits are designed to allow multiplexing of up to five relevant targets that the customer can compile from a range of provided targets, including adding custom-designed primer/probe systems according to their requirements. Determination of the AAV titer can be combined with investigating residual DNA if desired or needed. For ease of use of both kits from the laboratory to the result, software will be provided, enabling the fast and reliable calculation of the AAV titer and the VCN determining the lentiviral integration into CAR T cells. The efficacy of gene and cell therapy therapeutics and the patient's safety relies on the exact determination of viral titers for in vivo and ex vivo approaches. Our quantification kits combine ease of use with innovative quantification methods and fast-forward results.

1024. Development Considerations in Transfection Process for Gene Therapy Viral Vector Production

Mukesh Mayani, Oliver Meade, Nicole Nohilly, Kevin Keating, Yvette Valenzuela, John F. Kerwin

Gene Therapy Development, National Resilience, Inc., Waltham, MA

Transient transfection represents a crucial yet complicated process in the manufacturing of adeno-associated (AAV) and lentiviral (LVV) vectors. The transfection process is often sensible and prone to high variability, particularly when conducted at a large scale for manufacturing

vector drug substance. Achieving higher vector productivity pivots on the precise and consistent preparation of transfection complexes involving plasmid DNA (pDNA) and transfection agent. Factors such as transfection cocktail composition, mixing time, hold time, particle size and stability significantly impact cell transfection, viral vector titer and infectivity attributes. In this presentation, we will highlight important distinctions and discuss crucial observations of transfection processes in manufacturing of both AAV and LVV vectors. Further, this presentation discusses essential small- and large-scale considerations for preparing the transfection cocktail, examining influential variables such as the pDNA to transfection agent ratio, pDNA quantity and HEK293 cell density that result in remarkable 4-6-fold increase in vector productivity, achieving titer values of upper e11 vg/mL for AAV and upper e7 TU/mL for LVV, respectively.

1025. Analysis of Recombinant Adeno-Associated Viral Vectors Using Charge Detection Mass Spectrometry

Anisha Haris[1], Rebecca D'Esposito[2], Kevin Giles[1], David Bruton[1], Chris Wheeldon[1], Alistair Schofield[1], Keith Richardson[1], David Eatough[1], Steve Preece[1], Matthew Lauber[2]

[1]Waters Corporation, Wilmslow, United Kingdom,[2]Waters Corporation, Milford, MA

Recombinant adeno-associated virus (rAAV) has emerged as a suitable delivery vehicle for gene therapy products. With AAV-based gene therapy continuing to evolve, there is an increasing need to provide improved analytical tools to better characterise the therapeutics and to facilitate their production. Analytical focus is placed on full, partial, and empty capsid characterization and impurities due to the potential impacts that they may have on clinical efficacy, safety, and immunogenicity. Charge detection mass spectrometry (CDMS), using an electrostatic linear ion trap (ELIT), is an ultra-high mass technique, which provides direct mass measurement of individual ions through simultaneous determination of their mass-to-charge ratio (m/z) and charge (z). We aim to demonstrate the potential of CDMS for analysis of rAAVs and their product-related impurities including the detection of empty and partially filled capsids, where it can be a challenge for current methodologies and conventional mass spectrometry. AAV empty and full capsid samples of various serotypes (AAV1, AAV2, AAV5, AAV6, AAV8 and AAV9) were purchased from different commercial suppliers. The samples were buffer exchanged into 200 mM aqueous ammonium acetate solution with 0.001 % Pluronic F-68 (Gibco) using size-exclusion columns (Bio-Rad). Ions were generated in positive ion mode using nanoelectrospray ionisation and mass analysis was performed using a prototype CDMS instrument. Data processing and visualisation was achieved using in-house software. AAV capsids consist of 60 copies of three viral proteins VP1, VP2, and VP3 which are assembled in a range of stoichiometries (nominally 50:5:5 respectively) with an average mass around 3.7 MDa. The average mass of a full capsid (empty capsid plus genome) is typically in the 4-6 MDa range. CDMS data were obtained for the empty and full capsid samples of various AAV serotypes. The measured average masses were in good agreement with the theoretical values of the empty and full AAV capsids. Interestingly, low molecular weight species with distinct ion populations (< 1 MDa) were observed for all the empty AAV capsids, with the exception of AAV5. Additionally, for the full AAV samples, empty particles were detected in AAV1, 2, 5, 8, and 9 with partial/intermediate particles detected in AAV1, 5, 6, and 9. These data highlight the ability of CDMS to readily distinguish between empty, full, and the partially filled/intermediate capsids. Partially filled capsids as well as an increase in the empty capsid ion population were detected for an AAV8 full capsid when stored at 4 °C instead of -80 °C, indicating that storage conditions of AAVs can have a significant impact on their stability, and ultimately affecting the potency of the product. Relative quantitation of empty and full capsids for AAV8 were also investigated for mixtures ranging from 100 % empty to 100 % full, in increments of 25 %. The data were plotted as mass peak area vs. % full capsids and a linear response and correlation were observed (R^2> 0.99), highlighting the utility of CDMS for this type of work. The results presented herein demonstrate the potential of CDMS to provide valuable mass information about large and complex biotherapeutic agents, going well beyond the realm of conventional mass spectrometry. Distinguishing between empty, partial, and full particles in manufactured AAV formulations can be readily achieved, including the relative quantification of empty and full particles based on the peak area ratios of the average masses detected. Characterization of additional critical quality attributes of AAVs are also provided, such as the presence low molecular-weight impurities.

1026. Unveiling the Prolonged Performance and Reusability of AVIPure Resin for rAAV9 Purification

Junping Li, Dandan Wang, Xiaoyan Wang, Xin Xin, Wei Hu

Hangzhou Exegenesis Bio Ltd., Hangzhou, China

The purification of Recombinant Adeno-Associated Virus (rAAV) heavily relies on affinity chromatography for capsid capture. However, the substantial costs associated with resins underscore the importance of maximizing their longevity and reusability to reduce the overall cost of goods for commercialization. While various commercially available rAAV affinity resins exist, Repligen's AVIPure AAV9 resin distinguishes itself through its alkali-tolerant synthetic peptide design, coupling chemistry, and high binding capacity of 1E14 vp/mL at a 1-minute residence time and >1E15 vp/mL at a 4-minute residence time. The pressure flow performance is 3 bar at >300 cm/hr. This comprehensive study evaluated the performance and cycle time of AVIPure AAV9. A total of 21 purification cycles were executed using the same rAAV9-containing clarified harvest as the load, with 0.1M NaOH clean-in-place after each cycle. The results showcase remarkable consistency in both rAAV recovery and impurity clearance, indicating minimal loss of binding capacity after 21 cycles (see Figure 1). The average recovery of viral particles was 97% (see Figure 2). Notably, levels of host cell DNA, residual packaging plasmids, and leachable peptide ligand remained consistently low throughout all cycles. These findings demonstrate the remarkable stability and cleanability of AVIPure AAV9's peptide ligand in the presence of the strong and effective cleaning reagent NaOH, thereby extending the operational life of the resin and contributing to a reduced cost of goods for commercialization.

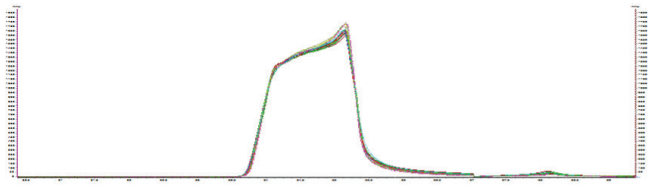


Figure 1: AVIPure affinity chromatography Elution Peak Overlay

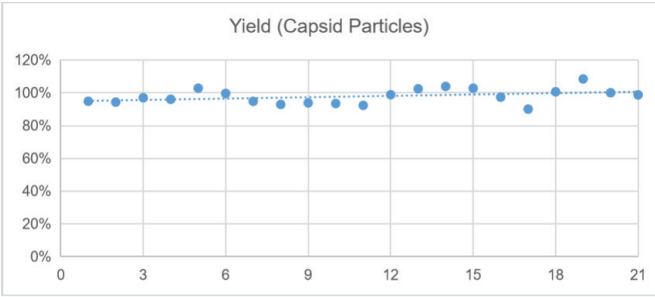


Figure 2: AVIPure affinity Chromatography Step Recovery in Capsid Particles

1027. A Commercially Producible Non-Lethal Virus Insect Cell Line

Shixian Zhang, Yan Wu, Yujing Liu, Yangyang Sheng, Xin'an Lu

Beijing Cygenta BioTechnology Co., Ltd, Beijing, China

The baculovirus expression system stands out as a robust tool for producing recombinant proteins, vaccines, and AAV vectors. In 2014, the FDA reported contamination of commercially produced baculovirus cell lines sf21 and sf9 cells with rhabdovirus, emphasizing the need for rhabdovirus-free insect cells to ensure the safety of biological products. In this study, we successfully established a rhabdovirus-free Sf9 (RVF-Sf9) insect cell line using a monoclonal screening method with antiviral reagents. The RVF-Sf9 cell line, cultured for 44 passages, exhibited no rhabdovirus detection, maintaining stable doubling time and cell viability across passages. Baculovirus titers at different passages showed no significant differences. Genomic copies of Adeno-Associated Virus Vectors (AAV2-VEGF) produced in RVF-Sf9 and SF9 (with rhabdovirus) cells were comparable. Further production of AAV-VEGF in 200-liter batches demonstrated a higher crude harvest yield of 5X10^14 vg/L using RVF-Sf9 cells compared to 3X10^14 vg/L with SF9, with no discernible differences in purity or product characteristics. Our results showcase the successful creation of a stable rhabdovirus-free cell line, providing a safer platform for commercial baculovirus system production.

1028. Rapid Optimization of AAV9 Production in Suspension HEK293 Cells to Develop a Scalable Process for Clinical Supply

Oliver Meade[1], Cuitla Chavez-Pena[1], Xiaotong Fu[1], Mukesh Mayani[2]

[1]Resilience, Inc., San Diego, CA,[2]National Resilience, Inc., Chelmsford, MA

Recombinant AAV has proven to be one of the most promising options for treatment of devastating neuromuscular rare diseases. While AAV platform processes can greatly reduce time to the clinic compared to bespoke approaches, transient transfection performance can vary within an established platform based on serotype and transgene attributes. In this study, a toolbox of rapid optimizations was used to augment the incumbent triple transient transfection platform and significantly improve production vg titer while maintaining % full particles of v1.0 of the process. Using high throughput tools such as the ambr250 bioreactor and Tecan EVO liquid handler, the vg titer of AAV9 encoding a short-length transgene (< 3 kb) for the treatment of a rare neuromuscular disease was improved 2.5-fold at small scale. The resultant process was successfully scaled to 10L pilot reactors in advance of engineering and GMP batches. The process development case study illustrates the effective interplay of platform unit operations with rapidly implementable process improvement levers, achieving clinical supply targets for rare diseases requiring high vg/kg doses.

1029. Analytical Comparability of Four Alternative AAV9 Downstream Processes

James Michael Hatfield[1], Rok Zigon[2], Austin Smith[3], Greg Boyd[4], Stephen Ollosi[3], Ales Strancar[5], Andrew Knudten[6], Maja Leskovec[2], Joseph Balleydier[1]

[1]Research and Development, Advanced Medicine Partners, Durham, NC,[2]PC3, Sartorius BIA Separations, Ajdovscina, Slovenia,[3]Analytical Development, Advanced Medicine Partners, Durham, NC,[4]Process Science, Advanced Medicine Partners, Durham, NC,[5]Sartorius BIA Separations, Ajdovscina, Slovenia,[6]Advanced Medicine Partners, Durham, NC

Improved analytical methods allow us to better understand the capsid heterogeneity in AAV therapeutic preparations. This better understanding allows us to develop manufacturing processes that produce AAV therapeutics with fewer non-functional capsids. We will present the results of a four-arm AAV-9 comparability protocol that includes three alternative 2-column downstream processes at 50-L scale and one ultracentrifugation (UC) process at 200-L scale. We consider the UC process the gold-standard for functional-capsid purification. Simplified process descriptions for the capture and empty/full (E/F) separation steps are listed below. 1. **Capture:** CEX monolith (NaCl gradient), AEX filter (flow-through) **E/F:** UC 2. **Capture:** CEX monolith (NaCl gradient), **E/F:** AEX monolith (NaCl gradient) 3. **Capture:** CEX monolith (pH gradient), **E/F:** AEX monolith (NaCl gradient) 4. **Capture:** Affinity resin (pH step gradient), **E/F:** AEX monolith (NaCl gradient). The analytical-comparability of the drug substance lots will include: Vector yields (vg/mL and capsids/mL), host-cell protein (HCP), plasmid-DNA (pDNA), and host-cell DNA (hc-DNA); Empty, partial, and full capsid content by SV-AUC (Sedimentation-velocity Analytical Ultracentrifugation), CDMS (Charge Detection Mass Spectrometry), and Mass Photometry; vector protein purity by SDS-PAGE and CE-SDS (capillary electrophoresis); aggregation by SEC (size exclusion chromatography); vector DNA purity by DNA gel, long-read and short-read Next Generation Sequencing; potency by *in vitro* eGFP expression; and capsid protein charge heterogeneity by icIEF (capillary isoelectric focusing). AUC analysis will show that the UC method produces highly purified AAV9 Drug Substance with > 90% full capsids while the two-column processes with AEX empty-full separation produce Drug Substances with > 85% full capsids.

1030. Low Endotoxin Control Strategy for an AAV-Based CNS Gene Therapy

Stephen Stephansky, Li Chen, Ying Cai
Ultragenyx Pharmaceutical Inc., Bedford, MA

Bacterial endotoxin is known to cause low to severe inflammatory reactions in patients. Health authorities has established guidelines for the endotoxin test methods and limits in the injectable drug product and medical devices. Medicinal products delivered to the central nervous system (CNS) especially demand a very low level of Endotoxin, which has become a major challenge for the manufacturing process. In developing AAV CNS gene therapies, it is important to understand the potential sources of endotoxin in able to establish a comprehensive control strategy. Raw materials, process solutions, product containers and ancillary supplies must all be scrutinized for low endotoxin levels to minimize potential introduction to the process or product. A robust downstream process with adequate endotoxin clearance capability is equally important to mitigate potential material risks. The endotoxin test method could be optimized to improve its sensitivity and limit of quantification (LOQ). In this work we present a new process improvement to enhance endotoxin control for an AAV based CNS gene therapy. The rAAV gene therapy is produced with the platform Producer Cell line (PCL) process at Ultragenyx. The upstream process consists of cell expansion into a 2000L bioreactors and infection via helper virus adenovirus Type 5 (Ad5). The downstream process consists of depth filtration, chromatography steps, heat inactivation and final formulation and concentration. While the process was demonstrated successful to deliver several 2000L batches, it was noted that solutions used in the final formulation and concentration step require extremely low level of endotoxin, nearly 10-fold lower than assay LOQ. To improve overall endotoxin control strategy, several endotoxin reduction techniques were evaluated, with one endotoxin reduction filter selected and studied. The study demonstrated several logs of additional endotoxin clearance capability for process solutions. Implementing this endotoxin reduction filter has minimal impact to the existing AAV manufacturing process, yet significantly improved process robustness to meet low endotoxin limit required for the CNS Gene Therapy.

1031. Increased Reproducibility in a Critical Downstream Processing Step Allows for Speed to Commercialization of Gene Therapy Product to Treat Leber's Hereditary Optic Neuropathy (LHON)

Scott A. Jeffers
GenSight Biologics, Paris, France

Gene therapy is one of the promising areas in the search for treatments for rare diseases like Leber Hereditary Optic Neuropathy (LHON). Without gene therapy, patients may find themselves afflicted with a disease with few or no options for efficacious treatment. In LHON, this means progressing to legal blindness within a year of first noticing defects in one's central vision. But to be approved, a gene therapy must not only prove itself in clinical trials; it must also have a manufacturing process that can be reliably executed to the highest level of quality. The affinity step in the downstream portion of the manufacturing process is particularly critical for the final drug substance: the step must deliver the recovery and purity of viral vector and must not fail, thereby nullifying the previous steps that had been executed successfully. Choosing the right equipment is a key success factor in getting the affinity step right. For Lumevoq (GS010), this has meant taking specific factors into account, such as reproducibility, monitoring of critical parameters, and ease of use. This talk will focus on this critical downstream step and how GenSight has solved this issue.

1032. Unraveling Impact of Manufacturing Process-Related Stresses on AAV Stability, Aggregation, and DNA Release

Saba Ghazvini, Michelle Jones, Michael Everton, Benson Gikanga, Madhura Som, Sandeep Yadav
Sangamo Therapeutics, Brisbane, CA

As gene therapy use continues to expand, substantial investment has been directed toward the adeno-associated virus (AAV) platform, recognized as one of the most promising delivery vehicles. Since material availability and cost are two of the main challenges of early-stage development, there is a significant need to clearly define the specific assays needed for stability evaluation to minimize material, time, and resources. In this study, we have investigated the impact of some common stress types on the stability of a model AAV6, including thermal, freeze-thaw, exposure to ambient and UV light, agitation, pH variation, and oxidation. To gauge AAV6 stability comprehensively, we employed a diverse array of analytical techniques, including Size Exclusion Chromatography coupled with Multi-Angle Light Scattering (SEC-MALS), Field Flow Fractionation coupled with Multi-Angle Light Scattering (FFF-MALS), Subvisible Particle Characterization using laser obscuration technique and Micro-Flow Imaging (HIAC and MFI), Dynamic Light Scattering (DLS), Free DNA detection via dye binding, and fluorescence measurements during thermal ramping to assess aggregation (Tagg). Results showed that thermal stress had the most significant impact on monomer loss, followed by UV light and low and high pH. Agitation created subvisible particles but not measurable aggregates. Freeze-thaw and exposure to visible light had a minor impact on the monomer loss or aggregate formation. Incorporating separation techniques with MALS furnished additional insights, enabling the quantification of capsid and vector genome (vg) titers suitable for stability screening. Our data provide a deeper understanding of the intricate interplay between stress conditions, aggregation, and particle formation. Notably, detecting aggregation or subvisible particles by HIAC/MFI or DLS in isolation proved insufficient to resolve the overall stability impact. Lastly, the addition of free DNA assay and correlation with SEC-MALS/FFF-MALS provides a more comprehensive picture of the release of DNA and aggregation to enable risk ranking different stresses on AAV stability.

1033. Development and Scale Up of Adeno Associated Virus Transient Transfection Manufacturing Process with Improved Productivity and Product Quality

Ying Liu

CMC, Genomic Medicine Unit, Sanofi, Waltham, MA

Rapidly increasing growth potential of gene therapy requires scalable, efficient and cost-friendly AAV production process. However, manufacturing AAV is not simple, with multiple complex challenges ranging from critical raw material (e.g. plasmids) generation and ensuring high titers of viral vectors, to purification and full capsid enrichment, where not all AAV particles may contain the intended genetic payload. In this presentation, we report our recent advances tackling these challenges. The first improvement was made via changing the packaging system from traditional triple plasmids (Adhelper, Gene of Interest, and Rep/Cap) to a two-plasmids system. This change allowed for 2-3 fold improvement in productivity and an increase of the full vectors generated in cell culture. Interestingly, coupling with an optimized cell feed, we achieved 10-fold increase on AAV productivity across all scales. To ensure a scalable transfection procedure, we employed single use mixing container in the procedure with optimized timing of DNA complex formation and addition. The recent large-scale batch demonstrated the consistency and scalability of the optimized process. Overall, recent advances led to significant improvements on multiple challenges, which enhances our process to yield a high-titer and high-quality AAV. Collectively, these technological breakthroughs will lead the next generation of AAV vector production process which can accelerate the timeline to the clinics and increase accessibility of gene therapy product through reduced COGs.

1034. Analytical Comparison of AAV Manufactured with Triple-Plasmid Transfection/HEK293 and Double-Baculovirus Infection/Sf9 Processes

Shreya Ahuja, Stuart Nelson, Adnan Arnaout, Mary Ng, Garrett Daniels, Andrew Pla, Shital Kakkar, Priyam Raut, Kaavya Maganti, Diogo de Oliveira Pessoa, Sitharthan Kamalakaran, Ilan McNamara, Mansuo L. Shannon, Jorge F. Haller

Prevail Therapeutics, a wholly owned subsidiary of Eli Lilly and Company, New York, NY

Adherent HEK293 triple-plasmid transfection and Sf9 double-baculovirus infection are two widely used GMP AAV manufacturing processes. However, direct comparison between the two platforms, using clinical manufacturing processes with the same vector sequence, has not been well studied. We recently completed an *in vitro* comparability exercise in which these two manufacturing processes were evaluated based on product quality attributes, including release testing, characterization testing, and stability. Final products from both processes showed comparable analytical attributes regarding safety, purity, identity, achievable strength, biological activity, and stress stability. Furthermore, both processes yielded AAV product comprised of viral capsid proteins with identical mass and similar post-translational modifications. In addition, differences in the process residuals, which are inherent to the processes, were observed. Overall, the baculovirus/Sf9 process consistently presented higher levels of full viral capsids, with significantly less AAV partials and empty viral particles. Additionally, the products derived from the baculovirus/Sf9 platform contained less total DNA impurities compared to triple-plasmid transfection/HEK293 process. The main contributor to residual DNA in the triple-plasmid transfection/HEK293 process was plasmid DNA. In contrast, no detectable infectious baculovirus or particle associated Sf9-Rhabdovirus RNA were present in the Sf9 produced drug substance. In conclusion, the double-baculovirus infection/Sf9 process was able to upscale AAV production and deliver a comparable AAV product to the AAV material manufactured from the triple-plasmid transfection/HEK293 adherent process.

1035. Development of HEK293 Cell Line for Optimal Production of Novel Capsids with Enhanced Brain Tropism

Zeynep Guillemin, Varshini Venkatesan, Amanda Ibe-Enwo, Christian Gagnon, Shamik Sharma, Kumar Dhanasekharan

Voyager Therapeutics, Inc., Lexington, MA

Voyager has developed-novel AAV capsids with significantly enhanced brain tropisms and achieved transgene expression that is superior to conventional AAV serotypes in non-human primates. These capsids have the potential to increase the safety and effectiveness of AAV gene therapy. In order to accelerate clinical studies of therapeutic candidates using novel capsids, rapid process development leading to a cost effective manufacturing process is the next challenge. HEK293 cells are the most widely used cell-line for rAAV production and Voyager has internally developed a HEK293 cell line to support our portfolio of AAV gene therapy assets. The VYGR-293 cell line was developed by adapting a serum-fed adherent cell line to a serum-free suspension culture. Further, VYGR-293 cells were optimized for maximum productivity throughout the production process from cell seeding to lysis. Triple transfection parameters of total plasmid amount, cell density at transfection, plasmid to transfection reagent ratio and molar plasmid ratio (Transgene: Rep-Cap: Helper) were tested using Design of Experiment (DOE) studies. Optimal parameters that enabled the highest productivity and %Full capsids were thus selected. The upstream process was further developed by examining optimal N-1 to N split ratios, amino acid utilization during production and time of harvest studies. In addition, multiple additives and commercial transfection additives are explored. VYGR-293 cells achieved 2-4x increase in volumetric titer after these optimization studies, reaching above 2E11 vg/mL. rAAV obtained from VYGR-293 cell line had good product quality profile including high % full capsids. In summary, this cost-efficient cell line provides a good foundation for the development of an AAV expression platform to produce drug candidates for neurological disorders.

1036. An Innovative Affinity Chromatography Approach for Effective Removal of Empty Capsids in Recombinant AAV Products

Tao Wu, Lijun Wang

CoJourney Bio Ltd., Lower Gwynedd, PA

Ensuring the safety and regulatory compliance of recombinant adeno-associated virus (rAAV)-based gene therapy requires the meticulous removal of product-related impurities, particularly empty capsids, from rAAV preparations. While ultra-centrifugation using density gradient is an effective polishing step for rAAV, its scalability is limited. Anion exchange chromatography (AEC) offers satisfactory separation of empty and full capsids, but conventional affinity resins typically lack effectiveness in removing empty capsids. This study introduces a novel affinity chromatographic approach designed for the efficient removal of empty capsids from genome-containing viral particles. The method leverages the differential binding affinity of empty capsids compared to full capsids. We present a comprehensive method for the purification of the rAAV9 vector utilizing POROS CaptureSelect AAVX affinity resin, which holds promise for application across various rAAV serotypes to achieve consistent empty and full separation. Chromatographic enrichment of rAAV9 full particles is accomplished through linear gradients based on Tris buffer at an optimal pH, supplemented with additives. The composition of the affinity column fractions is evaluated using UV and analytical ultracentrifugation, revealing a substantial enrichment of full capsids. This innovative affinity chromatography approach not only enhances the downstream purification process but also holds potential for improving the overall quality and safety of rAAV-based gene therapy products.

1037. Comparing CsCl Density Gradient Ultracentrifugation and Anion Exchange Chromatography for Full Capsid Enrichment of a Novel Adeno-Associated Viral Vector

Roberto Facendola[1], Kavitha Bodige[1], Jacob Guzman[1], Patrick Carroll[1], Kevin Nguyen[1], Tom Elich[1], Shamik Sharma[1], Kumar Dhanasekharan[2]

[1]Voyager Therapeutics, Cambridge, MA,[2]Voyager Therapeutics, Inc., Cambridge, MA

A key challenge in manufacturing recombinant Adeno-Associated Viruses (rAAV) for gene therapy applications is the removal of capsids which do not contain the complete transgene of interest, otherwise known as "empty" or "partially full" capsid impurities. It is common for triple transfection processes using Human Embryonic Kidney (HEK) 293 cell lines to produce more empty capsids than full, which lead to low percent full ratios in the cell culture harvest. Since empty capsids cannot transduce any target cells in a patient, their presence lowers the efficacy of an rAAV mediated genetic therapy. Therefore, robust processes are needed to remove empty and partially filled capsids from rAAV drug product. One technique is cesium chloride density gradient ultracentrifugation which separates empty and full particles by mass. This process is agnostic to capsid structure and can therefore separate full rAAVs from empty rAAVs regardless of the serotype (including modified or engineered serotypes) using standard protocols. The major drawbacks to this method are long process times, lack of scalability, manual product collection, and low step yields. Recently, chromatographic techniques have been emerging as a more efficient method to separate empty capsids. One common chromatographic mode used for this is Anion Exchange (AEX) chromatography. This approach exploits slight charge differences between full and empty capsids to separate them from one another in a bind-and-elute mode. AEX chromatography methods are more scalable and require less process time than ultracentrifugation. However, since AEX purification depends heavily on capsid surface charges, development time is needed to optimize a specific protocol for each serotype and/or engineered capsid. Furthermore, in addition to capsid surface properties, characteristics of the packaged transgene may also impact AEX purification performance. Here, we present data for both ultracentrifugation and chromatographic separation methods for rAAV polishing, and compare performance in terms of full capsid enrichment, process yield, residual impurity clearance, and process time. The cumulative data helps to identify the appropriate purification strategy for a range of biomanufacturing scenarios. Additionally, a review of data generated for multiple capsids and transgene constructs offers interesting insights to the relative influence of capsid and transgene properties on AEX purification.

1038. Separation of Empty and Full Engineered Adeno-Associated Virus Capsids Using a Weak Anion Exchanger

Varun Gejji[1], Hari Acharya[2]

[1]Capsida Biotherapeutics, Thousand Oaks, CA,[2]Capsida Biotherapeutics, Inc., Newbury Park, CA

Gene therapy, using recombinant adeno-associated virus (rAAV) as the delivery vehicle, has shown promising results to treat genetic disorders. To expand the therapeutic space, engineered AAV capsids with improved tissue tropism and transduction efficiency are being developed. As the need to manufacture highly specific engineered capsids grows, several challenges to process scale up persist. One of the key challenges is separating full capsids containing the gene of interest (GOI) from empties. Currently, full and empty capsid separation often relies on ultracentrifugation, however, scaling ultracentrifugation is not feasible. In this work, we explore full enrichment of engineered capsids using anion exchange chromatography. We examine several process parameters including binding conditions (pH and salt), elution conditions (pH, salt concentration, gradient elution, step elution, and hybrid elution profile), loading rates, and resins. Resins screened range from weak to strong exchangers with varying charge densities. Our studies indicate that the binding strength of engineered capsids can significantly differ from the wild type. Elution over different anion exchangers in the presence of various salts or over a range of pH did not yield better separation, likely due to the nature of engineered capsids. We identified several control variables that enabled full capsid enrichment.

1039. Next-Generation Sequencing as a Multi-Attribute Method for Monitoring Critical Quality Attributes of Gene Therapy Products

David Killen[1], Thipa Asvarak[1], Sarah Berger[2], Thomas Hartsch[2]

[1]Genedata Inc., Lexington, MA,[2]Genedata AG, Basel, Switzerland

Developing and producing Advanced Therapy Medicinal Products (ATMPs), such as gene therapies, is a challenging task that involves designing complex vectors, using high-quality cell lines, and complying with strict regulatory guidelines. While these therapies have shown remarkable results, some have caused unexpected problems and delays during clinical trials. To prevent this, it is essential to test the Critical Quality Attributes (CQAs) of the desired therapeutics, which include identity, integrity, and safety. Regulatory agencies, such as the FDA or EMA, can request thorough information from the biopharmaceutical companies about the CQAs of the product during the IND application. For this reason, having a simplified set of tests and the proper technology to measure simultaneously various CQAs of these therapeutics would be highly beneficial. Next Generation Sequencing (NGS) has become a powerful tool for biopharmaceutical companies to automate CQAs testing of gene therapy products. By harnessing the power of NGS technology, high-quality gene therapy products that meet both good manufacturing practice (GMP) requirements and clinical standards can be developed. With automated NGS workflows, biopharmaceutical organizations can speed up the development and manufacturing of gene therapy products and lower the cost of testing their CQAs.

1040. Recombinant Adeno Associated Virus Full Particle Enrichment Using Affinity Chromatography and Scalable Continuous Flow Ultracentrifugation: A Scalable, Flexible and Cost Effective Approach

Gael Mbele[1], Eleonora Leggiero[1], Sara Fabbricino[1], Miriam Centrulo[1], Fabio Perini[1], Bill Vestal[2], Sandra Merino[3]

[1]Innovavector, Pozzuoli, Italy,[2]Alfa Wassermann B.V, Ijsselstein, Netherlands,[3]Alfa Wassermann B.V., Ijsselstein, Netherlands

Adeno-associated virus (AAV) is one of the most promising gene therapy vectors due to their low immunogenicity and large tropism. A robust, reliable and scalable purification process for AAV vectors is essential to the gene therapy industry. To date, manufacturing purification methods for AAV vectors are mostly based on affinity chromatography, for rAAV particles capture, and ion-exchange for full particles enrichment. Purified rAAV vectors, resulting from such biomanufacturing process, still vary according to the packaged genome sizes, including full-genome, intermediate, and empty particles. As empty capsids are thought to reduce transduction efficiency and can induce unexpected immune responses, there is an exponentially increasing demand for improved purification strategy with the aim of lowering the empty particles content to properly support rAAV-based preclinical and clinical studies. In addition, IEX ion exchange chromatography steps can be time-consuming to develop because they depend on the serotype being used. Alfa Wassermann scalable continuous flow ultracentrifuges meet all the demands of cGMP manufacturing for viral vaccines and viral vector gene therapy products and provide a scalable solution to move from laboratory tube rotors to industrial scale ultracentrifugation. Here we report an approach, based on density gradient ultracentrifugation that combines concentration and purification in a single step, reducing the total number of process steps and process time. This directly increases the overall yield, production capacity and product quality with the benefit of remaining flexible for multi-product purification as the separation methodology is serotype independent. Therefore, we reasoned that coupling affinity chromatography with a centrifugation polishing step can be a fast, efficient and cost-effective approach to provide high yield of purified rAAV with a high content of full particles. Here, we report a side-by-side comparison of rAAV8 product profiles obtained either by; 1) 2 steps chromatography (affinity and IEX) based approach and 2) affinity chromatography followed by continuous flow ultracentrifugation.

1041. Bioprocess Optimization for Improved AAV Titer and Percent Full Capsids with a Sf-Rhabdovirus-Negative Insect Cell Line and CD Insect Cell Medium

Sandra McNorton

MilliporeSigma, Lenexa, KS

The resurgence of gene therapies is providing lifesaving options to patients with otherwise untreatable diseases, leading to increased demand for large amounts of high-quality viral vectors. Adeno-associated virus (AAV) is one of the leading viral vector platforms. The insect cell based Baculovirus Expression Vector System (BEVS) based on baculovirus infections for production and Baculovirus Infected Insect Cell system (BIICs) based on infected cells for production are being utilized by biopharmaceutical companies to achieve large volumes of high-quality recombinant adeno-associated virus (AAV) to treat genetic diseases. *Spodoptera frugiperda* (Sf) cell lines are widely used as hosts for BEVS and BIIC. However, the majority of Sf9 and Sf21 cell lines contain a Sf-rhabdovirus which is considered a process contaminant and must be eliminated from the product. To improve the safety profile of the BEVS and BIIC production methods, we developed a high performing Sf-rhabdovirus-negative (Sf-RVN®) Platform, composed of an Sf9 rhabdovirus-free cell line with an optimized chemically-defined medium (EX-CELL® CD Insect). In this study, we optimized the MOI and RepCap:ITR-GOI ratio for AAV production with the BEVS and BIIC systems using our GMP Sf-RVN® insect cell line (developed by GlycoBac LLC) cultivated with our chemically defined medium to achieve high titers in bioreactor tubes, and further increased AAV production and percent full capsids with the BIIC system using post-infection feeds.

1042. Comparison of AAV Downstream Purification Process Using Chromatography and Density Gradient Ultracentrifugation

Smriti Mishra[1], Matthew Kuc[2], Lori Mullin[2], Joe S. Orlando[2]

[1]Virus and Gene Therapy Bioprocessing, MilliporeSigma, Bedford, MA,[2]MilliporeSigma, Bedford, MA

Recombinant adeno-associated viral (rAAV) vectors provide an efficient and effective tool for therapeutic gene delivery. During genome packaging in host cells, a heterogeneous population of vectors is produced consisting of full, empty, and partially full capsids. An excess of empty capsids in a therapeutic dose can trigger unintended immune responses; therefore, therapeutic potency requires the removal of empty capsids for final formulation. Empty and full capsids are similar in their physical characteristics and vary slightly in isoelectric point (pI) and density which makes separation challenging. Here we compare two methods for the separation of empty and full capsids based on the mentioned characteristics. A cesium chloride (CsCl) density gradient ultracentrifugation (DGUC), that is AAV-serotype independent and a chromatographic method utilizing AEX resins. We demonstrate the capabilities, efficiencies, and challenges of two methods using models AAV2 and AAV5 in downstream vector processing. Both methods are evaluated from similar start material using affinity capture chromatography feed. Our findings demonstrate the deeper understanding and importance of the two methods in terms of their usage depending on the requisites of the final formulation. Each method provides flexibility based on the therapeutic drug product's production scale, availability of instruments, raw materials, and technical expertise.

1043. Optimization of Scalable rAAV Production for Gene Therapy - Leveraging At-Line Amino Acid Measurements for Bioprocess Modeling-Driven Approaches

Prasanna Srinivasan[1], John Joseph[1], Tam N. T. Nguyen[2], Graziella Piras[3], Ji Young Anderson-Czajkowski[3], Milla Neffing[3], Scott Miller[3], Anthony J. Sinskey[4], Richard D. Braatz[5], Jackie Wolfrum[1], Paul W. Barone[1], Stacy Springs[1]

[1]Center for Biomedical Innovation, Massachusetts Institute of Technology, Cambridge, MA,[2]Research and Development, BioNTech, Cambridge, MA,[3]908 Devices, Boston, MA,[4]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA,[5]Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA

Impact: Utilising mechanistic models for HEK293 rAAV production and at-line spent media analysis for data-driven process intensification and yield improvement by cell culture media and feed optimisation. **Introduction:** rAAV viral vectors (VV) used for *in vivo* gene therapy are commonly produced using transient transfection of HEK293 cells. Although this method is well established, challenges remain in achieving high VV titer and a high ratio of full-to-empty capsids. Our aim is to build a mechanistic understanding and knowledge of amino acid (AA) consumption to guide the development of advanced manufacturing processes. The resulting model-driven approach is used to design, predict, test, and optimize VV production processes - scaling up and transferring to a continuous process at bioreactor scale. Media optimization can be a laborous process involving multiple DoEs. Rapid and simple analytical tools at the point-of-use are required to quickly assess key-nutrient consumption and provide feedback to optimize feed and supplement addition strategies. In this study, we performed data-driven media screening and optimization experiments leveraging a fast, at-line amino acid analyser, REBEL. **Methods/Approach:** For initial experiments, rAAV was produced via transient transfection in a fed-batch 300 mL mini-bioreactor and in the shake flasks. Samples were collected to identify potential AA depletion that may impact capsid titre. **Results:** Asn is an important AA building block and constitutes ~10% of capsid protein composition. Results show clear differences in fluxes of Asn and Asp, which were reported previously as candidate AAs for producing viral particles. Other essential AA also exhibited strong depletion, indicating opportunities to optimize cell culture conditions. With subsequent experiments, the impact of AA addition to the cell culture was assessed with the goal of improving vector titre. **Conclusion:** This work is relevant to addressing key challenges of rAAV production platforms via process engineering and optimization of cell culture conditions to scale-up vector production and development of continuous bioprocessing of rAAVs. The technology used uniquely enables at-line AA measurements, leading to deeper insights and informed feeding strategy optimization.

1044. Harnessing the Power of High-Throughput Virology and Design of Experiment for Rapid Vector Production Process Optimization

Jean-Simon Diallo, Jondavid De Jong, Keara Sutherland, Tianxiao Yang, Andrea Vervoort

Virica Biotech, Ottawa, ON, Canada

Introduction: Biomanufacturing of cell and gene therapies is a complex process, hampered by variability in yield, quality, and scalability of manufacturing platforms. Production parameters can be key drivers for vector quality, quantity and cost, making them prime process optimization targets. However, assessing all possible variables and understanding their interactions with product output and each other can be a convoluted and time-intensive task when using traditional one-factor-at-a-time (OFAT) process optimization strategies. **Methods/Approach:** A high-throughput, 96-well format virology platform using transduction-based quantification of luciferase reporters allows for rapid quantification of various produced viral vectors. We developed transfection-based production and quantification assays for AAV (suspension 293) and lentivirus (adherent 293T) and used DoE statistical methods to optimize up to six different production parameters simultaneously. This approach of combining high-throughput virology assays with DoE is amenable to various vector production platforms, including replicating viruses, reverse genetics and inducible systems. **Results:** AAV production in suspension HEK293s was optimized, with the methodology allowing simultaneous optimization of up to six factors, including total DNA, nucleic acid type, cell count, transfection reagents, and addition of small molecule enhancers for optimization of AAV production. Additionally, LV production optimization in adherent HEK293T by full factorial combination of various plasmid ratios was performed. Importantly, the optimized conditions identified at small scale were translatable to larger-scale formats, demonstrating scalability. **Conclusion:** We demonstrate that combining high-throughput assays with the statistical power of DoE enables process optimization of multiple factors in time-frames not achievable through traditional OFAT methods. Furthermore, we exemplify how the output data can be used to optimize processes for various factors, including transduction signal, TU/mL,

vg/mL or even cost/vg. The flexibility of optimizing different outputs from a single data set unlocks the potential for data-driven decisions to maximize production processes based on strategic needs.

1045. Increased Viral Vector Yield: Development and Scale-Up Validation of Small Molecule Enhancers

Jean-Simon Diallo, Jondavid de Jong, Keara Sutherland, Andrea Vervoort

Virica Biotech, Ottawa, ON, Canada

Manufacturing gene and cell therapy vectors are complex and expensive, often resulting in insufficient yields to satisfy commercial demand. While advancements have been made in manufacturing strategies, the industry faces major challenges in production consistency and yield. It is also important to understand the key cost drivers of these production processes, which are crucial to creating more accessible and affordable therapeutics. An often overlooked bottleneck in cell culture-based manufacturing is the presence of cellular innate antiviral defenses that remain intact in producer cells. These well-conserved pathways are triggered via the detection of foreign nucleic acid and/or molecular by-products of viral replication/assembly, resulting in blunted viral vector yields in infection and transfection-based manufacturing strategies. Virica's Viral Sensitizers (VSEs™) encompasses a proprietary collection of small molecules that enhance the growth of viruses by transiently and efficiently dampening cellular antiviral defenses. Leveraging high-throughput methods, Virica has assembled a library of over 130 small molecules that enhance viral production by transiently antagonizing a broad range of innate cellular antiviral pathways. Owing to different molecular mechanisms, VSEs can be combined and formulated for specific uses across various viral and cellular platforms. Here we describe the development of custom small molecule formulations for enhanced production of therapeutically relevant vectors, including AAV (Adeno-Associated Virus) and lentivirus, using high-throughput methodologies. We present validation data supporting the scalability of AAV8-specific custom formulations yielding multi-fold increases (>2x) in genomic titer, capsid titer, as well as full to empty ratio in formats representative of large-scale commercial platforms, including the Ambr®15 microbioreactor system and bioreactor systems up to 5L working volume. Applicability of VSE technology in commercially relevant non-transfection based systems for lentivirus production is also shown; the addition of VSEs in a fully inducible suspension-based HEK293 system demonstrated over 3-fold enhancement in functional lentivirus titer. Finally, Virica uses bioprocess modelling to offer insights into manufacturing cost drivers to inform the long-term feasibility of various production strategies. Considering key modelling output metrics such as global, economic and environmental impact allows for more informed decision-making when considering multiple manufacturing strategies to increase yield. Ultimately, this data supports the broad applicability of small molecule enhancers, such as Virica's VSEs, and their use as a novel and economically feasible solution for increased production of viral vectors at scale.

1046. Finding the Needle in the HEKstack: Media Screening for AAV Production in HEK293

Pia Brinkert, Kathrin Teschner, Alyssa Buve, Vera Ortseifen

Sartorius Xell GmbH, Schloß Holte-Stukenbrock, Germany

Adeno-associated viruses (AAVs) are the leading platform for *in vivo* gene therapy. To date, transient transfection of HEK293 cells is state-of-the-art for AAV production. Despite extensive research, low productivity is limiting a broader clinical application. To overcome this, it is essential to approach and improve AAV production from multiple angles. For instance, the choice of culture medium has a major impact on AAV yield. Hence, we developed a cell culture medium tailored to the needs of AAV production, i.e., allowing for transient transfection, high growth rates, and high AAV titer. In addition, the medium was sought to contain a minimum number of components to reduce the variance and complexity in raw materials, to increase process robustness, and to reduce downstream interference. Initially, a minimal medium was produced which however did not allow for stable growth of HEK293 cells at shake flask scale. The medium was refined in a design of experiment (DoE) approach using MODDE®. Monitoring cell growth in shake flasks led to the identification of a group of essential vitamins. To assess the benefits for AAV production, three media variants were used in a transient transfection process with FectoVIR®-AAV. One media variant resulted in higher AAV genomic titer compared to the reference medium. To further increase AAV yields, this variant was supplemented with components selected based on AAV biology. Addition of a translation regulator led to a more than 100% increase in AAV genomic titer compared to the reference. Importantly, such titer increase was reproducible in a regulated system such as the Ambr®15. In conclusion, the combination of a DoE approach together with a rational design enabled the development of a lean HEK medium for stable HEK293 cell growth and high AAV titers. Thereby, it will contribute to overcome the current limitations of AAV production processes.

1047. A Novel Cell Lysis Solution with Advantageous Use Characteristics for rAAV Manufacturing That Can Lead to Greater Downstream Recovery by Minimizing Upstream Loss

Nandu Deorkar, Courtney O'Dell, Lori Fortin, Nima Sedaghat, Breanne Schwing, Rejean Wang, Jungmin Oh, Arvind Srivastava

Avantor, Radnor, PA

Many gene therapies and viral vaccines rely on a high dose of recombinant adeno-associated virus (rAAV) to deliver therapeutic DNA to patient cells, which may be challenging. Improving titer during the upstream process is important but recovering the titer during the harvest process is critical. Recovery of assembled rAAV particles can be challenging as their cytoplasmic localization requires release through cell lysis and separation from resulting cellular debris. Chemical lysis is the industry favored method of lysis to retrieve rAAV due to its scalability as well as other factors; however, variable recovery and

need for environmental regulatory compliance has led to continuous development and testing of chemical solutions. In this presentation, we will demonstrate the benefits of using a novel cell lysis solution which can rapidly rupture cell membranes across a wide range of operating conditions, is compatible with traditional DNA removal agents and also provides preferential yield and titer of rAAV. The features of this cell lysis agent, along with ease-of-use qualities such as relatively low viscosity and room temperature stability at storage conditions, make it the optimal solution to improve the overall viral vector manufacturing process by providing robust and efficient cell lysis that may allow for high titer rAAV recovery.

1048. In-Line and On-Line Monitoring of Key Cell Culture Parameters for Robust Cell and Gene Therapy Bioprocess

Christopher D. Brown[1], Colin Gavin[1], Stephen Driscoll[1], Wolfgang Kuennecke[2], Michael Hartlep[2], Milla Neffling[1], Boris Aleynik[1], Graziella Piras[1]

[1]908 Devices, Boston, MA,[2]908 Devices, Braunschweig, Germany

Over the last 20 years the industry has achieved remarkable success with mAb-based therapeutics, and extraordinary advances in mRNA-, cell- and gene-based therapeutics are becoming everyday news. However, predictive bioprocess development and control is (still) hard, especially with patient-specific therapies. Technologies for near real-time bioprocess characterization have rapidly advanced in recent years, expanding the panel of readily accessible process and product attributes. Fundamental chemical and physical engineering factors (dissolved gases, pH, temperature) have been measurable in/on-line for some time. Biological activity assays are conducted frequently, but are labor intensive, and some too slow to be actionable for improving therapy outcomes. Gene therapy manufacturing know-how has advanced in recent years due to increased efforts to tackle the many challenges in viral vector bioproduction. However, the HEK293 process is still dependent on key cell culture components and feeding strategy optimization. HEK293 nutrient (such as glucose) control to avoid the accumulation of toxic metabolites (such as lactate), is a key consideration in intensified and extended manufacturing processes. Parameters that influence cell culture viability, transfectability and resulting viral vector titer and quality are critical to understanding and control in an efficient manner. We discuss a novel Raman-based analytical solution that does not require any chemometric modeling for implementation. We describe the automated control of glucose with plug-and-play in-line monitoring and feedback loop. Cell therapy manufacturing needs efficiency and robustness to consistently produce products with improved treatment outcomes in patients. The cell therapy manufacturing process is dependent on multiple stages of cell manipulation, throughout which cell growth and health are imperative. Continuous, automated (on-line) measurement of process key components, lactate and glucose, enable data-driven decisions to be made for an effective cell product manufacturing process. However, this has been challenging due to the low level of analyte measurements required, small batches, and processing bioreactors and methods currently used. We discuss an automated on-line, biosensor-based analytical solution for various cell therapy bioprocess scales and processes. We will discuss recent advances in these cell and gene therapy bioprocess analytical technology regimes, approaches to real-time analytical modeling/calibration of these systems, and how their informing power can be leveraged for predictive bioprocess optimization.

1049. AAV-Based Gene Therapy Development: Some Real Concerns, Some 'Empty' Threats

Rajiv Gangurde

Cell & Gene Therapy, Regulatory Strategy, Parexel, Newton, MA

With five FDA-approved products and more than 130 ongoing clinical trials, AAV-based gene therapy has come a long way as a modality to challenge a wide variety of diseases. Long-term efficacy has been demonstrated in different organs, including liver, eye, and the central nervous system. However, some concerns, challenges, and threats continue to loom over the use of AAV vectors. Related to CMC, off-target transduction, immunogenicity, and residual impurities pose safety concerns. Manufacturing strategies for high-dose, systemic delivery products continue to be a challenge. The threat of empty capsids, whilst debate-inducing, remains elusive and poorly understood. Of the 33 clinical holds imposed by the FDA on cell and gene therapy products during 2020 - 2022, 15 are for AAV-based gene therapies. While majority of these holds are for clinical reasons, CMC deficiencies, such as lack of device compatibility, manufacturing inconsistencies, and product instability, are some of the main culprits according to the FDA. This talk focuses on these critical areas from a CMC and regulatory perspective, with specific examples of AAV serotypes in clinical trials, evolving regulatory guidance, and the need for analytical harmonization across the industry. Phase-appropriate CMC strategies to mitigate known risks for AAV gene therapy development are discussed.

1050. Enabling Commercial AAV Manufacturing by Xcite® AAV Stable Producer Cell Lines

Gang Li[1], Chien-Ting Li[1], Khanh Ho[2], Friedrich Michael Haller[1], Leila Aneke[1], Jiasong Jiang[1], Peng Wang[1], Tam Duong[1], Senthil Ramaswamy[1], Bingnan Gu[1]

[1]Research & Development, Lonza Houston Inc., Houston, TX,[2]Process Development, Lonza Houston Inc., Houston, TX

The superior safety profile and transduction efficiency make AAV an excellent viral vector for human gene therapy. However, current AAV manufacturing suffers from low productivity and scalability, high raw material cost and supply chain complexity, and high batch-to-batch variation. To overcome these limitations and industrialize AAV manufacturing, we have generated a high-performing, helper virus-free, Xcite® AAV stable producer cell line (PCL). The cell line is built upon our proprietary high-producer HEK293 cells used for AAV transient transfection platform, grown in chemically defined, serum-free, ADCF medium in suspension. The DNA expression vectors are designed to tightly control viral gene expression via engineered inducible promoters, and stably integrated into the cell genome in one step by our VS *piggyBac*™ transposon system, which markedly increase the efficiency of cell line construction. Upon small chemical doxycycline induction at regular cell density, our selected PCL clones outperform transient transfection method with even higher AAV

titer (>1E12 vg/mL) and comparable AAV genome packaging (>30% full capsids) at harvest. Moreover, these cells maintain the stability of AAV productivity and packaging over 30 cell passages corresponding the scale far beyond 2000 liters. Taken together, our Xcite® AAV stable PCL represents a next-generation platform that can enable high-quality commercial AAV manufacturing at large scale with significantly reduced cost and process complexity.

1051. Developing Faster and Easier AAV Purification Protocols with the AAVone System and Purpose-Built Ultracentrifuge Rotors

Cuiping Chen[1], Aparna Jasti[2], Yuyan Wang[1], Yu Liu[1], Leming Wang[1], Shawn M. Sternisha[2], Qizhao Wang[1], Daozhan Yu[1]

[1]AAVnerGene Inc., Rockville, MD,[2]Beckman Coulter Life Sciences, Indianapolis, IN

At AAVnerGene, we have developed a novel one-plasmid adeno-associated virus (AAV) production system called AAVone, which allows for greatly enhanced productivity, simpler protocols, higher consistency, reduced production time, and dramatically lower costs. It makes providing on-shelf AAV vectors for researchers more realistic. However, downstream purification is often the bottleneck in AAV production. Although chromatography offers some advantages, the purest and safest AAV products are generated using ultracentrifugation. Most common rotors can only hold 6 or 8 tubes, making sample purification throughput limited and tedious. Here, we developed faster and easier protocols using two high capacity rotors, the VTi 50.1 and Type 45 Ti. The VTi 50.1 can hold 12 tubes and each tube has 39.5 mL loading capacity, enabling more samples to be processed in a single run. The Type 45 Ti rotor can hold six tubes of approximately 100 ml each, which makes it perfect for processing large volume samples. We used both rotors to develop better protocols for small or large volume AAV productions with either cesium chloride or iodixanol gradients. The new protocols require fewer steps, simple sample preparation, lower ultracentrifuge run time and less effort. The purified AAV exhibits high yield, quality, infection and expression. In conclusion, optimization of the ultracentrifugation step can speed up the AAV production and reduce the cost, facilitating AAV gene therapy research and drug development.

1052. Process and Formulation Development for a Novel Blood-Brain Barrier Penetrant AAV Capsid

Taeho Kim[1], Zelong Xie[1], Saba Ghazvini[1], Jada Padilla[1], Alex M. Ward[2], Matthew R. Tiffany[2], David Ojala[2], Nikul Patel[1], Anthony Chikere[1], Phillip Ramsey[1], Sandeep Yadav[1]

[1]Sangamo Therapeutics, Brisbane, CA,[2]Sangamo Therapeutics, Inc., Richmond, CA

Recombinant adeno-associated virus (AAV)-based vectors for therapeutic gene delivery have shown great promise due to their excellent safety profile, ability to target a wide range of tissues, stable transgene expression, and may offer significant clinical benefits. However, conventional AAV serotypes have limited ability to cross the blood-brain barrier (BBB) and deliver therapeutic cargo to the central nervous system (CNS). Capsid engineering approaches have the potential to improve AAV delivery and tissue specificity including the ability to cross the BBB and deliver therapeutic cargo for CNS indications. The successful progression of these engineered capsids through clinical development will require a robust manufacturing process and stable formulation that enables large-scale production of these novel AAV vectors. In this study, we report the development of a scalable process for the production and purification of a recently identified engineered AAV capsid with the ability to cross the BBB in nonhuman primates. Further, a stable formulation was developed to enable optimal shelf-life and DP presentation aligned with the target product profile for CNS indication. Critical quality attributes such as titer, empty/full ratio, aggregation, purity, and potency were evaluated to demonstrate process and formulation performance. Both suspension HEK293 and *Sf*9 production platforms were evaluated and optimized to maximize capsid yield and purity. The HEK293 triple transfection process was optimized for various process parameters including transfection reagent to DNA ratio, cell density, total DNA amount, and transfection incubation time. Furthermore, a purification process was developed using a combination of scalable affinity capture and ion-exchange chromatography steps. Purification parameters such as pH, salts, and flow velocity were optimized to maximize recovery of the engineered AAV capsid variant and separate empty and full capsids. The optimized process parameters deliver a robust and scalable manufacturing process and formulation with product quality attributes critical to advancing novel viral vectors and genomic medicines in CNS applications.

1053. Evaluation of Cell Growth and AAV Production in a Fixed-Bed Bioreactor to Streamline Process Development

Anne MacIntyre, Nathan Hazi, Andrew Laskowski, Michelle Olson Peabody

Cytiva, Westborough, MA

The demand for viral vector-based gene therapies continues to grow as FDA approvals accelerate and the number of potential indications increase. To remain competitive, companies must streamline viral vector development timelines using advanced systems and efficient processes. For adherent cell culture processes, the iCELLis Nano bioreactor is an automated, single-use, fixed-bed bioreactor that facilitates excellent cell growth and volumetric productivity and offers high flexibility for gene therapy development. We evaluated how select process parameters affected cell attachment, cell growth and distribution, and recombinant adeno-associated virus serotype 5 (rAAV5) production in the iCELLis Nano bioreactor. We performed a series of growth and rAAV5 production cell culture runs using a HEK293 cell line. We evaluated the impact of linear speed and presence of Pluronic F-68 on cell growth and distribution. We also evaluated the impact of linear speed, cell density, and total DNA concentration during transient transfection on rAAV production. Pluronic F-68 exhibited no impact on cell growth, while linear speed was shown to influence cell growth and overall cell distribution. At the time of transfection, the cell density and total DNA concentration displayed an effect on rAAV production yield, while linear speed did not. The results highlight areas

of process robustness when using the iCELLis Nano bioreactor. They also suggest focus areas for process development when evaluating cell growth and rAAV production.

1054. Novel Solutions for Quality Control in Gene Therapy Viral Vector Development

Andrea O'Hara[1], Crystal Richardson[1], Chris Mozdzierz[1], Haythem Latif[1], Ginger Zhou[2]

[1]Azenta, Burlington, MA,[2]Azenta Life Sciences, Burlington, MA

Interest in gene therapy-based disease prevention and treatment has grown rapidly over the last decade. While lentivirus has historically been the viral vector of choice for gene therapy, recombinant adeno-associated viral (rAAV) vectors have seen widespread application in recent years due to the non-integrating ability. Baculoviruses are emerging as a highly adaptable vector with a broad tissue and host tropism. Regardless of vector, extensive quality control (QC) throughout the entire development and manufacturing process is essential. A robust QC process expedites safe and effective commercialization of the final product. While Sanger sequencing can be ideal to verify and validate plasmid sequences pre-packaging, next generation sequencing (NGS) combined with post-viral production methodologies such as CE analysis and transmission electron microscopy offer an effective high-throughput approach for monitoring AAV quality, from initial construct assembly to analysis of the encapsulated product. Both Illumina® short-read and PacBio® long-read sequencing technologies offer distinct advantages including sequencing of the entire viral genome, with detection of potential mutations, truncations, and contaminants. Here we describe our novel proprietary vector-agnostic workflows, for use in lentiviral, AAV or baculovirus settings. Starting with high quality synthesis of either vector plasmid or region of interest, full-length plasmid sequence is confirmed. Depending on these results, correction or new synthesis of plasmid options are applied. Following packaging within the viral vector system of choice, QC results using both short- and long-read NGS platforms are supported with a regulatory-compliant Sanger assay. State of the art synthesis reduces potential upstream errors. Sanger ITR sequencing and sequence correction upstream alleviates potential downstream issues in viral packaging. High-quality viral packaging ensures robust viral titers for downstream use. The combined NGS approach post-packaging alleviates current constraints for high throughput AAV sequencing and thereby enhance the overall QC process. Our Good Laboratory Practices (GLP) Sanger sequencing method extends read lengths through the entire ITR regions, allowing for rapid sequence confirmation of the final AAV product. The combination of these approaches enables a comprehensive solution, ideal for sequence confirmation of both transfer plasmid and final packaged product for improved viral vector gene therapy manufacturing in advance of FDA or EMA filings.

1055. Establishment of a Scale-Down Model for Robust and Cost-Effective Adeno-Associated Virus Process and Analytical Development and Characterization

Berenice Carrillo, Ran Li, Swathi Kuppireddy, Karina Cabrera, Gabrielle Huang, Alexa Truong, Weijie Yao, Liying Yang

PackGene Biotech Inc, Houston, TX

Adeno-associated virus (AAV)-based gene therapy has emerged as a promising approach for treating a wide range of genetic disorders. Despite advancement of innovative technologies and methodologies, AAV gene therapy is still facing a series of CMC challenges including those in upstream three-plasmid transient transfection and downstream empty/full viral particle separation, necessitating the development of robust and scalable manufacturing processes. For enhancement of process robustness, scalability and cost-effectiveness, we have established an integrative scale-down model for AAV manufacturing process development and optimization. The platform includes cell culture, transfection and lysis in a multi-well (MW) plate for upstream viral production, as well as cell lysate clarification, virus capture and elution with a spin filter column/plate for downstream crude purification. Multiple samples can be generated from different unit operations during the platform execution for AAV analytical characterization including titer, identity, purity, impurity and potency . We have shown that cell culture performance in MW plates have demonstrated a growth profile similar to that in shake flasks and bioreactors. Cell transfections performed in MW plates and shake flasks also result in comparable viral titers and empty/full ratios. After lysate clarification and optimized affinity purification with the spin filter column/plate, the purified viral samples can be subjected to more sensitive assays for characterizing critical quality attributes including process- and product- related impurities such as residual host cell DNA (HCD), protein (HCP), E1A oncogene and plasmid. In summary, the scale-down model developed in this study provides a cost-effective and efficient platform for process optimization and analytical characterization of AAV manufacturing process, and serves as a valuable tool for researchers and bioprocess engineers to investigate impact of various process parameters in AAV production process and critical quality attributes.

1056. Optimizing the Scalable Production of Recombinant Adeno-Associated Viral Vectors for Gene Therapies

Evan Tan, Patric Chua, Nurul Atiqah Herman, Alif Sufiyan Ali, Nur Izzati Soonaan, Wei Zhang, Say Kong Ng

Animal Cell Bioprocessing, A*STAR Bioprocessing Technology Institute, Singapore, Singapore

Background and novelty. Viral vectors based on adeno-associated virus (AAVs) have shown safety and efficacy as gene delivery vehicles. Viral vector production remains challenging as current production methods are not scalable. rAAVs are typically produced by the transient transfection of adherent HEK293 cells in serum-containing media and purified using ultracentrifugation. Upstream production

processes utilising serum-free suspension-based transfection process coupled with centrifugation-free downstream purification process allows for a scalable and efficient purification platform, essential for producing high-quality AAV vectors. Here, we describe an optimised serum-free suspension-based transient transfection process and a centrifugation-free downstream purification process capable of producing >10^{14} vg/L, post-purification. **Experimental approach.** rAAVs were produced by transfecting cells, in suspension, using PEI-MAX with plasmids pHelper, pAAV_RC2, and pAAV_GFP. Transfection process was optimised using DoE, with cell density at transfection and the concentrations of each plasmid as variables to be optimised. The optimised transfection process was validated in flask-scale production and the scalability of transfection process was demonstrated in 2L stirred-tank bioreactors. Lysis of the rAAV-containing culture was performed via a chemical-based approach utilizing Tergitol™ and nuclease. The lysate was then clarified using depth filters and then concentrated with ultrafiltration and diafiltration (UFDF). The primary capture step was performed using affinity column chromatography, whereas subsequent polishing was performed on anion exchange column chromatography for the separation of empty and full capsids. The final stage involved another round of UFDF to carry out buffer exchange, resulting in the final formulation of the AAV for use or storage. **Results and discussion.** rAAV titers obtained could be improved 2.3-fold after DoE optimization. In flask-scale productions, our current process yields rAAV titers comparable to commercially available workflows. Volumetric titers obtained in 2L STB are comparable to flask-scale productions. Similarly, we demonstrated a scalable, chromatography-based purification process as opposed to ultracentrifuge-based method. Enrichment of full capsids, assessed using a combination of TEM, PCR and ELISA assays, was up to 4-fold. Sufficient removal for impurities i.e., residual host cell protein and DNA, nuclease, and endotoxins, was also achieved. In addition, we have developed a streamlined and improved qPCR method for AAV quantification which performs comparably with dPCR.

1057. How to Resolve Vector-Based Therapeutics Major Manufacturing Pain Points by Innovation-Driven Collaborations

Rachel Legmann[1], Rene Gantier[2], Keen Chung[2], Kathleen Mihlbachler[3], Ganesh Krishnamoorthy[4], Frank Agbogbo[5]

[1]Gene Therapy, Repligen, Waltham, MA,[2]R&D, Repligen, Waltham, MA,[3]Systems, Repligen, Waltham, MA,[4]PD DSP, Forge Biologics, Grove City, OH,[5]PD, Forge Biologics, Grove City, OH

With increasing approvals of viral vector-based gene therapies, the industry is shifting its focus to the expansion of viral vector manufacturing to meet the demands of the growing number of therapies on the market. The higher complexity of viral vectors compared to monoclonal antibodies or recombinant proteins creates additional production and purification challenges to overcome. Therefore, collaboration, better fit innovative platforms and expertise are crucial for rapid continuous improvements in the efficiency of viral vector production to meet demand and get lifesaving therapies to patients quickly. This must be carefully balanced with consistency and quality of the final product. Multiple applications and technologies to address current manufacturing challenges and solutions to increase overall viral vector yield throughout development will be explored. Collaborative case studies will be presented to demonstrate the progress associated with entire process intensification and optimization from benchtop development to large-scale commercial manufacturing. Upstream process intensification strategies using the KrosFlo® tangential flow depth Filtration (TFDF®) technology, a perfusion platform for lentivirus and AAV high production and clarification will be presented. Two experimental upstream studies illustrate the next generation manufacturing process for AAV and LV viral vector intensification using HEK293 cells in suspension. In both experimental studies, the TFDF, a perfusion platform, was integrated into the bioreactor to intensify AAV8 viral vectors and in the second study lentivirus (LV) was intensified. The TFDF, a perfusion integrated platform, increased the HEK293 cell density by 3 X fold at time of transfection led to increase up to 10 X fold of total titer of rAAV8 and rAAV9. The cell retention filters enabled the continuous harvest clarification of secreted AAV8 particles into the media during the virus production, demonstrating the potential for continuous upstream-downstream processing of secreted AAV vectors. The TFDF platform increased lentivirus (LV) yield up to 86 X through synergism between high cell density, higher specific productivity, and continuous harvest approach. Overall, the TFDF platform enables manufacturers to meet high vector demand and potentially reduce the cost per dose. We will also present the collaborative effort with a gene therapy CDMO that involved a detailed evaluation of scaling up the affinity chromatograph step for AAV9 from a 1-L benchtop chromatography scale to a 167-L industrial scale using the innovative single-use advanced KRM™ Chromatography System. The large-scale tests were sequentially executed with the benchtop-scale tests in parallel. All process fractions were analyzed by Droplet Digital PCR. It also demonstrated high reproducibility between the scale-up runs without compromising quality product attributes such as empty and full ratio, endotoxin, residual host cell protein and aggregation. This successful AAV scaleup demonstrated a 240-fold increase in the overall viral recovery that is 1.4 X fold higher than predicted, as well as good reproducibility of the scale-up runs. The innovative KRM™ Chromatography Systems enabled high productivity and cost-effective manufacturing of viral vectors, including AAV and lentivirus, through novel design features like low hold-up volumes, gentle internal flow paths, and accurate pump performance. The results of this case study will further demonstrate the capabilities to scale-up form 500-L to 5000-L manufacturing scale bioreactors.

1058. Whole Genome High-Throughput Screen Identified microRNAs Enhancing rAAV Production

Bettina Finkbeiner[1], Madina Burkhart[2], Sabrina Reichl[1], Rupert Derler[1], Andreas Schulze[1], Florian Sonntag[1], Kerstin Otte[2], Markus Hoerer[1]

[1]Ascend, Planegg, Germany,[2]Institute for Applied Biotechnology, Biberach University of Applied Sciences, Biberach an der Riss, Germany

Recombinant adeno-associated viruses (rAAV) have become the leading vector for gene therapy in recent years. However, manufacturing bottlenecks pose a severe challenge to provide the required high quality rAAV amounts for increasing clinical and commercial demands. Scale-up of vector manufacturing and additional capacities are now commonly observed. However, further increase in cell-specific and

volumetric rAAV productivity are still neglected strategies to drive rAAV yields and beyond that potentially rAAV quality. MicroRNAs are short non-coding RNAs playing a crucial role in the regulation of gene expression during virtually all cellular processes and were already successfully exploited to boost the manufacturing of classical biologics. Therefore, microRNAs are considered a useful tool to overcome critical bottlenecks in rAAV vector production that, if addressed, could drive rAAV production efficiency towards the required levels. Based on the Ascend HEK293 cell line and split two plasmid system we have developed a robust modular suspension platform process for rAAV production, that is optimized towards yield at best possible and consistent quality from bench to 200 L scale. For a systematic identification of host cell factors that significantly impact rAAV productivity of our HEK293 cells, we performed a high-throughput screen using a genome-wide library of human microRNA mimics. With this approach, 160 microRNAs increased rAAV yields by more than 2-fold while over 1000 microRNAs decreased rAAV yields. The effect of the most promising microRNAs was confirmed using a multi-parallel microbioreactor system as down-scale model of our production platform. In summary, the top microRNAs identified from the genome-wide screen provide a very promising platform to further improve rAAV vector yields.

1059. AAV Capsid ELISA Automation for High-Throughput AAV Titer Determination

Silja Syrbe, Dana Holzinger, Caroline Odenwald, Christina Sonnek, Katharina Hammer

PROGEN, Heidelberg, Germany

The AAV gene therapy community is facing major challenges like the competitive go-to-market time pressure as well as increasing numbers of samples that need to be processed and analyzed within a very limited time span. Consequently, there is a growing demand for more time-efficient solutions with less hands-on time for analytical characterization of AAV samples. Enzyme-Linked Immunosorbent Assay (ELISA) is an industry standard in AAV gene therapy, well-known for its accuracy and reliability. Nevertheless, the time-consuming nature may hinder rapid analysis, which is essential for meeting tight production schedules or addressing urgent quality control issues. An automated ELISA processing system might significantly reduce potential human errors and variability while allowing fast and high-throughput analyses. To show that AAV capsid ELISA is suitable for high-throughput processing, we transferred the manual AAV9 ELISA (PRAAV9) to a fully automated system. We analyzed plate homogeneity, intra- and inter-assay variance, as well as recovery obtained by the automated system and compared the data to the same key performance indicators obtained by manual processing. Using a fully automated system significantly reduced hands-on time and allowed higher sample throughput, whilst maintaining robustness and accuracy of the conventional AAV ELISA. With this data we show a high-throughput protocol for our AAV ELISAs, thus can support the CGT users to accelerate efficiency of AAV titer determination.

1060. Determination of Full-Empty Ratio of AAV Standard Material by Orthogonal Methods

Lisa Mutz[1], Robert Mildner[2], David Goloka[2], Benjamin Draper[3], Catia Crespo[4], Katharina Hammer[1], Caroline Odenwald[1], Dana Holzinger[1]

[1]PROGEN, Heidelberg, Germany,[2]Waters | Wyatt Technology, Dernbach, Germany,[3]Megadalton Solutions, Bloomington, IN,[4]Refeyn Ltd., Oxford, United Kingdom

Today, AAV-based gene therapy is one of the most promising and fastest growing branches of modern medicine. Moving forward to a more clinical focus in this field, the use of reliable AAV standards and orthogonal methods is indispensable to meet current FDA guidelines. Since the production of comprehensively characterized, internal standard material is not feasible for a large number of companies and a lack of commercially available reference material, we now offer AAV standard material for the most commonly used AAV serotypes. We have established a comprehensive characterization process for these commercially available standards. Besides testing several characterization parameters (e.g. vg and capsid titer, filling grade, purity and aggregation), this process also contains the analysis with a set of suitable orthogonal methods ensuring the utmost level of accuracy and reliability of the data. Here, we present our study on current orthogonal methods for determining the full-empty ratio and their comparability using our eGFP-filled AAV standard material (AAV2, AAV5, AAV8 and AAV9). The study includes the full-empty analysis using Charge Detection Mass Spectrometry (CD-MS), Size Exclusion Chromatography - Multi-angle Light Scattering (SEC-MALS), Mass Photometry (MP), and a combination of Dynamic Light Scattering/ Static Light Scattering and Ultraviolet and Visible Light Measurements (DLS/SLS-UV/vis). Depending on the serotype and the method, some variations were observed regarding the filling grades. The average filling grades across serotypes were measured by DLS/SLS-UV/Vis, CDMS, SEC-MALS, and MP. The results obtained from CDMS and MP showed in average the highest correlation, while a little higher and lower full-empty ratios were observed using SEC-MALS and DLS/SLS-UV/Vis, respectively. The use of reliable standards within diverse orthogonal methods ensures not only the most accurate result but also the safety of the final product in compliance with current FDA requirements. Therefore, we present the reliability of our characterization process for AAV standards, thus demonstrating their reliability and suitability for biophysical and immunological applications, respectively.

1061. Quadplex dPCR as a Tool to Analyze Genome Integrity and Heterogeneity for Gene Therapy Drug Products

Ryan Massopust[1], Annabelle Phillips[1], Nejc Košir[2], David Dobnik[2], Sarang Brahma[1], Maria Belousova[1], Andrea R. Moore[1], Joshua Grieger[1]

[1]Asklepios Biopharmaceutical Inc., Research Triangle Park, NC,[2]Niba Labs, Ljubljana, Slovenia

Cell and gene therapies are transformational treatments with high numbers of IND-filings and product approvals in recent years. This rapid growth presents a need for improved product-specific analytics. Critical quality attributes, such as viral genome (VG) or viral particle (VP) titer, do not consistently correlate with drug potency or safety,

demonstrating a lack of genome-specific analyses. It is hypothesized that the heterogeneity of packaged genomes is a potential cause for this inconsistency. Therefore, there is a pressing need to improve characterization of packaged genomes for their heterogeneity and integrity. Our approach employs quadplex digital Polymerase Chain Reaction (dPCR), utilizing four primer probe sets that span the genome from promoter to poly(A) signal sequence, covering the critical elements of the AAV cassette. Novel genome integrity analysis utilizing the quadplex dPCR data allows calculation of both the absolute number and distribution (heterogeneity) of the target based AAV genome variants. The results presented here demonstrate that the number of intact genomes and the proportion of intact genomes relative to truncated variants changes across different lots of the same drug product. Thus, this novel assay may provide impactful insights into variable potencies and packaging efficiencies of gene therapy products. Results from dPCR genome integrity analyses may be utilized throughout the drug development pipeline, informing research and development all the way through patient dosing. We believe, in combination with orthogonal analytics such as Next Generation Sequencing and Analytical Ultracentrifugation, multiplex dPCR assays will serve as a core component of genome integrity and heterogeneity analytics packages.

1062. Production of AAV Vectors Using Synthetic, Enzymatically Produced Linear DNA

Celine Winckler, Florent Fordoxel, Angel Picher, Heikki Lanckriet, Amy Walker

4basebio, Cambridge, United Kingdom

Adeno-associated virus (AAV) remains a popular vector for gene therapy. However, AAV manufacture faces several challenges to keep pace with the demand for large scale, high quality batches. The standard plasmid triple-transfection presents several issues including availability and cost of GMP grade plasmid, sequence fidelity of the inverted terminal repeats (ITR), and safety concerns over non-specific encapsidations. 4basebio has developed a proprietary, scalable synthesis process for the production of linear closed DNA constructs via its Trueprime™ amplification technology. The hpDNA™ produced is devoid of any bacterial backbone and circumvents cumbersome fermentation processes required for plasmid DNA. Here, we compared the production of AAV vectors using hpDNA™ encoding for the typical Adenovirus helper functions, *rep* and *cap* genes, and an expression cassette consisting of AAV2 ITRs and a reporter gene driven by an ubiquitous promoter. Conventional plasmid triple-transfection was used as a control. We achieved equivalency in viral genome titres, full:empty ratios and infectivity between the two production methods. We have demonstrated that functional AAV vectors can be produced using hpDNA™, which could greatly accelerate therapeutic development of gene therapy programmes. The technology could overcome the difficulties associated with complex ITR structures required for AAV production, which are inherently difficult to synthesise via bacterial propagation systems. Moreover, the lack of plasmid backbone sequences such as antibiotic resistance genes enhances the safety profile of the AAV product.

1063. Transcriptomic Analysis Identifies Differential Expression Patterns in Cellular Stress Response, Signal Transduction, and Extracellular Matrix Proteins During AAV Production

Joshua Tworig[1], Francis Grafton[1], Markus Hörer[2], Chris A. Reid[1], Mo Mandegar[1]

[1]Ascend Gene and Cell Therapies, Alameda, CA,[2]Ascend Gene and Cell Therapies, Munich, Germany

Recombinant adeno-associated virus (rAAV) is a widely used viral vector for gene therapy. Despite its clinical efficacy, the manufacturing of rAAV faces challenges in productivity and vector quality, leading to high costs and limited availability of gene therapies. To meet the growing clinical and commercial demand, mechanistic understanding of the cellular response to rAAV production is required to develop next-generation rAAV production processes. In this study, we performed transcriptomic analysis at multiple stages of rAAV production to better understand the pathways altered during this process. RNA-sequencing was performed on suspension-adapted HEK293 cells originating from two polyclonal populations during rAAV9 production. Polyclonal and clonally derived cells were included in our analysis to identify robust transcriptional signatures independent of clonal differences. Differential expression analysis across timepoints revealed that heat shock and inflammatory proteins, as well as Golgi organization, spindle assembly, and other cytoskeleton-associated components were among the most significant upregulated genes, while inhibitors of DNA-binding proteins were most consistently downregulated during AAV9 production. A variety of extracellular matrix-associated proteins also exhibited significant changes in expression across AAV production. These data uncover novel and previously identified pathways that may affect rAAV productivity, potentially enabling a path to engineer improved processes and cell lines for higher yields and better quality rAAV production.

1064. Ministring DNA (msDNA™): Maximizing Quality of Novel DNA Vectors

Steven J. Hersch[1], Siddarth Chandrasekaran[1], Jamie Lam[1], Peyman Mokarami[1], Roderick A. Slavcev[1,2], Nafiseh Nafissi[1,2]

[1]Mediphage Bioceuticals, Toronto, ON, Canada,[2]School of Pharmacy, University of Waterloo, Waterloo, ON, Canada

Gene and cell therapy have elevated DNA vectors to crucial roles as starting or intermediate products in genetic medicine pharmaceuticals. The quality of DNA material is paramount in determining the ultimate product quality and its compliance with regulatory approvals. Ministring DNA (msDNA™) emerges as a novel DNA platform technology, generating a linear, covalently closed (LCC) DNA vector exclusively containing the sequence of interest. Produced through a simple *in vivo* reaction in *E. coli* cells, msDNA™ incorporates essential components for viral vector production, mRNA production, or non-viral gene therapy. Distinguishing itself from conventional plasmid-based systems, the msDNA™ is entirely free of undesirable contaminating bacterial backbone sequences. This positions it as an advantageous choice for a variety of gene and cell therapy applications.

In vivo manufacturing methods for msDNA yield pure, endotoxin-free, high-quality DNA. Many bacterial sequence-free or LCC DNA vectors are synthetically produced *in vitro* via polymerase-based manufacturing platforms. However, since bacteria encode mutation-reduction mechanisms, such as mismatch repair, our investigation into DNA production *in vivo* in *E. coli* focused on enhancing DNA fidelity. Commonly used next-generation sequencing approaches face limitations in sensitivity, making them insufficient to observe the low mutation rates predicted for *E. coli* in the literature. To address this challenge, we introduce a novel assay based on loss-of-function (LOF) mutations in the conditionally toxic sacB gene. Our findings reveal that DNA production in *E. coli* results in significantly fewer LOF mutations (80- to 3000-fold less) compared to enzymatic/polymerase DNA synthesis using PCR and rolling circle amplification (RCA). This study emphasizes that DNA synthesized *in vivo* in *E. coli* exhibits significantly fewer mutations than enzymatically-produced DNA, potentially enhancing the quality, fidelity, potency and yield of final pharmaceutical products. In summary, msDNA™ stands out as a scalable, high-quality, and high-fidelity LCC DNA vector with promising implications for advancing genetic medicine.

1065. Developing Fed-Batch Strategy to Optimize Plasmid DNA Production in Escherichia Coli DH5alpha for Cost-Effective Manufacturing

Jacob T. Hromyak, Carson G. White, Dylan J. Vasconcelles, Dennis O. Concepcion, Mahajoub B. Bello Roufai

Research and Process Development, Akron Biotech, FL, Sarasota, FL

Demand for high-quality plasmid DNA (pDNA) has greatly increased in recent years due to its myriad applications across therapeutic modalities - including gene therapies, vaccines, and mRNA. This increased demand poses a challenge to pDNA manufacturers, which must continue to increase yield and productivity without compromising on rigorous standards for purity, and ultimately, safety. Productivity in pDNA manufacturing is heavily influenced by host strain, plasmid type and size, growth medium and growth conditions. A widely used culture medium, TB (Terrific Broth) offers high cell density cultures but lacks the capacity to generate large quantities of pDNA (>60 ng/µl). In understanding the significance and effect of peptones in TB a proprietary chemically defined media was designed to increase pDNA titers. This abstract captures efforts to create a high yielding and scalable fermentation process, involving the evaluation of various strategies in Escherichia coli DH5α transformed cells. The development of the process was driven by a three-level single-factor experimental design focused on media composition, temperature, concentration of peptone and carbon source. Improvements in small- and large-scale pDNA yield during fermentation, resulting from media selection and optimization are summarized. The results revealed that a chemically defined media used in conjunction with a nutrient-dense feed supported high cell density cultures and increased pDNA yield and specific productivity when compared to conventional exponential fed batch cultures in TB media. The feed approach developed was optimal for the three plasmids tested and constitute a robust pDNA yield optimization platform established at Akron enabling the production of cost effective pDNA for advanced therapies. This feed-based optimization platform is being tested with various E. Coli Stains and plasmid sizes for fitness and possible strain specific optimizations.

1066. AAV-2 Production with Suspension HEK293 Cells in Scale-X Achieves High Productivity, High Full-to-Empty Capsid Ratio and Low Contaminants

Floriane Cohen

Univercells Technologies, Nivelles, Belgium

Adeno-associated viruses (AAV) are the vector of choice for a wide range of gene therapies, with a focus on rare diseases but increasingly shifting towards higher prevalence indications. This trend combined with varying dose requirements, sometimes exceeding 10E15 vg, means that manufacturing capacity will increasingly become a bottleneck. While stirred tank bioreactors (STRs) offer the hope of large-scale manufacture, they possess inherent limitations which means that scaling from the bench to commercial scale will always be an iterative and risky process with potential delays in the time to market. The next generation fixed-bed bioreactors such as the scale-X offer a low-volume, low-footprint, high-concentration alternative which not only reduces scale-up challenges for processes with suspension and adherent cell lines but also lowers the cost of goods. In this study, it is demonstrated that a suspension HEK293F cell line is successfully grown at high cell density in serum-free media in the scale-X bioreactor achieving the following results compared to the reference suspension process: - 3-fold increase in AAV-2 productivity - 3-fold increase in full-to-empty capsid ratio - A 10-fold reduction in HC-DNA and 5-fold reduction in HCP in the cell culture harvest A drug substance cost of goods reduction ranging from 25 to 52% process

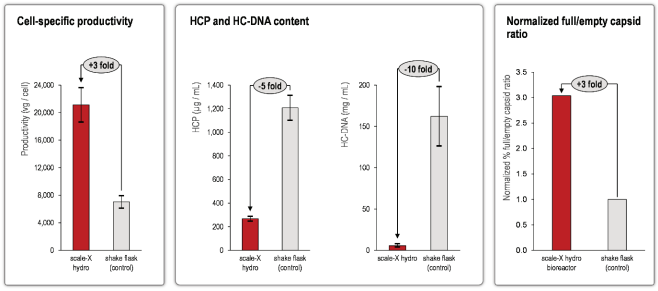


Figure 1. Cell-specific productivity (left), contaminants (centre) and full-to-empty capsid ratio (right) compared to reference shake flask

1067. A Novel, Synthetic DNA Alternative for rAAV Manufacturing

Ileana Guerrini, Roxanne Lourman, Martin Cusack, David Wilson, Luca Distefano, Ivana Pastierikova, Marco Guarrera, Alexander Pekarsky, Jorge Omar Yanez Cuna, Joel de Beer

Anjarium Biosciences AG, Schlieren, Switzerland

The number of FDA-approved gene therapy products using recombinant adeno-associated virus (rAAV) has grown rapidly in the last few years and highlights the key role this vector will play in future gene therapies. However, current manufacturing constraints

pose challenges in meeting the increasing demand for rAAV-based therapies. A fundamental challenge is the sourcing of large quantities of plasmid DNA, the essential starting material for conventional rAAV production, which entails long turnaround times and a high probability of alterations in the AAV-specific ITR sequences during the fermentation-based production processes. Furthermore, the bacterial sequences can potentially be packaged, risking the safety profile of the AAV product. We have developed a novel, optimized synthetic linear double-stranded DNA with customizable hairpin-ended structures for use in rAAV manufacturing. Our production process has several advantages over that of plasmid DNA since it is a one-pot, enzymatic, cell-free, scalable reaction with high yields and rapid production times. The final DNA product is characterized by superior purity and quality, and better product homogeneity. Interestingly, our customized hairpin-ended structures seamlessly integrate with the function of each synthetic DNA construct. To replicate and package the GOI without the need for any additional unwanted sequence, the hairpin ends of our GOI construct were designed as AAV2 ITR-like hairpins for single strand AAV (ssAAV) or self-complementary AAV (scAAV). To prevent the packaging of AAV structural and replication sequences, the hairpin ends of our RepCap and Helper were designed with synthetic hairpin structures. The absence of bacterial sequences in our DNA material reduces the risk of unwanted DNA sequences in the resulting rAAV product. Here we show that our synthetic DNA is suitable as input for ssAAV or scAAV manufacturing with a range of serotypes in a conventional triple transfection set. Triple transfection with plasmid constructs was used as reference. We show all rAAV produced from our synthetic DNA to be functionally equivalent to rAAV produced from plasmid. In addition, rAAV production from our novel DNA was comparable or better in yield, percentage of full:empty ratios, particles and infectivity. Altogether, our results point to our synthetic DNA as a superior alternative for starting material for AAV production compared to conventional plasmid DNA because of the speed and scalability of our process and the reliability and performance of our product.

1068. A Mechanistical Understanding of Producer Cell Line (PCL) Approaches for AAV Production Using Metabolomics

Swetha Kumar[1], Adhvait Shah[1], Laura Parisi[2], Lilu Guo[2], Bailin Zhang[2], Darrick Yu[1], Madhuresh Sumit[1], Jeong Lee[1]

[1]Genomic Medicine Cell Culture Process Development, Sanofi US Services Inc, Waltham, MA,[2]Research and Development, Sanofi US Services Inc, Cambridge, MA

With more than 30 CGT FDA product approvals, gene therapy is emerging rapidly and provides much promise in the treatment of rare neurological diseases. To have a state-of-the-art process that helps improve patient access to life-saving care, it is imperative that we gain a mechanistical understanding of manufacturing processes that increase productivity while maintaining acceptable product quality, particularly AAV based processes that are most employed for gene therapy manufacture. In the era of 'Big Data', we have employed metabolomics in our biopharmaceutical manufacturing process to enable data-driven process design and optimization. Using an in-house developed PCL, we employed metabolomics to identify differences in cellular response and AAV production upon wild-type Ad5 (wtAd5) infection. The conditions were run as biological replicates in batch and perfusion modes, at different cell densities in Sartorius Ambr250® systems. Supernatant samples collected at multiple time-points over the first 48 hours post infection were analysed on Flex2, Cedex BioHT, LC-MS/MS for metabolome profiling. AAV, Ad5 titer were quantified upon harvest. Differences in metabolite profiles between conditions with different AAV productivities, from pathways that are both cell specific and wtAd5 infection specific, were observed. Functional analyses, including KEGG and Gene Ontology (GO), of the metabolome data suggests accumulation of metabolite by-products that could potentially inhibit AAV production in batch cultures, removal of which could lead to increased productivity. We hypothesize that by gaining a deeper understanding of cellular response to wtAd5 infection, we can manipulate AAV processes to higher productivity and product quality. Using the power of 'Big Data', we can identify process switch-points, challenges and biomarkers that can guide scientists to design bioprocesses with superior cellular performance and process productivity.

1069. AAV Process Development in Culture Biosciences' 250mL Bioreactors

Rebecca Du, Hannah Monroe, Kris Tyner, Jean-Marc Guedon, Kat Kozyrytska, Elena Cant, Darcy Birse

Culture Biosciences, South San Francisco, CA

Adeno-associated viruses (AAVs) have emerged as an extremely promising and widely-used delivery vehicle for gene therapy. However, a major hurdle in the development of AAV-based gene therapy is the high cost of manufacturing AAV on the clinical and commercial scale due to the difficulty of scale-up and the inherently high variability during AAV production. Using triple transfection in suspension HEK293 has been shown to yield viral genome titers in excess of $1x10^{14}$ genomes/L. However, the percentage of filled AAV capsids and genome titers can range widely across batches and adjustments in upstream and downstream processing are required for each unique AAV serotype, making it difficult to standardize AAV production. Additionally, AAV production processes are not particularly robust; even after optimization, simply switching plasmids or transfection reagents can result in order-of-magnitude differences in viral titers, necessitating additional rounds of process optimization. These challenges are difficult to overcome when running just a few experiments at a time and are exacerbated by the high variability of AAV processes, which can mask the effect of the parameters being tested. However, they can be addressed through high-throughput, intentional exploration of design space using sufficient replicates to confidently identify critical process parameters that drive efficient and robust AAV production. Here we show production of multiple AAV serotypes in our 250mL bioreactors and demonstrate how running multiple small-scale bioreactors in parallel enabled us to quickly identify transfection reagents, complexation conditions, and bioreactor process parameters to improve overall yields.

1070. UpTempo℠ AAV Platform: Enhanced Process Development to Produce High Quality, High Yield AAV

Elena Andreeva-Gurnev, Casey Vanderheyden, Janna Q. Wisniewski, Mourad Mellal, Arthur Rostovtsev, Gunjan Andlay, Qin Guo, Brian Howard, Elena Prikhodko
Catalent Cell & Gene Therapy, Baltimore, MD

Catalent's UpTempo AAV platform provides a streamlined path from process development to drug product manufacturing for AAV gene therapies, addressing a bottleneck in time to a clinic for early-phase products. Multiple aspects drive product quality and speed, including a platform manufacturing process, Catalent's off-the-shelf (OTS) plasmids, and Catalent's clonal HEK293 cell line (Cell Line). In the UpTempo AAV process, recombinant AAVs are produced via transient transfection of HEK293 cells, cell lysis, and clarification, concentrated via tangential flow filtration (TFF), and purified by affinity chromatography, anion-exchange chromatography, and final formulation. UpTempo AAV process development includes product-specific optimization for yield and quality. Data will highlight recent platform updates including a Design-of-Experiments (DOE) approach to establish optimal transfection parameter design space, an improved harvest strategy to reduce processing time and reduce feed stream impurity levels, and screening of additional compounds that increase production titer up to 3-fold. In addition, 2L production and purification data were obtained across an extended set of AAV serotypes (1, 2, 5, 6, rh10) using Catalent OTS plasmids and Cell Line. Regardless of serotype, 2L yields measured up to 3 x 10^{11} vg/ ml, purity up to 99%, full capsids in the range of 75%, and empty capsids in the range of < 10%, assessed by analytical ultracentrifugation. Improved quality attributes were observed including reduced residual host cell DNA compared to AAVs produced with competitor tools. Scaling data indicates comparable yield, quality, and recovery, confirming that the UpTempo℠ platform process offers a robust solution for AAV manufacturing.

1071. How Does ICH Q5A R2 Impact Viral Clearance Studies for Non-Enveloped Gene Therapy Vectors?

Danielle Hickman
MilliporeSigma, Rockville, MD

Viral contamination of a manufacturing process for a biological product has wide implications from the manufacturer to the patient, resulting in many consequences such as drug shortages and the high cost of investigation, cleanup, and corrective actions. To avoid viral contamination, manufacturers implement a three-fold viral safety strategy: 1) Preventions: sourcing safe raw materials, other starting materials, and facility design; 2) Detection: testing cell banks and process intermediates; and 3) Removal/Reduction: evaluating downstream purification steps for their potential to inactivate or remove a contaminating virus. This has been a successful strategy, because although contamination events have occurred, viral contaminants have not been detected in the drug product. ICH Q5A R2 includes guidance for non-enveloped gene therapy vectors that are amenable to viral clearance. The scope of a viral clearance study is dependent on the stage of process development, with studies to support early-stage clinical trials smaller in scope than studies to support a late-stage clinical trial or a BLA. In this presentation, stage specific viral clearance study requirements and strategies will be discussed. In addition, data will be presented that demonstrates that non-enveloped gene therapy vector products have virus removal or inactivation capacity in their manufacturing processes.

1072. Development of a Serum-Free Suspension HEK293 Cell Line for AAV Production with High Yield and Low Encapsidated DNA Impurities

Hairui Yang
OBIO TECH, Shanghai, China

Adeno-associated virus (AAV) has emerged as a successful tool in gene therapy, with different serotypes exhibiting remarkable efficiency in targeting specific organs and tissues. The escalating demand for large-scale manufacturing of various AAV serotypes, particularly for pre-clinical and clinical trials, underscores the need to enhance the packaging capacity of producer cells, thereby optimizing the productivity of AAV vectors. This study delves into the growth characteristics and AAV packaging titer of a high-yield monoclonal cell line derived from HEK293. The findings reveal a substantial increase in the packaging titer of these high-yield HEK293 monoclonal cells—ranging from 17% to an impressive 435% when compared to the VPC2.0. Concurrently, the residual host cell DNA was notably reduced by 50% to 70% in comparison to the VPC2.0. To validate the scalability of the selected high-yield monoclonal HEK293 cell line in a manner more representative of current Good Manufacturing Practice (cGMP) standards, AAV production was assessed in bioreactors ranging from 15L to 50L to 200L. Consistent with the trends observed at 15L and 50L, the results from the 200L bioreactors demonstrated a remarkable 1.6-fold increase in genome productivity, coupled with comparable capsid production and consistent levels of residual host cell DNA. These data affirm that the chosen high-yield HEK293 cell line exhibits linear scalability across diverse production scales, maintaining consistent results. The demonstrated consistency in up to 200L bioreactors establishes the viability of cell line development for commercial supply, opening avenues for applications in indications with larger patient populations. More Importantly, this method is not only applicable to AAV production but can also be extended to other types of cell screening, demonstrating its broad application value.

Thursday Posters: Metabolic Diseases

1073. Modeling ABCD1 Deficiency in Adrenocortical Cells by CRISPR and Correction viaAAV-Mediated Gene Delivery

Yi Gong[1], Vasileios Kreouzis[1], Ann Moser[2], Casey A. Maguire[3], Florian Eichler[1]
[1]Massachusetts General Hospital, Boston, MA,[2]Hugo W Moser Research Institute, Baltimore, MA,[3]The Massachusetts General Hospital, Charlestown, MA

X-linked Adrenoleukodystrophy (X-ALD) is a debilitating monogenic disease caused by pathogenic variants in *ABCD1* that affects both males and females. The *ABCD1* gene localizes to Xq28 and encodes an ATP-binding cassette (ABC) transporter that mediates the transport of very long chain fatty acids (VLCFA) from cytosol to peroxisome where β-oxidation takes place. Thus, accumulation of VLCFA is the biochemical hallmark of X-ALD. With a birth incidence of about 1:14700, X-ALD is considered the most common peroxisome disorder. There is no clear genotype phenotype correlation in X-ALD, and the clinical spectrum ranges from devastating cerebral inflammatory demyelination (CALD) to a slowly progressive myeloneuropathy affecting spinal cord and peripheral nerve. The adrenal glands are commonly affected with a lifetime risk of adrenal insufficiency in ~80% of ALD males with VLCFA accumulation being the likely culprit of pathology within the adrenal cortex. In the present study, significant accumulation of VLCFA was detected in adrenocortical cells (HAC15) following ABCD1 silencing with a consequent notable reduction of cortisol levels in vitro. Lipid treatment has no clear impact on adrenal cortisol function in nontargeting (NT control) HAC15 cells. In contrast, ABCD1 deficient cells displayed further inhibited adrenal cortisol production following VLCFA treatment, suggesting the additive impact of genetic and environmental factors. In HAC15 cells we employed 2sgRNAs to mediate a large fragment deletion in *ABCD1* using CRISPR-Cas9. This led to profound accumulation of VLCFA in vitro. Most importantly, AAV9 mediated ABCD1 gene delivery to HAC15 knockout cells at 1E6 gc/cell significantly alleviate the VLCFA accumulation. While AAV9-mediated ABCD1 intrathecal delivery in the X-ALD mouse model has demonstrated efficacy in treating neurological features of ALD and has led to a current clinical trial to address the myeloneuropathy of X-ALD, our proof of concurrent AAV9-ABCD1 transduction in adrenal cortex provides a rational for AAV mediated gene therapy in the treatment of adrenal insufficiency.

1074. Correction of Arginine:Glycine Amidinotransferase (AGAT) Creatine Deficiency through a Novel Bicistronic AAV9 Construct

Tesla A. Peretti[1], Troy B. Webster[1], Robyn T. Binsfeld[1], Ilona Tkachyova[2], Michael B. Tropak[2], Andreas Schulze[2,3], Jagdeep S. Walia[1,4]

[1]Queen's University, Kingston, ON, Canada,[2]The Hospital for Sick Children, Peter Gilgan Center for Research and Learning, Toronto, ON, Canada,[3]Departments of Pediatrics and Biochemistry, University of Toronto, Toronto, ON, Canada,[4]Departments of Pediatrics, Queen's University, Kingston, ON, Canada

Creatine is a small molecule that plays an important role in energy metabolism and is required for overall health and neurodevelopment as seen from creatine deficiency syndromes (CDS). CDS are inborn errors of metabolism which result in neurological manifestations including intellectual disability, developmental delay, and seizures emphasising the importance of creatine in brain function. Endogenous production of creatine begins through the enzymatic function of arginine:glycine amidinotransferase (AGAT) to form guanidinoacetate (GAA) which is then converted to creatine by guanidinoacetate methyltransferase (GAMT) enzyme. AGAT-deficiency (AGAT-D) is an autosomal recessive disorder caused by loss of function mutations in the *GATM* gene encoding the AGAT protein preventing endogenous creatine production. Currently, the only treatment for AGAT-D relies on daily creatine supplementation, however this is not curative and does not restore AGAT function or normal creatine levels in the brain, leaving many symptoms untreated. We have performed a proof-of-concept *in vitro* study using two plasmid constructs- *phGATM* and *phGATM-GAMT*, through transfecting AGAT knockout HAP1 cells. Results demonstrate restoration of AGAT protein and creatine production. HAP1 cells were cultured in specialized media so that any creatine quantified would have to be produced by the cells. The cell lysates were analyzed for guanidino compounds such as creatine and GAA through liquid chromatography tandem mass spectrometry. Both constructs were able to restore creatine production, however in the *phGATM* treated cells, GAA levels were significantly elevated which could lead to potential neurotoxic effects likely due to relative GAMT-D, while the *phGATM-GAMT* treated cells received both enzymes for the pathway preventing GAA accumulation. These results have inspired the *in vivo* proof-of-concept study that is currently underway using *AAV9.hGATM* and *AAV9.hGATM-GAMT* vectors in murine models of AGAT-D. Further studies will aid in determining optimal efficacy and safety of the vectors for translation into clinical use for AGAT-D and potentially other diseases.

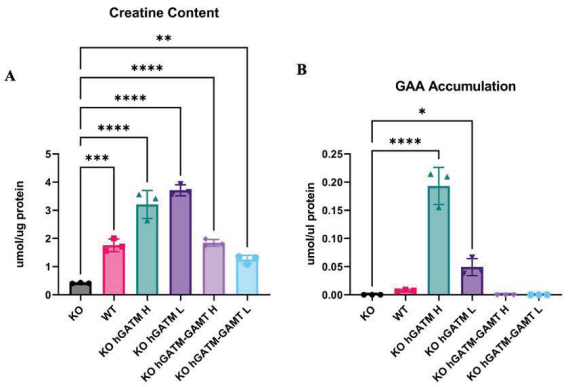


Figure 1. Intracellular creatine and GAA content of knockout (KO) *GATM* cells following high dose and low dose transfection of *phGATM* and *phGATM-GAMT*. Untreated (GFP transfected) KO cells represent the negative control and untreated (GFP transfected) wildtype (WT) cells represent the positive control. **A.** Treatment of *GATM* KO cells with high (3.75μg) and low (1.25μg) doses of *phGATM* and *phGATM-GAMT* significantly increased the intracellular creatine levels compared to KO levels (**** $p < 0.0001$, ** $p < 0.01$). Treatment with high dose (3.75μg) of *phGATM-GAMT* restored intracellular creatine levels to that of untreated WT HAP1 cells. **B.** Treatment of *GATM* KO cells with a high dose (3.75μg) of *phGATM* significantly (**** $p < 0.0001$) raises GAA levels to that of *GAMT* KO cells (unpublished Walia Lab data) leading to potentially toxic effects. Treatment of *GATM* KO cells with a high dose (3.75μg) and low dose (1.25μg) of *phGATM-GAMT* does not result in any GAA accumulation.

1075. From Rare Challenges to Results: Lessons Learned in Gene Therapy Development for Ultra-Rare Disorder

Julia Taravella

Rare Trait Hope Fund, New Olreans, LA

Aspartylglucosaminuria (AGU) (OMIM #208400) is an ultra-rare lysosomal storage disorder caused by pathogenic mutations in the AGA gene. This leads to a deficiency in aspartylglucosaminidase enzyme responsible for breaking N-linked glycoproteins. The deficiency results in the accumulation of these compounds, impairing bodily functions, particularly in the central nervous system. Patients present with speech/language and cognitive developmental delays, often diagnosed as autism, followed by a slow and steady decline in motor function

and cognitive abilities. Individuals remain dependent on caregivers for the entirety of their lives before succumbing to premature death around 35 years of age. Currently, there are no curative therapies for AGU. The inheritance pattern of AGU is autosomal recessive, and its prevalence varies across different populations. A significant majority of the research was conducted in Finland in 1970-1990s due to the disease higher presence in the Finnish population. These early efforts to develop a cure for AGU included promising gene and enzyme replacement therapies studies in animals. However, challenges such as securing sufficient funding and scaling up production posed significant obstacles, resulting in stalled research efforts. By 2013, while funding remained a hurdle, scientific advancements had led to a more standardized approach in the development of gene replacement therapy using AAV (Adeno-Associated Virus). This scientific progress marked a significant step forward in the potential treatment of AGU that was recognized by Rare Trait Hope Fund. This presentation discusses the development of gene therapy for AGU, from diagnosis, to pre-clinical development, to clinical trials, highlighting the unique challenges and strategies needed to make gene therapy a feasible option for ultra-rare disorders such as AGU. Emphasis will be placed on cost management, the need for multi-party collaboration, and understanding the nuances of developing treatments for rare diseases. This comprehensive overview aims to illustrate the process of bringing gene therapy from concept to clinical application for an ultra-rare diseases. The presentation will further discuss key takeaways from each stage of AGU treatment development. This includes insights from pre-clinical studies, the pre-IND phase, the nuances of gene therapy drug manufacturing, GLP toxicology studies, and the processes involved in IND submissions and related activities. Additionally, the keys to success for a patient foundation driving treatment development will be covered, including: - Hands on and active project involvement. - Maintaining regular communications with researchers/developers. - Adoption of standard(s) (vector, manufacturing, etc.) and common(s) with other developments (models, cultures, RoA) to save time, resources, and funding. - Frequent advice-seeking to reviews of key assumptions, leveraging collaborations and free research tools.

1076. Preclinical Assessment of the Efficacy and Safety of AAV9-HGSNAT Gene Replacement Therapy for MPS IIIC

Xin Chen[1], Thomas Dong[1], Merve Emecen Sanli[1], Anjala Jiji[1], Srikanth Singamsetty[2], Jill Wood[2], Steven J. Gray[1]

[1]UT Southwestern Medical Center, Dallas, TX,[2]Phoenix Nest Biotech, Brooklyn, NY

Mucopolysaccharidosis type IIIC (MPS IIIC or Sanfilippo syndrome Type C) is caused by the mutations in the heparan-alpha-glucosaminide N-acetyltransferase (HGSNAT) gene. It is an autosomal recessive disorder characterized by the lysosomal storage of heparin and heparan sulfate in the central nervous system that causes progressive destruction of nerve cells, with no specific treatment. The severity of the disease can range from mild to severe, even among affected individuals in the same family. MPS IIIC patients manifest with neuropsychiatric problems (hyperactivity, aggressiveness, and autistic features) followed by developmental delay, hearing loss, and childhood dementia. Most patients become paraplegic during adolescence and die before adulthood, but some survive until the fourth decade of life with progressive dementia and retinitis pigmentosa. Our group is evaluating the efficacy and safety of an adeno-associated viral 9 (AAV9)/HGSNAT vector in a mouse model of MPS IIIC *Hgsnat*^{P304L} developed by the team of Dr. A. Pshezhetsky, CHU Ste-Justine, Montreal, Canada. These mice were reported to show some biochemical and histological features of the disease starting around 2 months of age, behavioral phenotypes starting at 4 months of age, and average time to humane euthanasia endpoint around 10 months of age. Mice were randomized into treatment groups (vehicle, 1E11 vg/mouse, or 4E11 vg/mouse) and injected by a lumbar intrathecal route of administration at 7-10 days old or 120 days old. Study mice are weighed weekly up to 1 month old, then monthly thereafter. Urine is collected from all mice at 3, 6, 9, 12, 15, and 18 months of age, as well as at necropsy. A blood sample is collected at 2 months post-injection and will be collected again at necropsy for evaluation of markers of liver and kidney toxicity, as well as for evaluation of biomarkers of MPS IIIC. At 4, 6, 9, and 12 months old, all mice undergo behavioral phenotyping (open field, elevated plus maze, and novel object recognition). All mice are tested on the Y-maze at 6 months old only. Interim study results will be presented on treatment outcomes of the mice up to 6 months old.

1077. Enhancing Islet Isolation Techniques to Improve Digestion Efficacy: A Breakthrough for Pediatric Islet Auto-Transplantation in Fibrotic Chronic Pancreatitis

Masoumeh Asadi, Krishna K. Samaga, Ahad A. Kodipad, Chandrashekar B. Revanna, Jaimie D. Nathan, Appakalai N. Balamurugan

Islet GMP Facility, Center for Clinical and Translational Research, Nationwide Children's Hospital, Ohio State University, Columbus, OH

Introduction: Among improved islet isolation techniques, utilizing ideal combination of collagenase-protease enzyme blend is important. In this study, our primary objective was to utilize recombinant collagenases (rC1rC2) based on percent parenchymal mass for effective digestion of chronic pancreatitis pancreas to release islets followed by pediatric islet cell auto-transplantation. The dose and composition of the collagenase enzyme blends (CEB) used in the islet isolation process is a critical factor that impacts the number and quality of islets released from chronic pancreatitis pancreases (CPP). In this study, recombinant Clostridium histolyticum class I (rC1) and class II (rC2) collagenase on human pancreas digestion efficacy in combination with P. polymyxa protease (BP-protease) enzyme blend (rCEB) was assessed. **Methods:** No animal components were used in the generation of the raw material or purification of rC1, rC2, or BP-Protease. In our initial studies, we tested rCEB in 22 human research pancreases utilizing split lobe and full lobe models. In the current study, we introduced rCEB for clinical islet auto-transplantation (n=11). we also tested this enzyme blend in two validation islet isolations (n=2) perior to introduction in the clinic clinical. Collagenase was used at >19 Wunsch units/gram pancreas (20-40 unit) and proteas were used at >14.000 unit/gram pancreas (15,000-53,000 unit). Dosing of rCEB was based on percent parenchymal tissue mass in each chronic pancreatitis pancreas. Human islets were isolated according to our standard clinical islet isolation protocol. rCEB enzyme blend was injected into the pancreatic duct and digestion was done in

the Ricordi chamber. Pancreas digestion profiles (islet number, IEQ, islet/acinar morphology, tissue pellet volume, undigested pancreas) were carefully monitored and compared to isolations performed with traditional enzyme formulations. **Results:** Before introducing the rCEB for clinical islet auto-transplantation procedure, the effectiveness and optimal dosage of the recombinant enzyme (rCEB) were determined, and the functions of the islets were closely monitored to ensure their proper performance. Assessing percent parenchymal mass by visual and histology methods guided us to select the correct enzyme dosing. The digestion efficacy of rCEB in all five isolations were >90%, the undigested portion of pancreases were <10%. The digestive profiles of the pancreas exhibited normal characteristics and the final islet viability was >70% in all preparations. The final islet per gram pancreas was comparable to our previous islet isolation performed with traditional collagenase enzyme blends. **Conclusions:**. In summary, dosing rCEB based on percent parenchymal mass successfully digested the entire fibrotic pancreas and released all islets. The application of purified P. polymyxa protease in combination with rC1rC2 collagenases proved effective in maximizing islet yield for clinical islet auto-transplantation in fibrotic chronic pancreatitis cases.

1078. Macrophage-Based Therapy to Improve Surgical Outcomes of Urogynecologic Mesh Implantation in Patients with Diabetes

Rui Liang

University of Pittsburgh, Pittsburgh, PA

Synthetic meshes are often used to augment surgical repairs of pelvic floor disorders, prevalent conditions in aging women. We previously showed that diabetes induces macrophage dysfunction in the vaginal host response to mesh, possibly accounting for the prolonged and increased inflammation at the mesh site. Here we aimed to define the role of macrophages in the mesh-associated inflammation and address whether macrophage-based therapies can rectify the adverse effect of diabetes on the host response to mesh. Middle-aged inbred female Lewis rats were used for diabetes induction (n=14) with a single doze streptozotocin injection via tail vein (35 mg/kg), or as non-diabetic controls (n=11). Subgroups underwent mesh implantation without interventions (n=3-4 per group), with/without monocyte/macrophage ablation (n=3 per group), and with/without healthy monocyte treatment (n=4-7 per group). For mesh implantation, a light weight polypropylene mesh was implanted on the vagina via sacrocolpopexy following supracervical hysterectomy. For cell ablation, lyposome-encapsulated clodronate (LEC) solution (0.5 ml ip and 0.5ml iv for consecutive 3 days before surgery) was used. The effect of LEC on eliminating circulating monocytes and tissue residential macrophages was pre-tested and verified in a non-diabetic rat. Healthy monocytes (~1 - 2 x 10^6) isolated from healthy donor rats were infused to the mesh-implantation sites during surgery and injected via tail vein immediately following surgery. Inflammation outcomes were obtained via histomorphology analysis (Hematoxylin & Eosin and Trichrome staining) and flow cytometry. A mixed-effects model was used for statistics. We found that macrophages directly contributed to the scales of mesh-associated inflammation under normoglycemic conditions. Specifically, the cell ablation significantly decreased macrophage recruitment to the mesh site and the inflammation scale, while monocyte therapy restored the cell number and inflammation back to normal (all $p<0.05$). Under diabetic conditions, the positive relationship between mesh load and inflammation was disturbed, while the cell ablation and monocyte therapy reinstated the relationship (Figure). These findings suggest that macrophage-based therapies may normalize the host inflammatory response to medical devices (e.g., meshes), thus improving surgical outcomes in patients with diabetes.

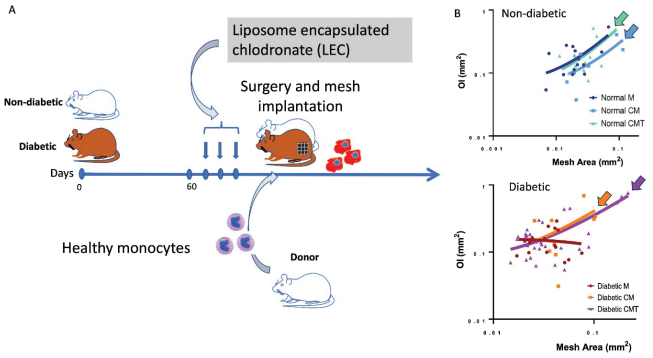


Figure. (A) Schematic drawing showing the flow of healthy monocyte therapy (CMT) following macrophage/monocyte ablation with chlodronate (CM) and mesh implantation (M) in diabetic vs. nondiabetic rats. (B) Inflammation analysis: Under normoglycemic conditions, the overall inflammation (OI) increased with mesh load (dark blue). Macrophage/monocyte ablation decreased the OI (light blue) while monocyte treatment (Green) returned the response back to normal (all $p<0.05$), proving that macrophages play determining roles in the mesh-elicited inflammation. Under diabetic conditions, the normal positive OI-mesh load relationship was disturbed (brown), which were restored by macrophage/monocyte ablation (orange) as well as monocyte treatment (purple).

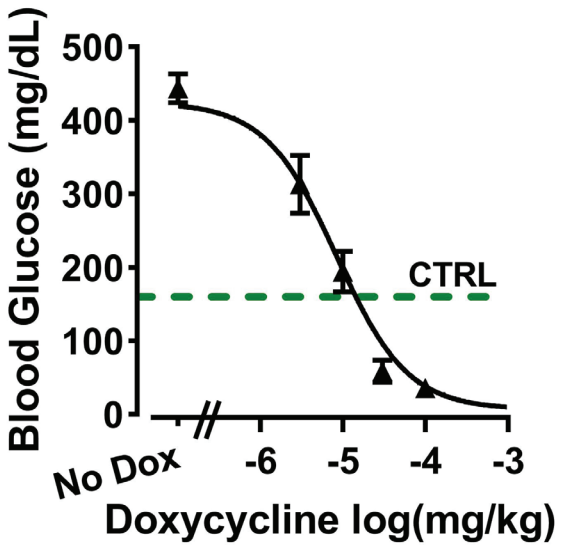
1079. Insulin Replacement Gene Therapy Using a Novel Glucose- and Drug-Inducible Adeno-Associated Virus

Aleksandra Maciejczuk[1], Stefan Hargett[1], Igor R. C. Rocha[1], Iuliia Vitko[1], Jun Xie[2], Guangping Gao[2], Thurl Harris[1], Edward Perez-Reyes[1]

[1]Pharmacology, University of Virginia, Charlottesville, VA,[2]UMass Chan Medical School, Worcester, MA

Objective: To develop AAV-based gene therapies that can be activated by the FDA-approved drug doxycycline (Dox). We present proof-of-concept studies using diabetic mice that show effective delivery and pharmacological regulation of an insulin gene replacement therapy. **Methods:** Glucose-inducible promoters have been developed by incorporating the DNA sequences that bind the carbohydrate response element binding protein, ChREBP. We developed a novel promoter with multiple repeats of ChREBP binding motif 1 and motif 2 (Jeong et al., 2011). For Dox regulation we engineered a single AAV to contain both components of the 3G Dox-on system (Clontech): 1) the reverse tetracycline transactivator (rtTA), whose expression is controlled by the glucose-inducible promoter; and 2) the tet operator, where the rtTA binds in the presence of Dox to induce the expression of recombinant insulin. Recombinant AAV8 pseudovirions were injected into the tail vein of diabetic mice (streptozotocin model). Blood glucose levels were monitored after a 4 hour fast and after an intra-peritoneal glucose tolerance test (GTT). Insulin production was monitored using serum samples and an ELISA directed against the human C-peptide produced by the rAAV. Expression of recombinant insulin mRNA was also validated using RT-qPCR. AAV delivery was confirmed by either qPCR or ddPCR on liver DNA. **Results:** Design of the AAV targeting plasmid was guided by in vitro studies measuring the expression of the fluorescent reporter, Fusion Red. In vitro experiments showed 2- to

5-fold regulation of expression by glucose and robust regulation by doxycycline (Dox) with a large dynamic range (100-fold). All in vivo experiments were blinded and included 4 groups of mice (C57Bl/6, n=4-5 each): Group 1 were control (CTRL) mice (not diabetic, no AAV); Group 2 were negative controls (diabetic, null AAV); while Groups 3 and 4 tested variations of the rAAV-Insulin gene therapy (1.0E+13 VG/kg). Baseline studies established that STZ-treated mice lost their ability to control blood glucose. Strikingly, administration of Dox normalized glucose handling in groups 3 & 4 to similar levels as observed in the control group. We then performed dose response studies varying the concentration of Dox in the chow (Figure). Very high doses of Dox (100 mg/kg) actually lowered blood glucose below levels observed in CTRL Titration down to 10 mg/kg Dox in chow restored blood glucose in diabetic mice to the control level. Taking into account chow consumption, this dose is half of that used in human patients during antibiotic therapy. **Significance:** The human insulin replacement market has been estimated at $42 billion dollars. Most commonly used strategies rely on the patient monitoring of their blood glucose and daily subcutaneous injections of recombinant insulin. These studies demonstrate that a drug-inducible AAV therapy can be used successfully to titrate insulin replacement by varying doxycycline dosing.



1080. The Highly Immunodeficient SRG Rat for the Development of Cell-Based Diabetes Therapeutics

Grace Walton, Fallon Noto, Diane Begemann, Cynthia Dunn, Lauren Keach, Darrell Robertson, Cassandra Scutchfield, Chris Brenzel, Michael Schlosser
Hera BioLabs, Lexington, KY

Immunodeficient rodents are vital for pre-clinical development of cell-based diabetes therapeutics. The SRG rat (Sprague Dawley Rag2-/-, Il2rg-/-) is normoglycemic under standard conditions and lacks mature B, T, and NK cells. The SRG rat offers several size-based advantages compared to mice, including more human-like physiology, easier surgeries, and ability to collect larger blood volumes on a more frequent basis. Compared to athymic nude rats, the SRG is more permissive to a wider range of human xenografts due to a lack of B and NK cells, which are still present in the nude rat. Given these advantages, we determined whether the SRG could serve as a model of streptozotocin-induced (STZ) Type 1 Diabetes or diet-induced obesity (DIO) Type 2 Diabetes. We compared blood glucose in SRG versus wild-type Sprague Dawley (SD) male rats following STZ treatment. Rats were administered vehicle, STZ 40 mg/kg, or STZ 65 mg/kg. Six days after low dose STZ, non-fasting glucose was 262 ± 41 (SEM) mg/dL in SD rats and 565 ± 64 (SEM) mg/dL in SRG rats. Six days after high dose STZ, non-fasting glucose was 679 ± 23 (SEM) mg/dL in SD rats and 505 ± 72 mg/dL in SRG rats. Compared to SD rats, weight loss associated with hyperglycemia was more pronounced in SRG rats. To induce obesity, insulin resistance, and T2D, SRG rats were fed control or high fat diet (60% kcal from fat). Body weight and food intake were recorded weekly. Glucose tolerance testing was performed after 10 and 24 weeks of high fat diet and insulin tolerance testing was performed after 11 weeks of high fat diet. Compared to low fat diet, DIO rats had increased caloric intake and body weight throughout the study. After 10 weeks, DIO rats had impaired glucose tolerance without elevated fasting insulin. Insulin tolerance did not differ at 11 weeks. At 24 weeks, glucose tolerance did not differ between diet groups, but DIO SRG rats displayed elevated fasting insulin ($P < 0.01$). In conclusion, SRG rats are more sensitive to 40 mg/kg STZ versus SD rats, with rapid weight loss and hyperglycemia. The SRG rat glycemic response to DIO showed temporal changes. These data demonstrate that the SRG Rat, a model with high-take rates for human xenografts, is a potential model for development of cell-based diabetes therapeutics.

1081. SKG0702, an AAV Gene Therapy Product for the Treatment of Wilson Disease

Li Wang, Yanqun Shu, Yanjun Shen, Ruojie Wang, Yuwen Du, Liuye Yao, Ruoyan Hu, Huimin Kong, Peilu Li, Bing Wu, Jinxin Deng, Lingling Zhao, Hao Luo, Zheng Luo, Yue Zhang, Shihua Cao, Yu Wu, Xia Liu, Yao Qiu, Xiaorui Bian, Hui Liu, Carol Huang, Jay Hou
Skyline Therapeutics, Cambridge, MA

Wilson disease, an autosomal recessive disorder caused by mutations in a copper-transporting P-type ATPase (*ATP7b*) gene, causes severe liver damage. Currently this disease is treated with the lifelong copper chelation therapy, which has many side effects and does not fix copper metabolism. To address these challenges, we developed SKG0702, a one-time gene therapy with adeno-associated virus (AAV) vector expressing an engineered codon-optimized version of the human mini-*ATP7b* transgene driven by a liver-selective promoter. AAV5 is the common capsid for liver targeting gene therapy. On the other hand, a different AAV (AAV_L) capsid has been drawing some attention in non-clinical/clincal stage liver targeting gene therapy programs recently. Here, we packaged mini-*ATP7b* transgene into AAV5 (SKG0701) and AAV_L capsid (SKG0702), respectively and did a head-to-head comparison of the transduction efficiency of the vectors in HepG2 cells, *Atp7b*$^{-/-}$ mice (Wilson disease model), and cynomolgus monkeys. We demonstrated that SKG0702 is superior in transducing hepatocytes compared to SKG0701 in both *in vitro* and *in vivo* studies. SKG0702 drove ~25-fold higher mini-ATP7b protein expression than

SKG0701 in HepG2 cells at the same multiplicity of infection. The dose range finding study in *Atp7b*$^{-/-}$ mice showed dose-dependent vector distribution and mini-*ATP7b* expression in the liver. However, intravenous (IV) delivery of SKG0702 mediated higher vector genome (vg) copy number in the liver and was more efficient in reducing hepatic copper accumulation than SKG0701. In addition, SKG0702 resulted in mini-ATP7b expression in >70% of hepatocytes and ~50% reduction of hepatic copper level in *Atp7b*$^{-/-}$ mice at the dosage of 2E12 vg/kg. Furthermore, IV delivery of SKG0702 in cynomolgus monkeys led to more vector distribution and transcription products in the liver than SKG0701. At the dosage of 5E12 vg/kg, SKG0702 mediated mini-*ATP7b* transcript expression in >60% of hepatocytes and was well-tolerated without any histopathological findings. In summary, SKG0702 is clinically advantageous with efficient hepatocyte transduction and is a promising gene therapy candidate for reducing liver damage and correcting copper metabolism in Wilson disease.

1082. Determinants of AAV Persistence in a Mouse Model of Glycogen Storage Disease Type III

Justine Nozi[1,2], Hanadi Saliba[1,2], Youssef Krimi Benchekroun[1,2], Jeremy Rouillon[1,2], Gianni Tavella[1,2], Valle Montalvo-Romeral[1,2], Bérangère Bertin[1,2], Louisa Jauze[1,2], Helene Jammes[3,4], Alice Jouneau[3,4], Coralie Pecquet[1,2], Louis Bergantz[1,2], Andrés F. Muro[5], Giulia Bortolussi[5], Sylvain Fisson[1,2], Nathalie Daniele[1], Laetitia Van Wittenberghe[1], David A. Gross[1,2], Tiziana La Bella[1,2], Antoine Gardin[1,2], Giuseppe Ronzitti[1,2]

[1]GENETHON, Evry, France,[2]Université Paris-Saclay, Univ Evry, Inserm, Genethon, Integrare Research Unit UMR_S951, Evry, France,[3]Université Paris Saclay, UVSQ, INRAE, BREED, Jouy en Josas, France,[4]Ecole Nationale Vétérinaire d'Alfort, BREED, Maisons-Alfort, France,[5]International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy

Liver-directed gene transfer with Adeno-associated viral (AAV) vectors demonstrated long-term efficacy in a large number of clinical trials and relevant preclinical animal models (D'Avola D, 2016; Weber ND, 2023). However, transgene expression loss was reported for some individuals without any evident correlation with the development of an immune response (Konkle BA, 2021) suggesting that other mechanisms might be responsible of this phenomenon. Importantly, this loss of transgene expression is poorly recapitulated by the current rodent models, since long-term expression is usually achieved in mouse liver in absence of significant liver damage (Ronzitti G, 2016). In this study, we hypothesized that subclinical liver conditions, such as fibrosis or hepatocyte metabolic impairment, may have an impact on the persistence of AAV liver gene transfer. To model such conditions, we used a mouse model of glycogen storage disease type III (GSDIII), which displays features of chronic liver damage as well as a mild progressive fibrosis. The relatively low level of fibrosis observed in this model did not impact gene transfer efficacy at short-term while, at long-term efficacy loss was observed with a kinetic similar to the one observed in humans. Importantly, the reduction of CpG dinucleotides in the transgene sequence and in the promoter of the expression cassette resulted in longer transgene expression stability and correction of the metabolic phenotype in the majority of the AAV-treated GSDIII mice. Interestingly, despite this optimization some of the mice showed robust transgene expression loss associated with transcriptional inactivation that was further characterized. Among mechanisms potentially affecting vector stability and persistence over time, we showed that hepatocytes proliferation, if not corrected, can lead to transgene expression loss due to AAV dilution starting from three months after treatment. Interestingly, in the same experimental setup, we identified differential methylation of the promoter sequence occurring specifically in GSDIII mice suggesting a deregulation of the epigenetic mechanisms in this mouse strain. Finally, adaptive immune responses triggered by the capsid or by the transgene were investigated highlighting an enrichment of non-conventionally activated effector/memory CD8+ T cells and an increase of CD8+ T cell infiltrates in the liver of Agl-/- mice one month after vector injection. The characterization of the role of these T cell infiltrates in the loss of correction observed over time is still undergoing in our model. In conclusions, our work suggests the involvement of multiple mechanisms in the long-term persistence of AAV genome and transgene expression in the mouse GSDIII liver. These findings support the hypothesis that the variable efficacy and long-term persistence observed in the clinical trials may be related to the presence of an underlying subclinical metabolic impairment, proliferation associated with fibrosis and/or the presence of a pro-immunogenic environment. These mechanisms should be investigated in patients enrolled in liver-targeted gene therapy clinical trials to better understand their contribution in humans and improve the outcome of liver gene therapy.

1083. LY-M003: Advancing Wilson Disease Treatment through Copper-Responsive Gene Therapy

Yuzhe Peng, Guofang Yan, Yixiong Chen, Qing Lin

Lingyi Biotech Co., Ltd., Shanghai, China

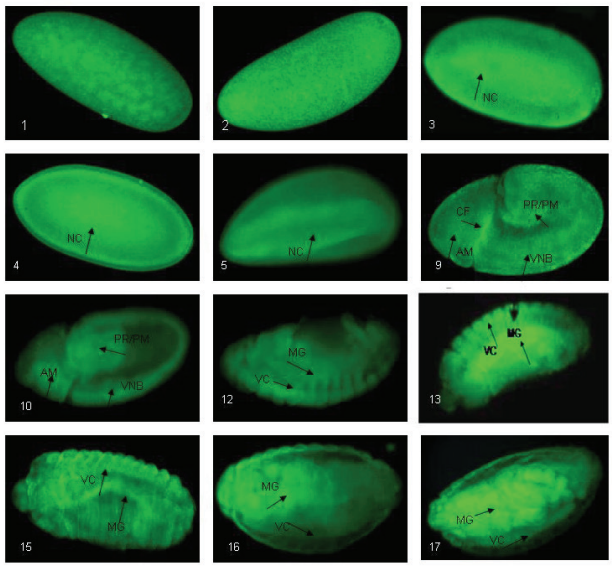
Wilson disease (WD), a rare autosomal recessive disorder, stems from mutations in the ATP7B gene, culminating in diminished ATP7B activity and the subsequent systemic accumulation of copper, predominantly affecting the liver, brain, and kidneys. This accumulation leads to severe organ damage, highlighting the critical need for therapeutic interventions that restore ATP7B protein function. LY-M003, an innovative adeno-associated virus (AAV8)-based gene therapy, is at the forefront of this challenge, designed to synthesize a modified ATP7B^{LY} protein in a copper-dependent manner through its Copper Responsive Engineering of ATP7B Expression (CREATE) system. The ATP7B^{LY} protein, engineered to mirror the functionality of its wild-type counterpart, forms the core of LY-M003's therapeutic strategy. In vitro studies showcase LY-M003's proficiency in expressing ATP7B^{LY}, coupled with a robust capacity for copper exportation. Preclinical efficacy evaluations in *Atp7b*$^{-/-}$ and *Atp7b*$^{M1/M1}$ mouse models—characterized by detrimental hepatic copper accumulation, heightened urinary copper excretion, and compromised serum ceruloplasmin activity—revealed LY-M003's potential. Notably, a 13-week treatment regimen in male *Atp7b*$^{M1/M1}$ mice with LY-M003 correlated with a dose-dependent decrease in tissue copper levels, normalization of urinary copper and serum ceruloplasmin levels, and a marked alleviation of liver lesions. Moreover, extensive toxicology assessments of LY-M003 in both CD-1 mice and Cynomolgus macaques underpin its safety and tolerability, offering a promising therapeutic

outlook. The amassed preclinical data robustly advocate for LY-M003's safety and therapeutic efficacy in both mice and non-human primates (NHPs), providing a solid foundation for the forthcoming human clinical trials.

1084. Aminopeptidase P (DAP) Expression Pattern in *Drosophila Melanogaster*

Suneeta Gireesh Kumar
Microbiology, Dr. D. Y. Patil Arts, Commerce and Science, College, Pimpri, Pune, Maharastra, India

Aim: Aminopeptidase P (APP) is an enzyme that has specificity for proline, can specifically cleave Xaa-Proline peptides and is a metallo-aminopeptidase. The bonds nearby to the imino acid proline are tough to be cleaved by many peptidases but APP can specifically break peptide bonds engaged with proline. Membrane-bound form and a cytosolic form are the two forms in which this enzyme exists. The exact physiological function of APP remains unclear and hence the present work attempts to determine it. Methods: In present study, expression pattern of cytocolic Aminopeptidase P (DAP) was determined in all the embryonic stages and larval stages of wild type Drosophila, by using polyclonal monospecific antibodies. To show the presence of DAP RNA in embryonic and larval stages, RNA in situ hybridization was performed. DAP *promoter-LacZ* fusion reporter gene vector was used to construct transgenic embryos to study the regulation pattern of DAP. To study the DAP expression profile, a transgenic fly consisting of DAP promoter with β-gal and GFP reporter genes in front of it was constructed. Results: DAP protein expression was observed in neuroectodermal cells, posterior midgut primordium, proctodeum, ventral neuroblast and primordial stomatogastric nervous system. It was observed in the ventral cord and midgut in the stage 12. The completely developed embryos showed intense occurrence of it in the ventral cord and gut region. The eye-antennal disc, wing disc and leg disc also showed the presence of DAP protein. LacZ expression in transgenic embryos also showed same pattern. Conclusion: Similar to various known multiple functional proteins, DAP could be one with different functions at different stages and in different cells. Data presented here designates DAP functions in the early embryonic and imaginal disc differentiation and development, suggesting that it may be required for the metabolism of proteins like neuropeptides, and tachykinins.



Thursday Posters: Liver Genetic Diseases

1085. Engineering of a High-Activity FVIII-BDD Gene Therapy Vector with High AAV-Packaging Efficiency for Hemophilia A

Qu Bin, Na Li, Xi Zhu, Wenjing Luo, Hongyu Li, Xingyu Yang, Shin-Shay Tian, Xiaoping Zhao
Shanghai Vitalgen Biopharma Co Ltd., Beijing, China

Hemophilia A is an X-linked recessive genetic disease caused by a deficiency or dysfunction of the clotting factor VIII (FVIII). We have developed a highly active FVIII-BDD AAV expression cassette and demonstrated its superior efficacy in mouse and non-human primate models The FVIII-BDD coding sequence was first codon optimized for enhanced human hepatic expression with reduced CpG to minimize silencing *in vivo*. Then, different regions of FVIII-BDD were mutated, such as the F309S mutation, the deletion of the furin recognition site 1645-1648 in the SQ sequence, the insertion of 5 glycosylation sites in the middle of the SQ, and combination mutations of the above described to further optimize hepatic protein expression. Since the A1 domain sequence affects the secretion and expression of FVIII-BDD, we replaced the A1 domain sequences with those of the pig FVIII, An53 and other proteins known for their high expression and secretion. We designed a series of X2 (two amino acid mutations), X4 (four amino acid mutations) and X5 (five amino acid mutations) to further improve the hepatic expression and secretion of FVIII-BDD. In addition, we also performed extensive promotor screening to obtain a short 175bp liver-specific promoter. By combining the above optimized elements, we generated several AAV expression cassettes and packaged into AAV8 for validation *in vivo,* together with two FVIII-AAV benchmark controls, BMN-270 and SPK-8011. While the benchmark BMN-270 group was found to have an average FVIII activity of 85.9 IU/mL,

our optimized expression cassettes were all performed significantly better, with the best one R216 showing an average FVIII activity of 1149.2 IU/mL. R216 expression cassette was selected and packaged into AAV5 for evaluation in non-human primates, using BMN-270 (also packaged in AAV5) as a benchmark control. We again observed significantly higher FVIII expression by R216 in comparison to BMN-270. The large size of FVIII has always presented a major challenge to develop an effective AAV gene therapy. After optimizing our vectors and selecting the smallest elements, we managed to reduce the R216 vector size to 4,917nt. At this size, there were still significant packaging and purification challenges. To address this, we have implemented measures such as inserting the lambda II filling sequence outside the ITR sequence, screening for the optimal RC and helper plasmid combination, and extensive optimization of the purification processes. We have successfully increased the packaging efficiency and virus yields and in addition, controlled the residual plasmid and Host Cell DNA (HCD) at very low levels, laying a solid foundation for advancing our FVIII gene therapy into clinical trials.

1086. The Combination Strategy of Lipid Nanoparticle-Encapsulated rAAV and Triamcinolone Acetonide Enables Enhanced and Successful rAAV Multi-Administration

Chunmei Gan[1], Mi Leng[1], Yu Liu[1], Zhaoyue Zheng[1], Siyuan Dong[2], Lei Gao[2], Jingya Ye[2], Hoi Yee Chow[1], C. Alexander Valencia[2], Biao Dong[2]

[1]National Clinical Research Center for Geriatrics and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China,[2]Sichuan Real and Best Biotech Co., Ltd., Chengdu, China

The multi-administration of recombinant adeno-associated virus (rAAV) is limited, in part, by immunological barriers in vivo. Herein, a novel strategy, named lipid nanoparticle-encapsulated rAAV combined with triamcinolone acetonide (LNP-rAAV + TAC), has been described. We demonstrated that both lipid admixing and LNP encapsulation enhanced liver-targeted transduction at initial administration. Also, successful tri-trafficking was achieved by LNP-rAAV2; however, weak expressions were observed following second and third transductions. In addition, triamcinolone acetonide administration, which modulated the immune response, permitted rAAV2 re-administration yet deaths were observed at higher-dose groups. Furthermore, the combined strategy of LNP-rAAV + TAC was tested in the tri-trafficking of rAAV2, which enabled powerful achievements in eradicating anti-rAAV antibodies and enhancing expression in the second and third transductions with a safe performance compared to their independent approaches. Thus, this combination strategy could be an attractive candidate for reducing immunogenicity and enabling multi-dosing of rAAV vectors.

1087. CRISPR Base Editing for *In Vivo* Correction of Severe Hemophilia a Missense Variant

Yuanyi Xu[1], Chun-Yu Chen[1], Meng-Ni Fan[1], Xiaohe Cai[1], Barbara A. Konkle[2,3], Carol H. Miao[1,4]

[1]Seattle Children's Research Institute, Seattle, WA,[2]Division of Hematology and Oncology, University of Washington, Seattle, WA,[3]Washington Center for Bleeding Disorders, Seattle, WA,[4]Department of Pediatrics, University of Washington, Seattle, WA

Hemophilia A (HA) is a genetic bleeding disorder in which patients are not able to produce sufficient functional coagulation factor VIII (FVIII, *F8* as the gene of FVIII). The identification and correction of the patient's *F8* pathogenic variant represents a personalized gene therapy approach to rescue FVIII production and therefore treat HA. We aim to use CRISPR cytosine base editing (CBE) to target the HA missense variant to achieve high editing efficiency and low bystander effects, resulting in phenotypic and genotypic improvements. Among *F8* variants identified in our patients with HA, we selected the T535C variant and designed sgRNA with mutation site T·A to C·G on C8. To examine the in vivo base editing, we constructed a B-domain-deleted mutant human FVIII (hFVIII) expressing plasmids encoding the T535C variant and used a wild-type hFVIII as a control. Subsequently, we proceeded with cloning the CBE plasmid by incorporating the U6 promoter and specific sgRNA targeting the mutant site into pCMV-BE4m vector backbone. The CBE plasmid, along with T535C mutated FVIII plasmid were hydrodynamically injected into an exon 16 deletion-HA murine models. The FVIII activities were determined by activated partial thromboplastin time assay. Treated mouse livers were harvested for genomic DNA extraction, followed by PCR of the target region and Sanger sequencing to evaluate the editing efficiency. We observed 18-28% overall editing rate and a wide editing window from the initial testing of base editing of the specific *hF8* variant using the parent CBE plasmid. Subsequently, we enhanced our CBE constructs by switching the CMV promoter to the stronger CAG promoter and explored existing CBE variants to increase targeting specificity. New combinations of mutated T535C hFVIII and CBE variants plasmids were administered by hydrodynamic injection and the editing efficiency was examined with the same methods. One of the highly active CBE variants driven by CAG promoter demonstrated the highest FVIII activity, exceeding 50% FVIII activity of the wild-type FVIII plasmid treated mice on day 7. DNA sequencing result also indicated 50% of *in vivo* correction of mutant C·G to normal T·A on day 7. Minor bystander effects did not significantly impact the functional FVIII activity recovery. The results suggest CRISPR base editing tools in combination with specific sgRNA can efficiently correct the missense mutation and provide effective phenotypic correction of mutant FVIII plasmid *in vivo*. We are currently testing the base editing tools to correct point mutations in primary human cells.

1088. Development of an AAV Gene Therapy Vector FT-004 for Hemophilia B

Zhong-Dong Shi[1], Chinmay Patkar[1], Yiling Fang[1], Chao Ren[2]

[1]Frontera Therapeutics, Bedford, MA,[2]Frontera Therapeutics, Shanghai, China

Hemophilia B is an inherited X-linked rare recessive genetic bleeding disorder caused by insufficient levels of a blood protein called factor IX (FIX). Using AAV vectors to deliver FIX expression cassettes to the patient's liver is a promising method to treat the disease. However, several early programs have failed likely due to low activity of wildtype FIX or loss of transgene expression. A high number of CpG dinucleotides in the transgene could stimulate innate immune response which might result in loss of durability. To develop a novel durable AAV gene therapy vector for treating as many hemophilia B patients as possible, we designed 6 vectors in either scAAV or ssAAV form containing a codon-optimized human FIX Padua variant driven by different liver-specific promoters. FIX-Padua is a natural hyperactive FIX variant whose activity is ~7-fold that of wild-type FIX. To reduce potential immunogenicity, the CpG count on the codon-optimized FIX Padua variant was reduced to 0. AAV5 capsid was used to target a broader patient population given its low seroprevalence. We tested the vectors for FIX expression and activity in vitro using the Huh-7 cell line, followed by further comparing them in an *F9* KO hemophilia B mouse model. We nominated the design that gave the highest potency according to one-stage clotting assay as our drug candidate - FT-004. Further in vivo preclinical study showed that FT-004 specifically targeted *F9* KO mouse liver and provided a high level of FIX expression, and the FIX antigen concentration in the plasma was stable from week 4 to week 8. Both FIX activity and aPTT time changes displayed a clear dose-dependent manner. FT-004 could reduce bleeding duration, decrease bleeding area, and increase survival rate. The minimum effective dose (MED) was determined to be 5E12 vg/kg. FT-004 produced at different scales (from 0.2 L, 50 L, and 500 L) showed a comparable potency in the hemophilia B mice. A 13-week GLP safety study in NHPs (cynomolgus monkeys) showed that FT-004 had no local or systemic toxic effects at all tested doses up to 9.7E13 vg/kg (NOAEL). Furthermore, administration of FT-004 also resulted in potent hFIX protein expression in NHPs in a dose-dependent manner. Vector genome copies were persistent from Day 29 to Day 92 post vector injection in NHPs, which may indicate that codon-optimized and CpG-removed FIX-Padua gene sequence did not elicit a strong immune response. Overall, the vector durability, efficacy, and safety data we generated from preclinical studies support further clinical development for FT-004.

1089. Optimized F8-X5 Variants Improve hFVIII Activity and Durability of Expression

Shiyong Wu[1], Matthew Chrzanowski[2], Kentaro Yamada[1], Sreevani Arisa[1], Jiahe Tian[3], Roland W Herzog[1], Weidong Xiao[1], Junping Zhang[1]

[1]Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN,[2]Nikegen LLC, Wynnewood, PA,[3]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY

Previously we developed a human FVIII variant FVIII-X5, which significantly improves AAV based gene therapy efficiency by enhancing hFVIII secretion. Due to AAV packaging capacity limitation, only shorter promoters can be used to regulate FVIII expression. Here we sought to develop better promoter/enhancer/intron cassettes to further improve AAV based gene therapy efficiency. New expression cassettes were constructed based on B domain deleted factor VIII (BDD-FVIII). The expression plasmids were tested by hydrodynamic injection into Hemophilia A mice. The hFVIII activity was measured by APTT assay. Five of these constructs showing significant better factor VIII expression level were further tested in AAV8 vector system using one of the BDD-FVIII, BDD-FVIII-X5 and a new BDD-FVIII-X5-LK genes. They were compared with AAV8-TTR-BDD-FVIII-X5, AAV8-hATT-codon-optimized BDD-FVIII (CoFVIII), and AAV8-TTR- CoFVIII. All AAV8 vectors were injected into HA mice (B6;129S-F8$^{tm1Kaz/J}$) at the dose of 4×10^{11} or 1×10^{11} vg/mouse, 5-7 mice/group. hFVIII activity was detected by chromogenic assay. Anti-hF8 IgG and anti-AAV8 IgG in serum were measured by ELISA. Even with a smaller promoter less than 200bp, the hFVIII activity reached a peak of 130% after one week. The newly designed mini-intron significantly enhance factor VIII expression. The hFVIII activity approached 220% at two weeks with an intron addition. We observed that hFVIII activity dramatically dropped after achieving a peak at later time points. Meanwhile, the anti-hFVIII IgG expression greatly increased accordingly. These results suggest that the expression cassette with an efficient mini-promoter-enhancer-intron combination lead higher hFVIII expression but does result in increased inhibitor formation in mice. For the factor VIII with a new heavy chain and light chain link FVIII-LK, the expression exhibited a unique pattern, the hFVIII activity showed a slight increase from day 7 to day 14 and achieved a dramatically high level at 220% from day 21 to 28. Interesting, no anti-hFVIII-IgG expression was detected in this vector group. In addition, we systemically characterized the genome integrity of the new AAV-FVIII produced. Alkaline gels showed that the packaged genomes size in 4906 bases, 4874 bases, 5080 bases exhibited an intact band while the packaged genomes of 5493 base and 5210bases show varying truncated bands. In conclusion, these optimized hFVIII-X5 expression variants are promising candidates for hemophilia A gene therapy.

1090. Development and Optimization of Factor VIII Transgene Cassettes for Hemophilia A Gene Therapy with AAV Vectors

Kentaro Yamada[1], Miguel Gonzalez[1], Zhouji Chen[2], Maite Munoz[1], Sreevani Arisa[1], Bridget Brian[1], Ype De Jong[3], Weidong Xiao[1], Randal J. Kaufman[2], Roland W. Herzog[1]

[1]Herman B Wells Center for Pediatric Research, Indiana University, Indianapolis, IN,[2]Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA,[3]Weill Cornell Medical College, New York, NY

Liver-directed adeno-associated viral (AAV) vector-based gene therapy drugs have recently received regulatory agencies for the treatment of the X-linked bleeding disorder hemophilia. However, large vector doses are used to treat hemophilia A (deficiency in coagulation factor VIII, FVIII). Furthermore, FVIII is not normally expressed in hepatocytes and may induce cellular stress responses. More efficacious vectors expressing better secreted forms of FVIII are desirable. To generate superior FVIII transgene cassettes, we tested AAV8 vectors harboring different cassettes expressing CpG-depleted, codon-optimized, B domain-deleted (BDD) human FVIII in hemophilia A mice (C57BL6/129 F8e16-/-). AAV vectors were administered iv at the dose of 1.0E+11 vg/mouse. Initially, we compared a limited number of published elements and found a construct utilizing the hepatocyte-specific HLP promoter, which drives FVIII expression in the commercial product roctavian, achieved ~55% of normal FVIII

activity at week 2. We next introduced amino acid changes that may enhance FVIII secretion. These variants included F309S, X5 (a variant in which 5 amino acids had been replaced with porcine residues for improved secretion), and a combination of these. While F309S failed to improve FVIII activity in circulation, introduction of X5 showed about 4-fold higher FVIII activity (~190%). Combination F309S/X5 showed the similar FVIII activity (~221%) compared to X5 alone. Analysis of AAV copy numbers and hF8 mRNA levels measured in livers at 6 weeks showed overall a good correlation between AAV copy numbers, F8 mRNA levels and FVIII activities for individual mice. Next, we replaced the HLP promoter with the transthyretin (TTR) promoter, which is utilized in a different product under commercial development and similar in size to the HLP promoter. This change consistently increased FVIII activity by ~2-fold for all constructs, resulting on average in 118% activity for WT FVIII and 112%, 414% and 354% for F309S, X5 and F309S/X5, respectively. When normalized for vector copy number in the liver, results suggested that inclusion F309S in WT FVIII or X5 variant modestly improved systemic FVIII expression by 2- and 1.4-fold, respectively. Thus far, all constructs expressed the "SQ" version of BDD-FVIII. Since the QS linker sequence contains a proteolytic cleavage site that may decrease FVIII stability, we deleted the SQ linker, which increased FVIII activity by 1.5- to 1.8-fold, with the additional advantage of further reduced the size of the vector genome. We are now testing whether performance of the X5 variant can be further enhanced by combination with linker deletion or mutations that reduce FVIII inactivation in circulation. In conclusion, FVIII can be rationally engineered to develop AAV gene therapy that is effective at reduced vector doses. We postulate that improved secretion combined with reduced burden of FVIII per hepatocyte will result in more stable gene therapy for hemophilia A.

1091. A Novel Factor VIII Variant That Yields Improved Secretion and Reduced ER Stress for Second Generation AAV-Mediated Gene Therapy for Hemophilia A

Jonathan R. Lindgren[1], Marie Seleme[1], Allysen Henriksen[1], Giang N. Nguyen[1], Denise E. Sabatino[1,2]

[1]Division of Hematology and The Raymond G. Perelman Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA,[2]Department of Pediatrics, Division of Hematology Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

An AAV gene therapy product was recently approved for the treatment of patients with severe hemophilia A (HA). Ongoing clinical studies have shown promise, however, it is unclear whether long term FVIII activity within a therapeutic-to-curative range can be achieved for all patients. There remains a need to develop 2nd generation products that achieve durable levels of FVIII expression at lower vector doses. Most products in clinical development make use of a B-domain deleted (BDD) human factor VIII (hFVIII) transgene. Our group has identified a unique hFVIII-BDD protein variant, hFVIII-Δ3-SP/DE, that is secreted more efficiently and expressed at higher levels after gene therapy when compared to hFVIII(Nguyen et al 2023). In vivo studies of AAV delivery of the hFVIII and hFVIII-Δ3-SP/DE showed that the variant expressed 2-4-fold higher levels of protein in the plasma which inversely correlated with intracellular FVIII protein levels. To further understand the mechanism for the increased FVIII levels in the plasma after AAV gene therapy, we investigated the secretion of hFVIII-Δ3-SP/DE in vitro. Using established stable BHK cell clones, we determined the ratio of intracellular to secreted FVIII by comparing clones expressing hFVIII or hFVIII-Δ3-SP/DE. The ratio of intracellular FVIII to secreted FVIII from stable BHK clones expressing hFVIII (4.7 ± 1.3) was significantly higher than hFVIII-Δ3-SP/DE (1.6 ± 0.3, $p < 0.001$). This demonstrates that the hFVIII-Δ3-SP/DE variant is secreted more efficiently than hFVIII. Previous studies have shown that overexpression of FVIII may activate ER stress pathways. We hypothesized that hFVIII-Δ3-SP/DE may reduce cellular stress responses since it is secreted more efficiently. Stable BHK clones expressing hFVIII and hFVIII-Δ3-SP/DE were evaluated for transcriptional activation of ER stress response elements using the Cignal ERSE-Reporter Dual-Luciferase Assay. Two independent stable clones expressing the hFVIII-Δ3-SP/DE variant expressed significantly lower levels of ERSE-driven luciferase activity (0.53 ± 0.24 and 0.51 ± 0.25 RLUs) compared to clones expressing hFVIII (1.11 ± 0.34 RLUs) ($p < 0.05$, Dunnets multiple comparisons test). Stable clones were further assessed for relative mRNA expression of the unfolded response element marker, BiP. Clones expressing hFVIII had significantly increased levels of BiP expression compared to clones expressing hFVIII-Δ3-SP/DE, at similar levels of hFVIII mRNA (3.2 vs 0.95, respectively, 2-sided unpaired t-test $p = 0.0002$). Furthermore, the normalized amount of BiP expression per copy of FVIII mRNA was higher in BHK clones expressing hFVIII vs hFVIII-Δ3-SP/DE (2.8 vs 1.8, respectively, two-sided unpaired t-test $p = 0.044$). The hFVIII-Δ3-SP/DE variant has reduced ER stress induction, compared to hFVIII, possibly due to the improved secretion efficiency of the variant protein. In conclusion, the hFVIII-Δ3-SP/DE variant shows enhanced secretion both in vitro and in vivo, leading to higher FVIII levels in plasma and reduced ER-stress, showing that this variant overcomes a key barrier in AAV gene therapy for hemophilia A.

1092. The Impact of Anti-Platelet Antagonists on the Hemostatic Efficacy of Platelet-Specific FVIII Gene Therapy in Hemophilia A Mice

Hongyin Yu[1,2], Jocelyn A. Schroeder[1,2], Jeremy G. Mattson[2], Qizhen Shi[1,2]

[1]Dept of Pediatrics, Medical College of Wisconsin, Milwaukee, WI,[2]Blood Research Institute, Versiti Wisconsin, Milwaukee, WI

Hemophilia A is a prime model for gene therapy. Recently, the FDA has approved AAV-mediated liver gene therapy to treat patients with hemophilia A. However, pediatric and adult patients with severe liver disease or neutralizing antibodies to AAV, which are present in 30-50% of the population, are excluded from AAV liver gene therapy. Our previous studies have demonstrated that platelet-targeted FVIII expression (2bF8) is effective in rescuing the bleeding phenotype of hemophilia A ($F8^{null}$) mice even in the presence of anti-FVIII inhibitory antibodies (inhibitors). Currently, our gene therapy approach is in Phase I clinical trial. Since antiplatelet drugs are commonly used in the clinic, it is important to investigate how platelet antagonists affect the clinical efficacy of platelet-derived FVIII. Here, we evaluated two categories of platelet antagonists: one is the antibody (Leo.H4) that specifically blocks the αIIbβ3 complex, and the other is the drug (Clopidogrel, Plavix) that inhibits the $P2Y_{12}$ subtype of the ADP

receptor to investigate how anti-platelet medications impact the clinical efficacy of platelet-derived FVIII. Hemostatic properties of platelet-FVIII were assessed by *ex vivo* ROTEM and native whole blood TGA (nWB-TGA) analysis of whole blood and *in vivo* $FeCl_3$-induced carotid artery injury model, lateral tail vein transection (TVT) injury model, and tail tip bleeding test (TTT). In $2bF8^{Tg}/F8^{null}$ mice with a platelet-FVIII level of 6.89±0.65 or 12.32±0.73 mU/10^8 platelets, all parameters from ROTEM analysis were comparable to those in WT control mice. However, all parameters, including clotting time, clot formation time, maximal clot firmness, α-angle, and A10 were significantly impaired in the presence of Leo.H4 compared to native samples from 2bF8 mice. In contrast, in samples from WT mice, only α-angle and A10 were significantly tempered in the presence of Leo. H4. Interestingly, peak thrombin and endogenous thrombin potential determined by nWB-TGA were significantly enhanced in the presence of Leo.H4 compared to native samples from $2bF8^{Tg}/F8^{null}$ mice, but there were no significant differences in other parameters. Leo.H4 did not impact the hemostatic parameters determined by nWB-TGA in WT mice. Complete occlusion occurred in the $FeCl_3$-induced carotid injury in all 2bF8 and WT mice, but none occluded when Leo.H4 was infused. In the TVT injury model, when Leo.H4 was infused, none of the $2bF8^{Tg}/F8^{null}$ mice stopped bleeding in the 10-minute bleeding test, and animals lost a significant amount of blood (525.98±92.06 µL). In contrast, when animals were infused with IgG isotype control, the bleeding time was 0.99±0.05 min, and blood loss was 17.05±9.37 µL. There were no significant differences in the bleeding time and blood loss between the 2bF8 and WT groups infused with IgG in the TVT test. When mice were treated with Plavix, all 2bF8 and WT animals bled the entire 10 minutes during the TTT test, which was significantly different compared to the control vehicle-treated animals, in which the bleeding time was 0.88±0.39 and 1.43±1.19 min, respectively. The blood loss in the Plavix-treated animals was 84.1±68.18 and 56.74±49.16 µL in the 2bF8 and WT groups, respectively. The blood loss in these animals was 13- and 16-fold more than those lost in vehicle-treated control animals (6.4±6.19 and 3.5±4.67 µL, respectively, in the 2bF8 group and WT group). In conclusion, our results demonstrate that platelet aggregation is critical in maintaining the clinical efficacy of platelet-derived FVIII in hemophilia A and anti-platelet medications can impede the hemostatic efficacy of platelet-FVIII

1093. Peptide-Based Nanoparticles Mediated mRNA Rescue of Factor VIII; a New Approach to Treat Hemophilia A

Gilles Di Vita[1,2], Nathalie Durany[1,2], Veronique Josserand[3], Melanie Guidetti[4], Neil Desai[2]

[1]DIVINCELL, Nimes, France,[2]AADIGEN, Pacific Palisades, CA,[3]IAB, Grenoble, France,[4]IAB, Genoble, France

Hemophilia A is an X-linked bleeding disorder caused by the deficiency of coagulation Factor VIII. Hemophilia A patients are currently treated with costly and inconvenient replacement therapy of short-lived factor VIII (FVIII) protein. Although safe and efficient delivery of therapeutic mRNAs for protein replacement therapies offers great promise, it remains challenging. We have developed a new peptide-based platform that can potently deliver functional mRNAs encoding Factor VIII to the liver. ADGN technology constitutes a biocompatible and scalable system to efficiently package and deliver mRNA to the target site. ADGN technology is based on short amphipathic peptides that form stable nanoparticles with nucleic acids through non-covalent electrostatic and hydrophobic interactions. The chemistry of ADGN-nanoparticles can be tuned in order to target specific organs and to induce robust mRNA expression in the targeted tissue. Here we selected ADGN-peptide with a liver tropism and able to target liver and lymph nodes. ADGN/mRNA nanoparticles are stable both in solution and in dry powder formulations, with an average size of 100 nm and a high encapsulation efficiency (> 90%) We demonstrated that Intravenous (IV) and subcutaneous (SQ) administration of ADGN-carrying mRNAs encoding human FVIII (ADGN-F8) into a Hemophilia A mouse model, using different dosing regimens (ranging from 0.2 to 1.0 mg/Kg), results in a rapid pulse of FVIII protein expression (within 12h) and a recovery of the Factor VIII level in the blood. The expressed FVIII protein is therapeutically effective, like the recombinant human factor VIII protein, that is the current standard of care. The FVIII protein expression and activity remained above therapeutic levels up to 30 days post-injection (>50% of the reference range) and slowly declined thereafter. Repeated IV (0.2 mg/Kg) and SQ (1.0 mg/kg) injections of ADGN-F8 once every two weeks produced consistent expression of FVIII over time IV SQ , and maintained protein level and FVIII activity above clinically relevant threshold (>50% of the reference range). ADGN-F8 was redosed every 2 weeks over 3months without inducing liver toxicity, inflammation or chronic alteration as determined by histology. Cytokine, histology and liver enzyme profiling showed that repeated SQ and IV administrations of the ADGN-F8 did not cause any adverse innate or adaptive immune responses in immune-competent, hemophilic mouse model. Our study showed the potential of ADGN as a safe and effective platform of new mRNA therapies for Hemophilia A. ADGN-F8 treatment produced rapid and prolonged duration of FVIII expression that could be a therapeutic alternative for current Hemophilia A treatments. Similar strategies could be applied to other clotting disorders and for other hepatic diseases.

1094. *In Vivo* CRISPR Mediated Editing of PCSK9 Using Peptide-Based Nanoparticles Durably Lowers Cholesterol in Mice

Gilles Di Vita[1,2], Nathalie Durany[1,2], Veronique Josserand[3], Melanie Guidetti[3], Neil Desai[1]

[1]AADIGEN, Pacific Palisades, CA,[2]DIVINCELL, Nimes, France,[3]IAB, Grenoble, France

Atherosclerotic cardiovascular disease remains the leading cause of death worldwide. A major contributor to the development of atherosclerosis are elevated low-density lipoprotein cholesterol concentrations. Hypercholesterolemia is a highly prevalent genetic disorder and mutations *PCSK9 gene*, encoding proprotein convertase subtilisin/kexin type 9 (PCSK9) was identified to cause hypercholesterolemia. Inhibitors of PCSK9 have emerged as a promising new class of cardioprotective drugs *and b*ase editing strategies to permanently disrupt the *PCSK9* gene in the liver are currently being evaluated in Phase 1 clinical trial.By delivering an mRNA encoding for the Cas9 protein with guide RNAs (sgRNA) for a gene of interest it is possible to permanently knock out a pathogenic gene mutant in a target cell, curing otherwise incurable genetic diseases. However, *in vivo* administration CRISPR components remains a limitation to the overall potency and for the therapeutic application of

the CRISPR-CAS9 system. We have developed a new delivery platform that can potently deliver active CAS9 mRNA/gRNA complexes *in vivo* and selectively silence PCSK9 gene in mouse liver. ADGN technology constitutes a biocompatible and scalable system to efficiently package and deliver CAS9mRNA/gRNA to the target site. ADGN technology is based on short amphipathic peptides that form stable nanoparticles with RNAs. The molecular composition and sequences of ADGN nanoparticles have been rationally designed for selective organ targeting of functional RNAs and to overcome off target accumulation. *PCSK9* being preferentially expressed in the liver, we have selected ADGN-peptide with a liver tropism and able to target liver i*n vivo*. ADGN/RNAs nanoparticles exhibit stable physicochemical properties both in solution and in dry powder formulations, with an average size of 100 nm, a slightly negative charge surface and a high encapsulation efficiencies (> 90%).We demonstrated that intravenous administration of ADGN/Cas9mRNA:gRNA nanoparticles resulted in mRNA accumulation in the liver with CAS9 protein expression starting 6 hrs after administration with an optimal expression after 24 hrs, and slowly declined thereafter.ADGN has been used for the co-delivery of mRNA encoding for Cas9 and a guide RNA targeting exon 1 of mouse *PCSK9*. We showed that a single administration of ADGN-PCSK9 induced in a permanent alteration of PCSK9 in mice. We observed nearly 50 % of knockdown of *PCSK9* gene in the liver at various doses down to 0.5 mg/Kg. ADGN-PCSK9 treatment resulted in a stable reduction of circulating PCSK9 plasma levels by ca. 75% and reduction of plasma cholesterol level by ca. 55%. All of these alterations remained stable for at least 2 months after a single-dose treatment. Cytokine, histology and liver enzyme profiling showed that ADGN-PCSK9 administration does not induce liver toxicity, inflammation or chronic alteration and any adverse innate or adaptive immune responses in immune-competent mice. No off-target were detected with a 0.5 mg/kg dose and no modification in PCSK9 and cholesterol levels was observed when using control ADGN nanoparticles containing mRNA Cas9 and a control gRNA targeting GFP. ADGN nanoparticles constitute a new platform for CRISPR editing of PCSK9 in order to reduce low-density lipoprotein cholesterol as a treatment of atherosclerotic cardiovascular disease. Our results provide a proof-of-concept for how ADGN technology can be successfully applied to make precise CRISPR gene editing in therapeutic target genes in the liver.

Thursday Posters: Lysosomal Storage Disorders

1095. Preclinical Therapeutic Efficacy of Novel Bicistronic Adeno-Associated Virus Vectors for GM2 Gangliosidosis Gene Therapy

Ali Ramezani, Anna Leigh Lynch, Juliette Hordeaux, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Background: Tay-Sachs disease (TSD) and Sandhoff disease (SD) arise from mutations in the *HEXA* and *HEXB* genes, respectively, that result in reduced or complete loss of the heterodimeric enzyme β-N-acetylhexosaminidase (HexA). A deficiency of functional HexA prevents degradation of its substrate, GM2, meaning GM2 accumulates in neurons where it causes lysosomal dysfunction, neurotoxicity, and death. No cure or effective therapy is currently available for either TSD or SD. Research has shown that gene therapy can confer substantial therapeutic benefits in animal models, with adeno-associated virus (AAV) vector-mediated *HEXA* gene delivery and expression emerging as one of the most promising approaches for treating TSD and SD. Objective: Generating functional HexA heterodimers requires co-expression of the α- and β-subunits within the same cell; given the limited transgene capacity of AAV vectors, previous work has explored the co-delivery of two separate monocistronic AAV vectors, albeit with limited success. Our objective was to develop bicistronic AAV vectors that co-express both subunits of HexA and identify the optimal design. Methods: We developed novel bicistronic AAV-hu68 vectors co-expressing the *HEXA* and *HEXB* complementary DNA using a 2A peptide or bidirectional (BD) promoter strategy. We evaluated several different viral 2A peptides (2A-1 to 2A-5), as well as novel and naturally occurring short BD promoters with or without the cytomegalovirus enhancer, ranging in size from 131 bp to 869 bp (BD-1 to BD-6). We identified vectors with the most efficient 2A peptides following in vitro transfection and Western blot analysis of *HEXA-HEXB* double knockout (AB-KO) human embryonic kidney (HEK293) cells. To test the BD promoters, we constructed AAV vectors with enhanced green fluorescent protein and mCherry fluorescent biomarkers flanking the promoters and tested these vectors in vitro in 293T cells and in vivo in C57Bl6 mice. We evaluated the therapeutic efficacy of selected AAV-hu68 vectors with the best-performing 2A peptides or BD promoters in 4- to 6-week-old SD mice (*Hexb*$^{-/-}$) following intracerebroventricular (ICV) administration of 1E11 genome copies/mouse. Results: AAV vectors with 2A-1 and 2A-2 exhibited the highest expression, near-complete peptide cleavage, and highest enzyme activity in transfected AB-KO HEK293 cells. Among the BD promoters, AAV vectors with BD-1, BD-2, and BD-3 conferred the highest transgene expression and enzyme activity in transfected AB-KO HEK293 cells. Following ICV dosing in adult SD mice, we detected HexA enzyme activity in the blood of all dosed animals on day 7 at levels that reached or exceeded wild-type levels. Moreover, all AAV vectors significantly improved survival compared to untreated SD mice. The median survival increased post dosing from 10.3 weeks for untreated SD mice to 15.7 weeks for AAVhu68-HEXB-[BD-3]-HEXA vector-dosed SD mice ($p<0.01$), 17.6 weeks for AAVhu68-HEXA-[2A2]-HEXB vector-dosed mice ($p<0.005$), and an undefined survival for AAVhu68-HEXA-[2A1]-HEXB vector-dosed mice, as 50% of the treated SD mice in this group survived past 22 weeks post-dosing (the experimental endpoint). Histological analysis of SD mice brains stained with an anti-GM2 antibody demonstrated that all vector-treated SD mice exhibited decreased GM2 buildup compared with untreated SD mice; we observed the greatest reductions (85% and 67%) in GM2 accumulation in the brain in SD mice treated with AAVhu68-HEXA-[2A2]-HEXB and AAVhu68-HEXB-[BD-3]-HEXA vector, respectively. Conclusion: Upon ICV administration in SD mice, our novel bicistronic AAV vectors achieved high HexA expression and activity levels, ameliorated brain GM2 accumulation, and conferred significant survival benefits. These data highlight the potential efficacy of bicistronic AAV vectors for treating GM2 gangliosidoses such as TSD and SD.

1096. Development of Systemic AAV Gene Therapies for Neuronopathic Gaucher Disease

Tae Un Han[1], PamelaSara Head[2], Adenrele Gleason[1], Bahafta Berhe[1], Akhil Kulkarni[1], Tiffany Chen[1], Charles P. Venditti[2], Ellen Sidransky[1]

[1]Medical Genetics Branch, NIH/NHGRI, Bethesda, MD,[2]Metabolic Medicine Branch, NIH/NHGRI, Bethesda, MD

Introduction: Gaucher Disease (GD) is an autosomal recessively inherited disorder resulting in the deficiency of the lysosomal enzyme glucocerebrosidase (GCase). The disorder has a spectrum of both neuronopathic and non-neuronopathic manifestations, with varying levels of severity. Current treatments (enzyme replacement therapy and substrate reduction therapy) are effective for non-neuronopathic GD, but do not reverse neuronopathic symptoms, as these therapies do not cross the blood-brain-barrier. Our aim was to develop novel viral gene therapies and evaluate their efficacy in a murine model of neuronopathic GD (nGD). **Methods:** Codon adjusted and optimized *GBA1* genes were developed and tested for expression in human HEK 293T cells and a H4 neuroglioma *GBA1* knock-out cell line (H4 *GBA1-/-).* The *GBA1* construct, co*GBA1.1* demonstrated highest GCase expression was cloned as a transgene under the control of the EF1S promoter and produced supraphysiological GCase activity in H4 *GBA1-/-* cells. Next, *coGBA1.1* was used to produce single stranded AAV EF1s *coGBA1.1* HPRE vectors packaged in AAV9 and AAVrh10 capsids. The recovery of GCase expression was evaluated in H4 *GBA1-/-* cells via infection with the *GBA1* AAVs followed by Western Blot analysis. Then the AAV-*GBA1* constructs were tested *in vivo* using a K14-lnl skin-recovery knock-out mouse model of nGD. The AAV-*coGBA1.1* vectors were delivered at varying doses via facial vein (FV) infusions or retro orbital (RO) injections in P1 neonatal K14-lnl pups and survival was evaluated. We are also developing a model of delayed onset nGD using a tamoxifen-induced *gba1* knock-out strategy, where symptoms are induced by oral administration of tamoxifen in older mice. **Results:** Both AAV9 and AAVrh10 EF1s *coGBA1.1* HPRE vectors restored GCase levels in a dose dependent fashion in H4 *GBA1-/-* cells. K14-lnl animals treated with a 4×10^{11} GC dose of the AAV9-EF1s *coGBA1.1* HPRE prevented neurological and systemic manifestations of nGD and extended lifespan (>300 days), with grossly normal behavior, development, weight gain, clinical appearance, and fertility while the untreated K14-lnl mice had weight loss, an abnormal gait, seizures, Gaucher cells in visceral organs and severe neuroinflammation at two weeks of age. Treatment of three K14-lnl mice with AAVrh10- EF1s *coGBA1.1* HPRE the same dose also prevented the development of symptoms. The tamoxifen-induced *gba1* knock-out model developed neurological findings and neuroinflammations similar to those in K14-lnl mice at age 10~14 weeks, depending on when tamoxifen was administered. **Conclusions:** The EF1s *coGBA1.1* HPRE transgene, when packaged into an AAV9 or AAVrh10 capsid, demonstrated potent *in vivo* activity in a severe model of nGD, a devastating genetic neurodegenerative disorder currently without any effective therapy. After dosage optimization in mouse models of nGD, we will explore the feasibility of a clinical trial in patients while we further develop conditional, inducible models of nGD.

1097. Long-Term Functional Benefits of Treating MPS IH with a Compact Self-Complementary AAV9-ΔIDUA^{op} Vector

Tierra A. Bobo[1], Hyouk-Bum Kwon[2], Xinyu Cao[2], Michael Robinson[2], Allen Zheng[3], Haiyan Fu[1]

[1]Pediatrics Genetics and Metabolism, The University of North Carolina at Chapel Hill, Gene Therapy Center, Chapel Hill, NC,[2]Gene Therapy, The University of North Carolina at Chapel Hill, Gene Therapy Center, Chapel Hill, NC,[3]NeuroGT, Inc., Chapel Hill, NC

MPS I is a devastating LSD caused by mutations in the iduronidase (IDUA) gene. The disease manifests in all organs, with profound neurological impairments in the majority of patients. No treatment is currently available for the neurological disorders of MPS I. Addressing this unmet need, this study develops an effective self-complementary (sc) gene therapy vector for treating MPS I using the trans-BBB-neurotropic AAV9 via systemic delivery. We constructed a gene replacement vector, scAAV-Δh*IDUA*^{op}, containing the codon-optimized human *IDUA* cDNA (h*IDUA*^{op}) to enhance the transgene expression. To accommodate the limited packaging capacity of scAAV vector, we engineered the vector genome to 4,850bp by using a 228bp truncated miniatur CMV promoter, a 16bp SNRP1 Poly A signal, and miniaturized h*IDUA*^{op} cDNA (Δh*IDUA*^{op}). We tested scAAV-Δh*IDUA*^{op} vector *in vitro* to assess rIDUA expression, secretion, and scAAV packaging. *In vitro* studies showed that codon-optimization of h*IDUA* cDNA resulted in >2-fold increase in rIDUA expression. The deletion of 15 bp non-conserved 3' untranslated sequence from h*IDUA*^{op} not only enabled the packaging of scAAV-Δh*IDUA*^{op}, but also further enhanced rIDUA expression and secretion. The scAAV9-ΔhIDUA^{op} viral vector was tested in 1-2m-old MPS I mice via an IV injection at 2e12vg/kg, 5e12vg/kg and 1e13vg/kg to determine the therapeutic potential. The vector treatments resulted in rapid and persistent IDUA expression, the clearance of lysosomal storage pathology throughout the CNS, PNS and periphery tissues, and amelioration of neuroinflammation, leading to significant behavior improvement and extension of survival (ongoing) in MPS I mice. Notably, 2e12vg/kg was identified as the minimal effective clinical dose. This study demonstrates the promising clinical potential of the systemic scAAV9-ΔhIDUA^{op} gene delivery for treating neuropathic MPS I. The 4,850bp vector genome allows the packaging into scAAV vector, which is slightly beyond the previously defined size limit (~4,700bp).

1098. Development of a Best-in-Class AAV Gene Therapy for Fabry Disease with Enhanced a-Gal A Expression and Cellular Uptake by Protein Engineering and Promoter Evolution

Lu Guo, Zihui Zhao, Wenjing Luo, Jing Ma, Xi Zhu, Xiaoping Zhao, Shin-Shay Tian

Vitalgen BioPharma, Shanghai, China

Fabry disease (FD, OMIM 301500) is an X-linked, dominantly inherited genetic disorder caused by the deficiency or absence of lysosomal α-galactosidase A (EC 3.2.1.22, α-Gal A), resulting in pathogenic deposit of globotriaosylceramide (Gb3 or GL-3) and its derivatives globotriaosylsphingosine (lyso-Gb3) in heart, kidney and other tissues.

The enzyme replacement therapies (ERT) approved for FD require repeat dosing and patients run the risk of developing neutralizing antibodies that reduce the therapeutic efficacy over time, supporting the need for a better treatment. One of the main limitations for FD ERTs is the poor cellular uptake efficiency of the α-Gal A protein by the relevant tissues, e.g., heart and kidney. The limited expression of the mannose-6-phosphate (M6P) receptor (M6PR), which mediates α-Gal A cellular uptake, limits α-Gal A uptake and its efficacy. Based on the association of M6P with protein glycosylation, α-Gal A mutants with increased N-linked glycosylation sites were designed to increase the numbers of M6P in the expressed α-Gal A protein. The mutants were evaluated in several liver (HepG2, Hep3B & Huh7) and kidney (A-498, ACHN &769-P) cell lines for their secreted protein expression (liver cell supernatants) and uptake (kidney cells) levels. Among the 13 mutants evaluated, mutant M7 showed the highest levels of cellular uptake, increasing up to 6-fold over the wild type α-Gal A. M7 and the wild type control were packaged into AAV8 for further evaluation in GLA$^{-/Y}$ mice at the doses of 5×10^{11}, 5×10^{12} and 5×10^{13} vg/kg. The activities of α-Gal A in the plasma, liver, heart and kidney at day 28 were measured and the levels of plasma lyso-Gb3 were analyzed. The results confirmed that in comparison to the wild type control group, the M7 group achieved higher α-Gal A enzyme activities in the liver and heart and further more, significantly better lyso-Gb3 lowering in the plasma. In parallel, the AAV expression cassette was optimized by introducing various *cis*-regulatory elements to the promoter. Based on the *in vitro* liver cell protein expression and *in vivo* efficacy studies in the GLA$^{-/Y}$ mice, P18 was identified as the best candidate. A final AAV expression cassette P18-M7 was generated and packaged into AAV8 for evaluation in the GLA$^{-/Y}$ mice at the doses of 1×10^{11}, 1×10^{12} and 1×10^{13} vg/kg. The results showed that AAV8-P18-M7 effectively down-regulated the plasma, liver, heart and kidney lyso-Gb3 to the wild type levels at 28 days after AAV administration, significantly more effective than the several benchmark constructs used for comparison. To summarize, by optimizing α-Gal A protein cellular uptake and obtaining a chimeric promoter to drive stronger liver-specific protein expression, we have developed a significantly improved AAV-based gene therapy for treating Fabry Disease.

1099. Preclinical Development of ZS805, a Favorable Liver-Directed rAAV Gene Therapy for Fabry Disease

Fuqin Fan[1], Siwu He[2], Ming Zhang[1], Yu Liu[2], Zhaoyue Zheng[2], Siyuan Dong[1], Lei Gao[1], Jingya Ye[1], C. Alexander Valencia[1], Hoi Yee Chow[2], Biao Dong[1]

[1]Sichuan Real & Best Biotech Co., Chengdu, China,[2]National Clinical Research Center for Geriatrics and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, China

Fabry disease is a rare X-linked recessive lysosomal storage disease caused by dysfunctional mutations in the alpha-galactosidase A (*GLA*) gene, leading to systemic accumulation of the deleterious substrates, namely, Gb3 and Lyso-Gb3, within various tissues and organs. Progressive substrate buildup results in the development of cardiovascular and cerebrovascular symptomology, such as heart failure, stroke, end-stage renal disease and even premature death. The current standard treatment for Fabry disease is enzyme replacement therapy (ERT). While ERT has demonstrated the ability to slow the progression of the disease, its significant drawbacks, including limited efficacy, immune intolerance, immunogenicity of recombinant alpha-galactosidase A (α-Gal A) and need for lifelong infusions, have been observed by a number of studies. Herein, we present ZS805 as a rAAV5 vector harboring an expression cassette featuring a novel liver-specific promoter, a modified signaling peptide (SP21) sequence and a codon-optimized human GLA cDNA. In the investigational new drug-enabling preclinical study, we showed that in the α-Gal A KO mouse model a single intravenous injection of ZS805 (dose ranging from 2.0×10^{12} vg/kg to 2.0×10^{13} vg/kg) led to a substantial and persistent increase in the plasma α-Gal A protein and activity in a dose-dependent manner. Crucially, the plasma α-Gal A protein and activity reached supraphysiological level at a dose value of 2.0×10^{12} vg/kg, with about a 90% clearance rate of the Gb3 and lyso-Gb3 substrates in the plasma. At the end of study (Day 92), α-Gal A protein expression and α-Gal A enzymatic activity persisted, the levels of Gb3 and lyso-Gb3 significantly decreased in all examined tissues, including the liver, spleen, heart, kidney, lung, gastrocnemius muscle, brain, and dorsal root ganglia.

1100. Evaluation of AAV.GMU1 Biodistribution via Intravenous Administration for Treatment of Gaucher Disease

Swathi Ayloo[1], Shih-Ching Chou[1], Jae Ryu[1], Charles Miller[1], Jie Bu[1], Tracy Lacson[2], Mariana Gonclaves[2], Lilu Guo[2], Shelley Naas[1], Bradford Elmer[1], Catherine O'Riordan[1], Martin Goulet[1], Christian Mueller[1], Shyam Ramachandran[1]

[1]Genomic Medicine Unit, Sanofi, Waltham, MA,[2]Precision Medicine and Computational Biology, Sanofi, Cambridge, MA

Our previous work with AAV.GMU1 has demonstrated broad CNS (central nervous system) tropism in non-human primates (NHPs). We also observe that lower viral load of AAV.GMU1 capsid yields higher transgene expression compared to other capsids. Bi-allelic mutations in *GBA1*, the gene encoding glucocerebrosidase (GCase) are causative for Gaucher disease (GD). GD is a lysosomal storage disease characterized by excessive lipid build-up in macrophages across multiple peripheral tissues, including liver spleen, as well as muscle. GCase is a lysosomal enzyme that catalyzes the hydrolysis of glycolipids glucosylceramide (GL1) and glucosylsphingosphine (Lyso-GL1). Reduction or loss of GCase activity leads to the accumulation of substrates, disrupting lipid homeostasis. Here we evaluate biodistribution of AAV.GMU1 with intravenous (IV) doing in rodent and NHPs and establish efficacy of our engineered *GBA1* payload for effective lipid clearance for the treatment of type 1 GD. We first examined biodistribution of AAV.GMU1 in NHPs three weeks post IV dosing and observed robust transduction of several peripheral organs such as liver, spleen, heart, and muscles such as diaphragm, quadriceps, and gastrocnemius. We then examined biodistribution of AAV.GMU1 with IV dosing in rodents in the context of GD using our engineered lead *GBA1* variant. Consistent with broad transduction of several peripheral organs in NHPs, we observe similar transduction in rodents. We also performed functional efficacy studies in mice pre-treated with CBE, a covalent inhibitor of GCase. In contrast to vehicle-injected mice, we observe increased GCase activity as well as robust clearance of lipids in AAV.GMU1-GBA injected mice. Importantly, we observed reversal of lipid

accumulation even in tissues that received little to no AAV vector indicating robust cross-correction by our engineered lead construct. Collectively, our data demonstrate that AAV.GMU1 via IV dosing has broad biodistribution in multiple peripheral organs including muscle tissues. The biodistribution in combination with our lead *GBA1* construct engineered for cross-correction establishes a gene therapy platform for the treatment of GD Type 1 with IV dosing.

1101. Comparing Efficacy of AAV Capsid Serotypes for the Treatment of Feline GM1 Gangliosidosis

Courtney Garrett[1,2], Amanda L. Gross[1], Heather L. Gray-Edwards[3,4], Miguel Sena-Esteves[3,5], Douglas R. Martin[1,2]

[1]Scott Ritchey Research Center, Auburn University College of Veterinary Medicine, Auburn, AL,[2]Department of Anatomy, Physiology, & Pharmacology, Auburn University College of Veterinary Medicine, Auburn, AL,[3]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[4]Department of Radiology, University of Massachusetts Medical School, Worcester, MA,[5]Department of Neurology, University of Massachusetts Medical School, Worcester, MA

Introduction. Feline GM1 gangliosidosis is a fatal neurodegenerative disease caused by a deficiency of lysosomal β-galactosidase (β-gal). The feline disease faithfully emulates the pathology of GM1 in humans, making cats an ideal animal model for studying this disease. Adeno-associated viral (AAV) gene therapy for GM1 aims to restore β-gal activity and minimize disease progression. Choosing the appropriate AAV serotype and delivery route is critical for GM1 therapy since the vector must display tropism for cells that can achieve therapeutic effect and reach the areas most affected, such as the cerebellum. Intracranial injection of AAV into the thalamus and deep cerebellar nuclei resulted in a 7.5-fold increase in the lifespan of GM1 cats, but the procedure is invasive and requires a craniotomy for delivery to the brain parenchyma. Injection into the CSF via the cisterna magna (CM) is less invasive and could potentially improve biodistribution to certain parts of the CNS and peripheral organs. This study compares the effects of AAV serotype on survival, β-gal distribution, viral distribution, and disease progression in GM1 cats following CM injection using serotypes AAV9 and AAVrh10. Methods. Each cohort received 1.5e13 vector genomes/kg body weight at 2.2 ± 0.3 months of age (symptom onset occurs at ~4 months). 4-MU enzyme assays were used to assess β-gal enzyme activity in CNS tissue samples as well as several peripheral organs. The same CNS samples were also stained with X-gal to visualize β-gal activity. 7T Magnetic resonance imaging was used to track disease progression across cohorts. Immune response to the capsid was also evaluated using ELISA-based serum antibody titers. Results. β-gal activity increased in the cerebellum and the spinal cord for both treatment cohorts, but not the cerebrum - untreated animals exhibit little to no β-gal activity in the brain and spinal cord (Figure 1). Both cohorts also displayed improved β-gal activity in peripheral tissues, such as the heart and cervical dorsal root ganglion. MRI showed delayed neurodegeneration in both cohorts, which correlated with delayed clinical symptoms such as inability to stand (Figure 2). Ultimately, untreated GM1 cats (n=9) survived 7.9 ± 0.3 months, GM1+AAV9 (n=3) cats survived 13.9 ± 1.9 months, and GM1+AAVrh10 (n=3) cats survived 11.9 ± 0.9 months. Statistical significance was found between the survival of untreated GM1 cats treated cats in both cohorts (p=0.0089) but there was no statistical significance found between AAV9 and AAVrh10 treated cats (p=0.1966). Conclusions. This study indicates efficacy of AAV therapy in both treatment cohorts and suggests that CM-injected AAV9 delays disease progression compared to AAVrh10 for the feline model.

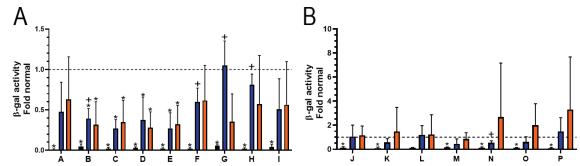


Figure 1. β-gal activity. Enzyme assays were performed to assess β-gal restoration in the CNS of treated cats when compared to untreated GM1 cats. * p<0.05 vs Normal, + p<0.05 vs GM1

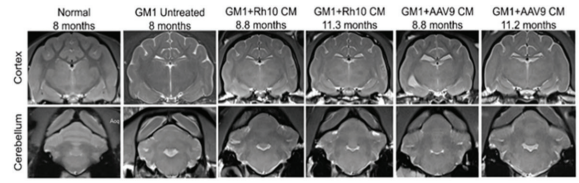


Figure 2. 7T MRI analysis. When compared to normal cats at 8 months, untreated GM1 cats have isointense (equal intensity) gray and white matter. The two treatment cohorts showed preservation of white matter at approximately 8.8 months. By endpoint, neurodegeneration and atrophy also progressed in both cohorts to a similar degree, as demonstrated by isointensity of the parenchyma and increase in CSF (bright white) around the brain, respectively.

1102. Pioneering Gaucher Disease Treatment: Preclinical Efficacy and Safety Study of LY-M001

Xiaojing Sheng, Yingying Cai, Tianqi Jiang, Chenming Lu, Yixiong Chen, Qing Lin

Lingyi Biotech Co., Ltd., Shanghai, China

Gaucher disease (GD), a prevalent lysosomal storage disorder, arises from mutations in the GBA1 gene, leading to deficient activity of glucocerebrosidase (GCase). This deficiency triggers the accumulation of glucosylceramide (GlcCer) and glucosylsphingosine (Lyso-GL1) in lysosomes, causing multisystemic organ damage. LY-M001, a novel rAAV serotype 8 vector-based therapy, aims to rectify this deficiency by delivering a codon-optimized *GBA1* gene, driven by a hybrid hepatic-specific promoter, and expressing a modified GCase ($GCase^{LY}$). This study evaluates LY-M001's potential as a therapeutic agent for GD. Employing the LY-Vector™ platform, over 500 AAV vector candidates were meticulously crafted and screened through rigorous in vitro and in vivo analyses, leading to the selection of LY-M001. Preclinical efficacy assessments in $Gba^{M1/M2}$ Gaucher model mice demonstrated remarkable restoration of GCase activity and substantial reduction of GlcCer and Lyso-GL1 in key organs post a single intravenous dose of LY-M001. Correspondingly, a reduction in Gaucher cells across vital tissues was observed. Long-term stability of GCase activity was confirmed in C57BL/6J mice over 10 months, with no adverse effects noted. Additionally, a 13-week GLP toxicology study in Cynomolgus monkeys endorsed LY-M001's safety profile, exhibiting exceptional tolerability and absence of toxicity. The convergence of these comprehensive preclinical data underscores LY-M001's profound potential in revolutionizing the therapeutic landscape for Gaucher disease, heralding a new era of treatment possibilities.

1103. Gutless Adenovirus-Mediated Gene Transfer of NEU1 Sialidase Rescues Sialidase-Deficient Mice

Suleiman A. Igdoura[1], Allyson Peek[2], Maria Athanasopoulos[2]

[1]Biology and Pathology & Molecular Medicine, McMaster University, Hamilton, ON, Canada,[2]Biology, McMaster University, Hamilton, ON, Canada

Sialidosis, an inherited primary deficiency of lysosomal sialidase, is an autosomal recessive lysosomal storage disease, characterized by the accumulation of sialylated glycopeptides and oligosaccharides in tissues and body fluids, leading to a range of clinical manifestations, including visual disturbances, ataxia, and myoclonus. Currently there is no treatment for Sialidosis, while there has been some success reported for the treatment of several lysosomal storage diseases using enzyme replacement therapy or bone marrow transplantation. Helper-dependent gutless adenoviruses (HD-Ad) have been studied as potential gene therapy vehicles for the treatment of genetic diseases. To this end, a full-length lysosomal sialidase gene and promoter was cloned into a HD-Ad vector and were tested in vitro and in vivo in sialidase-deficient cells and mice respectively. Following HD-Ad infection, lysosomal NEU1 sialidase enzymatic activity and mRNA levels in cultured deficient cells were dramatically increased and sustained for 20 passages. After an intravenous administration of HD-Ad NEU1 vector in sialidase deficient mice, mice were terminated at one, two and six- and 18-weeks post infection. Our in vivo results show that HD-Ad NEU1 demonstrated a sustained expression of sialidase beyond four months post-infection. The HD-Ad NEU1 also corrected sialidosis-associated phenotype. The in vitro and in vivo preclinical studies presented herein using helper-dependent adenovirus sets the stage for clinical trials in Sialidosis patients.

1104. Advancing Neuronopathic Gaucher Disease Modeling Using Microglia-Containing Brain Organoid

Yi Lin[1], Benjamin Liou[1], Jason Hammonds[2,3], Stuart Adler[1], Ying Sun[1,3]

[1]Division of Human Genetics, Cincinnati Children's Hospital, Cincinnati, OH,[2]Division of Infectious Diseases, Cincinnati Children's Hospital, Cincinnati, OH,[3]Department of Pediatrics, University of Cincinnati School of Medicine, Cincinnati, OH

Mutations in *GBA1*, encoding the lysosomal acid β-glucosidase (GCase), cause neuronopathic Gaucher disease (nGD). To model the impact of *GBA1* mutations on human midbrain development, we derived human 3D midbrain-like organoids (MLOs) from a nGD patient fibroblasts-derived induced pluripotent stem cells (iPSCs) and a gene-corrected isogenic control iPSCs created by CRISPR/Cas9 system, as well as healthy human iPSCs. The nGD MLOs closely resembled key phenotypes associated with nGD. However, the major challenge of a physiological nGD MLO model is the lack of resident microglial cells, which play pivotal roles in brain homeostasis and pathogenesis in nGD. To address this barrier in nGD modeling, a healthy human iPSC line with a knocked-in PU.1 inducible cassette was generated using CRISPR/Cas9 gene editing. PU.1 is a critical transcription factor for controlling myeloid/microglia lineage commitment. We have developed a culture strategy involving the combination of PU.1-knocked in iPSC and unmodified iPSC, resulting in the spontaneous generation of microglia containing MLOs (mcMLOs) with doxycycline induction. These mcMLOs contained microglia-like cells expressing typical microglia markers (Iba1+PU.1+), without apparent impact on the formation of midbrain enriched dopaminergic neurons in neuroectoderm differentiation conditions. Importantly, mcMLOs demonstrated a responsive chemokine system showing increased expression of *CD11b*, *LGALS3*, *IL-1β* to the inflammation stimulation induced by lipopolysaccharide, confirming microglial functionality. Pharmacological inhibition of GCase using conduritol B epoxide resulted in a complete loss of GCase activity in healthy mcMLOs, and consequently, elevated expression of microglia activation related genes, mimicking inflammation phenotype in nGD. These results provide compelling evidence supporting the modelling of nGD using mcMLOs. Meantime, we have knocked in an inducible PU.1 expression cassette into iPSCs derived from nGD patients, including both type 2 and type 3 GD, and corresponding isogenic lines. We aim to employ this innovative microglia-containing brain organoid platform using nGD patient and isogenic iPSC lines to study the role of microglia in neural development and neurodegeneration associated with nGD.

1105. Single vs Dual Transgene *Ex Vivo* Gene Therapy for Multiple Sulfatase Deficiency

Vi Pham[1,2], Lucas Tricoli[2], Parith Wongkittichote[2], Xinying Hong[1,2], Lars Schlotawa[3], Stefano Rivella[1,2], Rebecca Ahrens-Nicklas[1,2]

[1]University of Pennsylvania, Philadelphia, PA,[2]Children's Hospital of Philadelphia, Philadelphia, PA,[3]University Medical Center Goettingen, Goettingen, Germany

Multiple sulfatase deficiency (MSD) is an ultra-rare, inherited lysosomal storage disorder characterized by the functional deficiency of all sulfatase enzymes. MSD is caused by mutations in the gene *SUMF1*, encoding a sulfatase modifying factor that activates 17 downstream sulfatases. MSD patients present with combined multisystemic features of single-sulfatase disorders such as the mucopolysaccharidoses and metachromatic leukodystrophy (MLD), including neurologic regression, respiratory complications, liver enlargement, bone abnormalities, cardiac dysfunction, and hearing loss. Currently, there are no disease-modifying therapies for MSD. We aimed to develop *ex vivo* gene therapy with hematopoietic stem cell transplant for MSD, as this approach has proven successful for related lysosomal disorders. We designed lentiviral vectors encoding either 1) *SUMF1* or 2) a downstream sulfatase *ARSA* that is greatly implicated in the disease. *ARSA* ex vivo gene therapy is approved by the EMA for MLD and has the potential to be repurposed for patients with MSD. *In vitro*, MSD patient fibroblasts transduced with the lentiviral vectors demonstrated improved biochemical phenotypes including rescue of sulfatase activities and glycosaminoglycan accumulation. *In vivo,* the *SUMF1* lentiviral vector proved efficacious in rescuing biochemical and behavioral phenotypes in MSD mice that underwent hematopoietic stem cell transplant with *ex vivo* gene therapy. To further augment outcomes, we designed a novel lentiviral vector that co-expresses both *SUMF1* and *ARSA*. This dual vector greatly enhanced biochemical

rescue *in vitro* and augmented disease phenotypes *in vivo* as compared to the single vectors. We will present the results of our ongoing work optimizing the co-expression of both *SUMF1* and *ARSA* transgenes.

1106. Engineering and Characterization of CLN3 Disease (Juvenile Neuronal Ceroid Lipofuscinosis) iPSC Cells

Devin Burris[1,2], Livia Sitori[2], Jeet Singh[2], Rebecca Ahrens-Nicklas[1,2]

[1]University of Pennsylvania College of Medicine, Philadelphia, PA,[2]Children's Hospital of Philadelphia, Philadelphia, PA

CLN3 disease (also known as Juvenile Neuronal Ceroid Lipofuscinoses (JNCL)) is a form of lysosomal storage disease which arises from mutations in the *CLN3* gene, which encodes CLN3 protein. CLN3 disease is a rare, pediatric genetic neurodegenerative disorder characterized by progressive vision loss onset at ages 4-7, seizures at approximately age 10, parkinsonism, problems walking, difficulty with speech and language, and death between ages 15-30. Loss of CLN3 function leads to lysosomal storage, the accumulation of cellular debris, hypothesized to disrupt neuronal function. CLN3 is a lowly expressed transmembrane lysosomal protein with unreliable antibodies for protein analysis and no final 3D structure, so indirect perturbations of protein function mainly inform the field. CLN3 was recently hypothesized to be a lipid species transporter, but its function is yet to be robustly supported. Unclear mechanism makes *CLN3* a good candidate for gene-level therapeutics. We have generated a patient-derived iPSC line from a rare attenuated CLN3 disease patient. The patient had preserved cognition and only experienced vision loss well into their 30s. This patient is compound heterozygous for the severe 1.02 kB deletion of exons 7 and 8 seen in 70-80% of cases and an intron 6-exon 7 splice acceptor variant. This patient line demonstrates an intermediate level of CLN3 transcript between WT and KO cells. We are using genome editing tools to generate and validate an isogenic control for future characterization of disease intensity in various iPSC derived cell types. We aim understand the impact of CLN3 and its splice variants on disease progression, cellular electrophysiology, and lysosomal storage when considering Gene Therapy approaches.

1107. AAV-HEXA-HEXB-Mediated Gene Therapy Reverts Sandhoff and Tay-Sachs Diseases

Sara Marcó[1,2,3], Gemma Elias[1,2], Miquel Garcia[1,2,3], Xavier Sánchez[1,2], Carles Roca[1,2,3], Albert Ribera[1,2,3], Víctor Sánchez[1,2], Joan Bertolin[1,2], Jennifer Pérez[1,2], Maria Molas[1,2,3], Xavier León[1,2], Fatima Bosch[1,2,3]

[1]Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain,[2]Cbateg, Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain,[3]CIBERDEM, Madrid, Spain

Tay-Sachs (TSD) and Sandhoff (SD) diseases are two rare autosomal recessive lysosomal storage diseases that results from the deficiency of the heterodimer β-Hexosaminidase A (HEXA), encoded by *HEXA* (TSD) and *HEXB* (SD) genes. Defective HEXA enzyme leads to progressive accumulation of GM2 ganglioside within lysosomes. TSD and SD patients exhibit severe, progressive neurodegeneration with indistinguishable devastating pathology and premature death. Currently, there is no cure for these diseases. Here, using mouse models of SD and TSD, we demonstrated therapeutic efficacy at short- and long-term of a novel gene therapy approach based on a single intra-cerebrospinal fluid (intra-CSF) administration of an adeno-associated (AAV) vector encoding simultaneously both *HexA* and *HexB* genes (AAV-*HexA*-*HexB*). AAV-mediated gene transfer resulted in widespread, even expression of a new covalently linked β-Hexosamindase A in the whole Central Nervous System (CNS) of TSD and SD mouse models. In addition, AAV-treated SD mice showed reversion of CNS lysosomal pathology, leading to correction of myelinization, autophagy and neuroinflammation. Moreover, intra-CSF administered AAV-*HexA*-*HexB* vectors efficiently transduced the liver, allowing the secretion of HEXA enzyme into the bloodstream, which completely corrected peripheral lysosomal pathology of SD disease. Reversion of pathological alterations were paralleled to normalization of behavioral deficits. More remarkably, treatment of SD mice with the single AAV-*HexA*-*HexB* vector significantly extended lifespan, compared to the co-administration of AAV-*HexA* and AAV-*HexB* vectors or the delivery of AAV-*HexA*-P2A-*HexB* bicistronic vector to SD mice. Altogether, our pre-clinical data clearly demonstrated that a single intra-CSF administration of AAV-*HexA*-*HexB* vectors resulted in efficient production of the new covalently linked β-Hexosamindase A, leading to whole-body correction of both CNS and peripheral pathology. These results provide strong rationale for the future clinical translation of the AAV-*HexA*-*HexB* gene therapy to treat Tay-Sachs and Sandhoff patients.

Thursday Posters: Neurologic Diseases

1108. Characterizing the Phenotypic Abnormalities of a Mouse Model of Inborn Error of Polyamine Metabolism for Gene Therapy Development

Oluwaseun Akinyele[1], Anushe Munir[1], Marie Johnson[1], Megan Perez[1], Yuan Gao[1], Jackson Foley[2], Yijen Wu[1], Tracy Murray-Stewart[3], Robert Casero[3], Hulya Bayir[1], Dwi U. Kemaladewi[4]

[1]Pediatrics, University of Pittsburgh, Pittsburgh, PA,[2]Pediatrics, Johns Hopkins University School of Medicine, Baltimore, MD,[3]Johns Hopkins University School of Medicine, Baltimore, MD,[4]Children's Hospital of Pittsburgh, Pittsburgh, PA

The polyamines (putrescine, spermidine, and spermine) are essential for normal cellular functions and are subject to strict metabolic regulations. Mutations in the gene encoding spermine synthase (SMS) lead to the accumulation of spermidine in an X-linked recessive disorder known as Snyder-Robinson syndrome (SRS), which manifest with a spectrum of symptoms including intellectual disability, developmental delay, thin habitus, and low muscle tone with no available treatment. Development of therapeutic intervention for SRS would require a mutation-specific animal model that recapitulates many of the abnormalities observed in affected individuals. Here, we study the molecular, behavioral, and neuroanatomical features of a

mouse model with a missense mutation in the *Sms* gene that results in a glycine-to-serine substitution at a position 56 (termed G56S) of the SMS protein and evaluate gene therapy as a treatment approach for the disease. This mouse was modeled after patients with the severe form of the disease. We showed that the G56S mice exhibit almost complete loss of SMS protein and elevated spermidine/spermine ratio in the skeletal muscles and the brain, as well as increased anxiety, impaired learning, and decreased explorative behavior. Furthermore, these mice fail to gain weight over time and exhibit abnormalities in brain structure and bone density. Transcriptomic analysis of the cerebral cortex revealed downregulation of genes associated with ribosomal protein synthesis and mitochondrial oxidative phosphorylation, which were functionally recapitulated in embryonic mouse hippocampal cells and primary fibroblasts isolated from the mice. Collectively, our findings establish the first in-depth characterization of an SRS preclinical mouse model that identifies cellular processes that could be targeted for therapeutic outcomes. Current experiments focus on developing and evaluating gene therapy for SRS via a viral-mediated expression of WT *Sms* transgene in patients-derived fibroblasts and the G56S mice with the potential to rescue some of the abnormalities mentioned above. We anticipate that the successful completion of this study will open the door to a gene therapy approach for SRS and other polyamine-related genetic diseases.

1110. Alpha-Synuclein Artificial miRNA-Based AAV Gene Therapy for Synucleinopathies

Mei Zhu[1], Yian Khai Lau[1], Fares Bassil[2], Audrey Lee-Gosselin[2], Barbara Calamini[3], Ricardo Luis Sanz[3], Fiona Menzies[3], Benjamin Skykind[1], Anindya Sen[1], Mansuo Lu Shannon[1]

[1]Prevail Therapeutics, New York, NY,[2]Prevail Therapeutics, Indianapolis, IN,[3]Eli Lilly Neuroscience Research, Cambridge, MA

Synucleinopathies are neurodegenerative disorders characterized by neuronal or glial accumulation of aggregates of alpha-synuclein protein (α-Syn), encoded by the *SNCA* gene. α-Syn is primarily expressed in neuronal tissues, making up ~1% of total cytosolic protein of brain cells. In synucleinopathies, α-Syn aggregates may spread through a prion-like mechanism, with toxic aggregated forms being transmitted from cell-to-cell as the disease progresses. In this regard, facilitating the clearance of α-Syn aggregates may have therapeutic potential to reduce the spread of the pathology and to potentially slow or halt disease progression. Various approaches have been investigated to prevent and mitigate α-Syn toxicity, however, so far only some symptomatic therapies have been explored, and no disease modifying treatments are available for any of the synucleinopathies. Here we are developing an AAV-based gene therapy (PR014) to deliver an artificial miRNA (amiR) targeting the *SNCA* gene to reduce α-Syn mRNA and protein levels. We aim to knockdown ≥50% of α-Syn, which has been well-demonstrated to be neuroprotective in multiple preclinical models of synucleinopathies. We screened siRNA sequences targeting *SNCA* gene *in vitro* in human cell lines, and selected a lead α-Syn amiR candidate achieving robust knockdown efficacy in α-Syn transgenic mouse model and human Parkinson's disease patients' iPSC-derived neurons. Our lead vector, packaged in the AAV9 capsid, significantly decreased α-Syn aggregates in a human iPSC-derived neuron-based pre-formed fibril (PFF) seeding model. These results support the further development and the potential therapeutic value of PR014 gene therapy for the treatment of synucleinopathies.

1111. Advanced Therapeutic Intervention for GBA1-Associated Parkinson's Disease: Preclinical Evaluation of Efficacy and Safety of LY-N001

Yingying Cai, Guofang Yan, Xiaojing Sheng, Yixiong Chen, Qing Lin

Dept. of Discovery, Lingyi Biotech Co., Ltd., Shanghai, China

Parkinson's Disease (PD) represents a prevalent neurodegenerative disorder, predominantly characterized by static tremor, rigidity, bradykinesia, and postural instability. Notably, mutations in the GBA1 gene, responsible for encoding lysosomal enzyme glucocerebrosidase (GCase), have been implicated in enhancing susceptibility and exacerbating the progression of PD. This study delineates the development and preclinical assessment of LY-N001, an innovative adeno-associated virus serotype 9 (AAV9)-mediated gene therapy. LY-N001 is engineered to constitutively express a codon-optimized $GBA1^{LY}$ gene, aiming to rectify the intrinsic genetic defect leading to lysosomal malfunction, thereby altering the disease trajectory. The efficacy of LY-N001 was rigorously evaluated in a murine model, employing a chemically induced paradigm via conduritol-β-epoxide (CBE) to inhibit GCase activity, subsequently inducing accumulation of GluCer and GluSph, akin to the neuropathological phenotype observed in GBA1-PD heterozygotes. Administration of LY-N001 via intracerebroventricular (ICV) injection in neonatal mice facilitated extensive viral distribution within the central nervous system (CNS), with subsequent RNA-based in situ hybridization (RNAscope) affirming the vector's presence in critical cerebral regions including cortex, hippocampus, striatum, substantia nigra pars compacta (SNc), and cerebellum. Immunohistochemical analyses corroborated the pervasive distribution of GCase enzymes throughout the neuronal architecture. In CBE-administered cohorts, LY-N001 significantly augmented GCase activity, evidenced by a 40-fold amplification in cerebral tissue and a 300-fold enhancement in cerebrospinal fluid (CSF). Additionally, a marked decrement in glucosylsphingosine concentrations (approximately 90%) was noted in cortical structures, paralleled by observable ameliorations in motor functionality and behavioral indices. Notably, LY-N001 efficaciously attenuated CBE-induced reactive astrogliosis and microgliosis in a dose-responsive manner. In summation, the extensive preclinical dataset underscores the profound therapeutic potential of LY-N001 for GBA1-PD, potentially inaugurating a novel epoch in the therapeutic paradigm for this intricate neurodegenerative ailment.

1112. Proof-of-Concept Efficacy of BGT-PD, a Novel AAV-Based Gene Therapy for the Treatment of Levodopa-Induced Dyskinesia (LID) in Parkinson's Disease

Giulia Leoni, Simon Eaglestone, Leigh Shaw, Adrien Lemoine

Bloomsbury Genetic Therapies, London, United Kingdom

Levodopa-induced dyskinesia (LID) is a prevalent and debilitating side effect of chronic levodopa (L-Dopa) treatment, the current gold standard for the control of motor symptoms in Parkinson's Disease (PD). LID is characterised by abnormal, involuntary movements that can appear a few months after initiating L-Dopa treatment, with approximately 90% of PD patients developing LID in the advanced stages of the disease. LID can have a significant impact on the quality of life of PD patients, remaining a major challenge in PD treatment and only limited therapeutic options are currently available. While the pathophysiology of LID is complex, a key factor is the severe loss of dopaminergic terminals and in consequence loss of dopamine transporter (DAT) activity, which is critical for the physiological regulation of extracellular dopamine levels and dopaminergic neurotransmission. As the disease advances, most of the administered L-Dopa is instead converted to dopamine and released in an uncontrolled manner by serotonergic neurons, resulting in a sudden rise in extracellular dopamine levels and overstimulation of dopamine receptors. Here, we tested the hypothesis that reinstating DAT activity in the striatum by single administration of BGT-PD, an adeno-associated virus serotype-2 vector expressing the human DAT gene, could ameliorate or completely reverse LID in a PD model of severe LID. To test this hypothesis, 6-hydroxydopamine (6-OHDA) hemiparkinsonian rats, rendered dyskinetic by daily administrations of L-Dopa/Benserazide, were treated with a single unilateral intrastriatal injection of BGT-PD. LID severity was assessed by evaluating axial, limb and orolingual abnormal involuntary movements (ALO-AIMs) at 2-, 4- and 6- weeks post gene therapy. The data indicated a profound and sustained anti-dyskinetic effect of BGT-PD, with complete suppression of LID observed at 4- and 6- weeks post-treatment compared to vehicle-treated animals. Non-motor PD symptoms were also assessed at the end of the study indicating a potential effect of BGT-PD treatment on anxiety-like behaviours. Moreover, the treatment was well tolerated, with no overt negative side effects observed. In conclusion, this study provides initial evidence of the therapeutic potential of our gene therapy approach to completely reverse LID by reinstating DAT activity. Additional studies will be required to evaluate the long-term efficacy and safety of our treatment candidate and support its future clinical translation.

1113. AAV-Mediated Gene Therapy for GBA-PD

Swathi Ayloo[1], Jae Ryu[1], Shih-Ching Chou[1], Charles Miller[1], Tracy Lacson[2], Mariana Gonclaves[2], Lilu Guo[2], Shelley Naas[1], Bradford Elmer[1], Catherine O'Riordan[1], Martin Goulet[1], Christian Mueller[1], Shyam Ramachandran[1]

[1]Genomic Medicine Unit, Sanofi, Waltham, MA,[2]Precision Medicine and Computational Biology, Sanofi, Cambridge, MA

Mutations in *GBA1*, the gene encoding glucocerebrosidase (GCase) are one of the most common risk factors for Parkinson's Disease (PD). GBA-PD patients are a genetically well-defined subpopulation of PD that are carriers of heterozygous mutations in their *GBA1* gene. GCase is a lysosomal enzyme that catalyzes the hydrolysis of glycolipids glucosylceramide (GL1) and glucosylsphingosphine (Lyso-GL1). Reduction or loss of GCase activity leads to the accumulation of substrates, disrupting lipid homeostasis. In GBA-PD, this further cascades into accumulation of Lewy bodies containing α-synuclein, promoting neuronal cell death. Here, we propose one-time intra-CSF administration of AAV-mediated expression of engineered *GBA1* for treatment of both GBA-PD and demonstrate efficacy data in both rodents and non-human primates (NHPs). To enable broad distribution of *GBA1* throughout the brain, we engineered the WT *GBA1* to make it readily secretable to facilitate cross-correction. We assessed subcellular localization and GCase activity of these variants *in vitro* and observed that both localization to lysosomes as well as activity for all variants was comparable to WT *GBA1*. Subsequently we introduced the variants packaged in AAV.GMU1 into mice through intra cerebroventricular (ICV) injections. Consistent with our *in silico* analyses, histology assays revealed robust secretion of our *GBA1* variants throughout the brain four weeks post AAV dosing. We also performed functional efficacy studies by treating mice and NHPs with CBE, a covalent inhibitor of GCase. Mice that received *GBA1* variants effectively reduced substrate accumulation induced by CBE. Based on our efficacy data in both rodents and NHPs with intra-CSF dosing, we nominated SS3-GBA1 as our lead *GBA1* variant. Our future studies include dose-range finding studies in mice and NHPs and establishing minimally efficacious dose in the clinic. Collectively, our data establish AAV-based treatment modality for GBA-PD patients.

1114. Development of Conduritol-β-Epoxide Induced Lipid Flux Model in Non-Human Primates

Swathi Ayloo[1], Jae Ryu[1], Shih-Ching Chou[1], Jie Bu[1], Bill McCarty[2], Lilu Guo[3], Mariana Gonclaves[3], Bradford Elmer[1], Martin Goulet[1], Christian Mueller[1], Shyam Ramachandran[1] , Emily Crosier[1]

[1]Genomic Medicine Unit, Sanofi, Waltham, MA,[2]DMPK, Sanofi, Cambridge, MA,[3]Precision Medicine and Computational Biology, Sanofi, Cambridge, MA

Mutations in *GBA1*, the gene encoding glucocerebrosidase (GCase) is one of the most common risk factors for Parkinson's disease (PD) with 7-10% of PD patients carrying a mutation in GBA1. GCase is a lysosomal enzyme that catalyzes the hydrolysis of glucosylceramide (GL1) and glucosylsphingosphine (Lyso-GL1). Reduction or loss of GCase enzyme activity leads to the accumulation of these glycolipids, disrupting lipid homeostasis. This further cascades into the accumulation of Lewy bodies promoting neuronal cell death. In addition, bi-allelic mutations in *GBA1* are causative for a rare lysosomal storage disorder, Gaucher Disease (GD), wherein macrophages in multiple tissues have lipids built up that cause enlarged liver, spleen, and muscle weakness. Thus, lipid accumulation is a hallmark feature of mutations in *GBA1*. Owing to the lack of good rodent models for GBA-PD as well as GD, researchers have previously established conduritol-β-epoxide (CBE) induced lipid flux model to mimic lipid accumulation

seen in disease. CBE is an irreversible covalent inhibitor of GCase activity. Extensive research has now demonstrated that administration of CBE induces a dose-dependent lipid accumulation both *in vitro* in various cell lines as well in as rodents. In order to evaluate efficacy and rank order our engineered *GBA1* gene therapy constructs in non-human primates (NHP), we set out to develop a CBE-induced lipid flux model in NHPs. Here, we designed and implemented a pilot study to investigate changes in lipid substrates upon CBE administration in NHPs. First, we determined the doses of CBE using allometric dose-scaling from rodents to NHPs. We administered three different doses of CBE to NHPs and examined tissues and fluids post CBE dosing. At all three doses tested, we observed a robust increase in Lyso-GL1 in plasma indicating efficient target engagement by CBE. In addition, we also observed a dose-dependent increase in Lyso-GL1 in peripheral tissues such as liver and DRG as well as in the central nervous system including brain (64 brain punches encompassing representative grey matter and white matter regions) and spinal cord. Consistent with CBE testing in other model organisms, we also observed a concomitant dose-dependent decrease in GCase activity in all tissues tested. Importantly, the majority of the deep brain regions such as caudate, putamen, globus pallidus, thalamus, and subthalamus demonstrated robust CBE dose-dependent lipid accumulation and corresponding decrease in GCase activity. Collectively, our results are the first to establish a CBE-induced lipid flux model in NHPs. Our work provides a platform for efficacy studies in not only our gene therapy programs but also approaches with other modalities targeting *GBA1*.

1115. SNCA Reduction for the Treatment of Synucleinopathies

Bradford Elmer[1], Erik Wischhof[1], Tiffany Chan[1], John Bladon[1], Ye He[1], Thanga Aishwarya Mahendran[1], Brenda Richards[1], Robert Jackson[1], Catherine O'Riordan[1], Martin Goulet[1], Christian Mueller[2], Shyam Ramachandran[3]

[1]Sanofi, Waltham, MA,[2]Sanofi Genzyme, Framingham, MA,[3]Sanofi, Winchester, MA

Long-term neuroprotective therapy is of considerable interest for the treatment of neurodegenerative synucleinopathies like Parkinson's disease or multiple system atrophy (MSA). These diseases are characterized by the accumulation of alpha-synuclein protein in the brain with associated neurodegeneration. Multiple lines of evidence strongly implicate alpha-synuclein as a causal factor in neurodegeneration, and reduction of alpha-synuclein has been well studied in preclinical models of disease. RNA interference (RNAi) mediated by artificial miRNAs has shown increasing promise as a highly specific therapeutic modality to reduce levels of target mRNA and protein. Adeno-associated viruses (AAV) are well-validated and constantly evolving gene delivery vectors with the ability to express therapeutic constructs for years in the brain. Therefore AAV-mediated RNAi represents a promising approach for the long-term reduction of *SNCA* and treatment of synucleinopathy. Artificial miRNAs (amiRNA) were designed to target *SNCA* mRNA. In vitro screening demonstrated robust target knockdown in human cells. Partial reduction of *SNCA* in differentiated human dopaminergic cells was neuroprotective against rotenone-induced mitochondrial dysfunction. *SNCA* reduction in vivo was evaluated via AAV-mediated delivery of a tool sequence (amiR-SNCA) to the brains of wild type mice, with target engagement maintained for up to 6 months without behavioral alteration. To assess the potential for partial reduction of *SNCA* to prevent the spread of pathologic forms of synuclein protein, proof-of-mechanism studies in the pre-formed fibril (PFF)-model of neurodegeneration were run in wild-type mice treated with AAV vectors expressing amiR-SNCA or control miRNA. Partial, 25-60%, reduction of endogenous *SNCA* mRNA resulted in near complete mitigation of the spread of pS129 alpha-synuclein pathology to the substantia nigra. Importantly, reduction of SNCA also prevented the loss of TH+ neurons in this region, even in those animals with modest *SNCA* reduction. To support clinical translation additional, human-specific, sequences were screened in vitro and in vivo via multiple expression formats to optimize target engagement. Several candidates exhibited desirable in vitro and in vivo performance, supporting the continued development of *SNCA* lowering therapeutics as a safe and efficacious approach for treating patients with synucleinopathies.

1116. Successful Targeted Delivery of an AAV Reporter via MRI-Guided Surgery to CNS Tissue; Feasibility, Tolerability, and Challenges

Erin L. Slosarek, Rick Haas, James R. Nelson, Michael Taschwer, Jay K. Herman, Brian E. McIntosh

Labcorp, Madison, WI

Recent advances in clinical approaches to delivering targeted gene and cell therapies to the brain have necessitated *in vivo* safety studies applying the same procedures used in the clinical studies. Neurodegeneration in the putamen as indicated in Parkinson's and Huntington's disease, or in the hippocampus, as indicated in Alzheimer's disease, requires precise treatment for gene replacement or disease reversibility. As an alternative to achieving central nervous system (CNS) exposure, administration into the cerebral spinal fluid results in a broader distribution of these therapies, but often includes systemic exposure. By targeting current surgical approaches to disease-specific parenchymal structures, one can minimize broader distribution and focus the therapy to the intended area. In this work, we demonstrated the feasibility, tolerability, and challenges of direct targeting of parenchymal structures using magnetic resonance imaging (MRI)-guided Clearpoint catheter-based infusion in nonhuman Primates (NHPs). A 1.5T MRI scanner in conjunction with Brainsight software® was used to scan the brain and plan the bilateral trajectories to the putamen and hippocampus. Post dose MRI confirmation using 2% Gadoteridol allowed for target confirmation. In addition, 2.6×10^{12} GC/dose site AAV-9-mCherry was used for biodistribution analysis at necropsy, 28 days post-dose. MRI scanning after infusion confirmed the correct dosing of the targeted structure. After surgery, animals were monitored for signs of neurological impairment, clinical abnormalities, and alterations in body weight. The surgical procedures were well tolerated with no neurological or clinical impairments. Histological and biodistribution analyses were used to determine tissue response to the dosing procedure and indicated successful expression of mCherry transgene in the target tissue. In general, mCherry expression was significantly greater in the target CNS tissue compared to the liver or dorsal root ganglia, which are typically locations of AAV-9 expression using alternative methods of CNS dosing. Together, these data offer a path forward when testing the delivery of cells or cell specific gene

therapies directly to the putamen or hippocampus and can provide a clinically relevant method for targeted brain delivery with accuracy in NHPs.

1117. GBA1-Linked Parkinson's Disease AAV Gene Therapy

Raquel Nunez Toldra, Alex Dimokov, Eliza Bujor, Silvia Podda, Parisa Rahimnashat, Tihomir Dodev, Su Kit Chew, Mario Buono, Nisha Nair Dastidar, Eileen McNeill, Henning Stennicke, Rose Sheridan

Freeline Therapeutics, Stevenage, United Kingdom

Parkinson's disease (PD) is a chronic, progressive, neurodegenerative disorder characterised by α-synuclein accumulation, Lewy Body formation and loss of dopaminergic neurones within the substantia nigra pars compacta (SNpc) which controls movement and coordination. Non-motor symptoms include cognitive decline, sleep disturbance and psychiatric symptoms. Mutations in *GBA1* are linked to Gaucher disease (GD), one of the most common lysosomal storage diseases. In this population, approximately 9.1% of patients are likely to develop PD before the age of 80, compared to 3-4% for the wider population[1]. Heterozygous *GBA1* GD mutation carriers and a number of non-Gaucher PD associated *GBA1* mutations share a similar level of risk of developing PD. A proportion (5-15%) of people with PD carry mutations in *GBA1* which encodes the lysosomal enzyme glucocerebrosidase (GCase), resulting in dysfunctional enzyme and lysosomes[1]. AAV-mediated gene therapy providing sustained endogenous levels of fully functional GCase may reduce the early and accelerated rate of disease progression observed for *GBA1*-linked PD. Our GCase variant 85 ($GCase_{var85}$), with two amino acid substitutions to the mature human GCase ($GCase_{WT}$), has been established as a more stable enzyme[2] with clinical benefit in GD and offers the potential for effective delivery to and improved distribution across the brain in comparison to $GCase_{WT}$. *In vitro* transduction of brain derived cell lines, including SH-SY5Y (neuroblastoma), demonstrated up to 10-fold or more GCase activity in cell supernatants with AAV9-$GCase_{var85}$ compared to AAV9-$GCase_{WT}$ across all cell types, reflecting the improved stability. Expression and distribution were evaluated in C57BL/6J male mice of 9 weeks of age, injected unilaterally into the right-hand caudate putamen with vehicle or AAV9-GFP, AAV9-$GCase_{WT}$ or AAV9-$GCase_{var85}$ at a dose of 1.3×10^{10} vg per mouse and analysed four weeks post dose. Whole brains were coronally cryosectioned at the striatal site of injection and the substantia nigra and sections were immunofluorescently labelled for GCase or GFP. The AAV9-GFP reporter demonstrated effective retrograde transport from the caudate putamen, with GFP widely distributed in axons of the substantia nigra. Qualitative immunofluorescent staining for GCase gave a more intense signal in numerous somata and neuropil of the injected right hemisphere and signal in the left hemisphere for AAV9-$GCase_{var85}$ compared to AAV9-$GCase_{WT}$, with a clear GCase signal in tyrosine hydrolase-positive somata in the substantia nigra pars compacta of animals from both groups. Biochemical analysis of GCase activity extracted from the right hemisphere striatum or substantia nigra of treated animals demonstrated approximately 10-fold higher activity for AAV9-$GCase_{var85}$ compared to AAV9-$GCase_{WT}$ in agreement with *in vitro* observations. In conclusion, direct brain injection of AAV9 constructs to the caudate putamen is effectively distributed to the target cells of the substantia nigra. The more stable $GCase_{var85}$ results in a 10-fold higher GCase exposure with broader distribution across the murine brain. [1] Smith, L.; Schapira, A.H.V. GBA Variants and Parkinson Disease: Mechanisms and Treatments. Cells 2022, 11, 1261. https://doi.org/10.3390/cells11081261; [2] Comper, F et al., Poster #41 Generation of β-Glucocerebrosidase variants with increased half-life in human plasma for liver directed AAV gene therapy aimed at the treatment of Gaucher disease type 1. WORLDSymposium 8-12 February, 2021

1118. Transcriptomic Analysis of GDNF Treatment in MPTP Rhesus Monkeys

Kaya Ekin Ceyhan[1], Alessandro La Ferlita[2], Krystof Bankiewicz[1], Lluis Samaranch[1]

[1]Neurological Surgery, The Ohio State University, Columbus, OH,[2]Cancer Biology and Genetics, The Ohio State University, Columbus, OH

Parkinson's disease (PD) is a debilitating neurological disorder affecting millions globally. Characterized by the loss of dopaminergic neurons in the brain, particularly in the substantia nigra, PD leads to significant motor and cognitive impairments. Currently, there are no disease-modifying therapies approved that halt this disease's progression. Hence, multiple efforts to develop ultimate treatments for PD are currently ongoing. Today, certain therapeutic advancements in gene therapy exhibit significant promise in mitigating the manifestations of nigrostriatal degeneration by addressing and rescuing dopamine levels. Notably, the administration of glial cell-line-derived neurotrophic factor (GDNF) holds particular significance in these efforts, by which this potent neurotrophic factor demonstrates a selective enhancement of the survival of neurons in the dopaminergic nigrostriatal pathway. However, the molecular characterization of this treatment is poorly understood. To better characterize this treatment, seven young *Rhesus monkeys* were MPTP over-lesioned and divided into two groups according to their survival time. Group 1 received a striatal Convection Enhanced Delivery (CED) of either AAV2-GDNF (n=2) or PBS (n=1) and was followed for one month. Group 2 received a striatal CED infusion of either AAV2-GDNF (n=2) or PBS (n=2) and was followed for six months. Agilent Microarrays were performed on snap-frozen putamen tissue samples from all the animals. Transcriptomic analysis demonstrated that GDNF treatment resulted in several differentially expressed genes across each time point involving cell-to-cell signaling, cell proliferation, or cell growth. Bioinformatics analysis using Ingenuity Pathway Analysis demonstrated several significant pathways among the common shared upregulated differentially expressed genes based on MPTP doses and the different observational time points. In summary, this study provides clearer insight into the biological mechanisms behind GDNF's therapeutic effects in an MPTP-based PD animal model and highlights further areas of research.

1119. Neuronal-Type Specific *SNCA*-Targeted Epigenome Therapy for Precision Medicine in Synucleinopathies

Bernadette O'Donovan[1], Zhiguo Sun[2], Boris Kantor[3], Ornit Chiba-Falek[1]

[1]Neurology, Duke University, Durham, NC,[2]CLAIRIgene, Durham, NC,[3]Neurobiology, Duke University, Durham, NC

Synucleinopathies are neurodegenerative disorders that share a pathological lesion of intracellular protein aggregates largely composed of α-synuclein protein, known as Lewy Bodies (LB) and Lewy-neurites. Elevated SNCA levels are causative in synucleinopathy pathogenesis, particularly Parkinson's disease (PD) and dementia with Lewy bodies (DLBs), while normal physiological SNCA levels are essential to maintain neuronal function. While PD and DLB share some clinical and pathological similarities, each disease presents distinct characteristics including the primary affected brain region and neuronal type, *i.e.* dopaminergic vs cholinergic, respectively. We developed neuronal-type specific *SNCA*-targeted epigenome therapies precisely for PD and DLB. The system is based on *an all-in-one* lentiviral (LV) vector comprises of CRISPR/d*Sa*Cas9 and gRNA targeted at *SNCA*-intron1 fused with a synthetic repressor molecule of KRAB/MeCp2(TRD). To achieve neuronal-type specificity for dopaminergic and cholinergic neurons, the system was driven by TH and ChAT promoters, respectively. Transduction into human induced pluripotent stem cells (hiPSC)-derived dopaminergic and cholinergic neurons from a patient with the *SNCA*-triplication resulted in efficient and neuronal-type specific downregulation of *SNCA*-mRNA and protein. Furthermore, the reduction in *SNCA* levels mediated by the LV/d*Sa*Cas9-repressor vector rescued disease-related cellular-phenotypes including, Ser129-phophorylated α-synuclein, neuronal viability, and mitochondrial dysfunction. Next, we conducted *in vivo* validation studies using a PD-mouse model induced by an AAV/A53T-human (h)SNCA vector that contains the mutated human-SNCA coding sequence fused with its native promoter/intron1 region. We performed bilateral stereotactic injection of the AAV/A53T-hSNCA vector into the mouse substantia nigra (SN), the right SN was co-injected with the therapeutic LV/dCas9-repressor and the left SN was co-injected with the control vector (LV/dCas9 no repressor). Analysis of 8 mice demonstrated a significant reduction (33%) in hSNCA protein in the right SN. Pathological examinations showed a robust reduction in Ser129-phosphorylated SNCA (53%), the surrogate of α-synuclein aggregates. In addition, we observed significantly higher expression of tyrosine hydroxylase in the right SN and striatum (1.24- and 1.73- fold higher, respectively), suggesting a greater retention of dopaminergic neurons. We also collected safety measures. Monitoring daily behaviors showed no abnormalities in well-being criterions and no weight loss. Additionally, safety measures demonstrated no issues for blood counts, serum chemistry and liver histology. Biodistribution evaluation following unilateral injection of low and high viral-vector doses showed no distribution of the viral DNA in cortex, liver, or the uninjected side of the SN. In conclusion, we established a novel neuronal-type specific *SNCA*-targeted epigenome therapy and provided *in vitro* and *in vivo proof-of-concept* using human based and animal disease models. Our unprecedented therapeutics strategy effectively fine-tunes the reduction of SNCA expression in neuronal-type precision, sufficient for reversing disease-associated perturbations. Our results demonstrate the therapeutic potential of our technology for PD and DLB and provide the foundation to advance towards IND-enablement and clinical trials.

1120. SNCA Gene Repression Mediated by Zinc Finger Repressors (ZFRs) as a Therapeutic Approach for Parkinson's Disease

Andrew Young, Kenneth Kim, Jill Cifrese, Asa Hatami, Hoang-Oanh Nguyen, Qi Yu, Kimberly Marlen, David Paschon, Annemarie Ledeboer, Stephen Lam, Sarah Hinkley, Alicia Goodwin, Alex M. Ward, Kathleen Meyer, Bryan Zeitler, Amy M. Pooler

Sangamo Therapeutics, Inc., Richmond, CA

Molecular and genetic evidence implicate alpha-synuclein (*SNCA*) as a key mediator of Parkinson's disease (PD) pathogenesis; directly contributing to neuron loss. Given this, reducing alpha-synuclein expression in neurons has the potential to halt or slow PD progression. Zinc finger proteins (ZFs) are DNA-binding proteins that can be engineered to specifically bind any genomic sequence. When fused to transcriptional regulatory domain repressors (ZFRs), they can reduce the expression level of a targeted gene. We engineered ZFRs with human alpha-synuclein repression activity for use as potential therapeutics for PD. In a proof-of-concept study, we designed a library containing 100s of ZFRs targeting human sequences near the *SNCA* transcriptional start site. The library was initially screened in human and rodent neuronal cells lines. ZFRs were then packaged into AAV and screened in human iPSC-derived GABAergic and mouse primary cortical neurons *in vitro*. We identified ZFRs that repressed *SNCA* mRNA by more than 50% over a wide dose range with minimal detectable off-target activity as measured by global transcriptomics analyses. ZFR AAVs were identified *in vivo*, demonstrating successful repression of mutant *SNCA* in key brain regions of a humanized PAC SNCA disease mouse model. ZFR AAV repressed human *SNCA* transcript expression *in vivo* as detected in both bulk tissue from the thalamus and midbrain, as well as within individual, tyrosine hydroxylase+ neurons in the midbrain. The data presented here support continued development of *SNCA*-targeting ZFRs as a therapeutic approach for the potential treatment of Parkinson's disease.

1121. UBE3A Gene Activation Mediated by Zinc Finger Activators (ZFAs) as a Therapeutic Approach for Angelman Syndrome

Andrew Young, Julie Qiao, Daniel Chung, Siavash Fazel Darbandi, Esmeralda Ponce, Jason Eshleman, Patrick Dunn, Bob Radeke, Jisoo Lee, Sarah Hinkley, Gregory Davis, Bryan Zeitler, Amy M. Pooler

Sangamo Therapeutics, Inc., Richmond, CA

Angelman syndrome (AS) is a complex neurodevelopmental disorder often diagnosed in early childhood that primarily affects the central nervous system (CNS). Hallmark symptoms of AS include intellectual disability, severe speech impairment, developmental delays, movement issues and seizures. Although AS patients have a normal life expectancy, they require lifelong care and currently lack any disease-modifying treatment options. The genetic cause of AS is complex and involves the pathological loss of expression from a single allele of the *UBE3A* gene, coupled with the typical epigenetic silencing of the second *UBE3A* allele specifically in neurons, leading to a complete loss of *UBE3A* expression in neurons of the CNS. In addition, there are multiple UBE3A protein isoforms encoded by the human *UBE3A* gene, which are believed to have

distinct intracellular localization and functionality. Given this, there is a significant challenge with restoring physiological levels of UBE3A to neurons through standard gene therapy overexpression strategies. One approach that has the potential to restore UBE3A in neurons is targeting the healthy, but epigenetically silenced, *UBE3A* allele in order to "unsilence" its expression and restore physiological levels of UBE3A. Indeed, recent clinical trials using antisense oligonucleotides designed for this purpose have shown positive signs of therapeutic benefit in AS patients. To develop a one-time, neuron specific, disease-modifying approach we utilized our proprietary platform to design zinc finger proteins targeted to the endogenous *UBE3A* promoter and fused them with a novel transcriptional activator domain (ZFAs) to drive transcriptional upregulation of the healthy *UBE3A* allele. In a proof-of-concept study, we designed a library containing 100s of ZFAs targeting human sequences near the *UBE3A* transcriptional start site and selected those showing the highest fold change in neuronal *UBE3A* expression for packaging into AAV vector. Given that our approach is not designed to be allele specific, we demonstrated the utility of AAV ZFAs using human neurons derived from AS patients containing a pathological deletion in the active *UBE3A* allele. Under these conditions, any UBE3A expression would necessarily be from the healthy, epigenetically silenced allele. In patient-derived neurons selected AAV ZFAs increased both *UBE3A* transcript and UBE3A protein levels to meet or exceed those seen in WT neurons. Using surrogate reagents designed to target the endogenous Ube3a promoter regions in mice, we saw similar levels of AAV ZFA mediated *Ube3A* upregulation in primary neurons sourced from a disease mouse model of AS. Taken together these findings support continued development of ZFAs for the treatment of Angelman syndrome.

1122. AAV-Mediated APOE4 Gene Silencing to Treat Homozygous APOE4-Associated Alzheimer's Disease

Kalpita R. Karan, Mara R. Kebret, Neil R. Hackett, Ronald G. Crystal

Genetic Medicine, Weill Cornell Medical College, New York, NY

The homozygous APOE4 genotype is the major risk factor for the development of early Alzheimer's disease. Genetic studies in mouse models of human APOE4 dependent pathology have established that reduction of APOE4 expression can rescue the phenotype. There have been attempts to therapeutically suppress central nervous system (CNS) APOE4 in murine models using anti-sense oligomers or small interfering RNAs, but these approaches require repetitive CNS administration due to the short half-life of these drugs. We hypothesized that APOE4 could be persistently suppressed in the CNS of APOE4 homozygotes using adeno-associated virus (AAV) expression of microRNAs (miRNA) designed to hybridize to and suppress human APOE mRNA. To accomplish this, we conducted an initial screening of human APOE suppression by 9 different miRNAs following transfection in 293 and Huh7 cells. Optimal suppression in both cell types was obtained with miRNA2A (targeting coding region nt330-351, 66% suppression in 293 cells, 69% suppression in Huh cells) and miRNA N4 (3' untranslated region nt1142-1162, 57% suppression in 293, 82% suppression in HuH). miRNA expression cassettes were designed for delivery by AAV gene transfer vectors with two copies each of two different miRNAs (mir2A and mirN4) co-expressed from the CAG promoter with a mCherry reporter transgene. A neurotropic AAVrh.10 variant with an engineered capsid (AAV.S2) was used to deliver the suppression cassette to the hippocampus of TRE4 mice (murine ApoE knockout, human APOE4 knockin at the murine apoE locus). Two weeks after intra-hippocampus injection into TRE4 mice, 3 mm coronal tissue slices were collected. Regional expression of miRNA at the injection site was demonstrated both by direct assessment of miRNA level relative to an endogenous reference and by assessing mCherry level as a proxy. The AAV.S2 engineered capsid provided 2.31 ± 0.37-fold higher expression of miRNA over that provided by wildtype rh.10 ($p<0.05$). In the targeted region, a single intra-hippocampus administration suppressed hippocampal APOE4 mRNA levels by 76.5 ± 3.9% compared to 41.3 ± 3.3% with the same cassette delivered by the wildtype rh.10 capsid ($p<0.0001$). We conclude that an expression cassette with 2 different miRNAs targeting APOE4 and delivered by engineered AAV capsid will generate highly significant suppression of CNS APOE4. Since APOE4 heterozygote humans have lower risk of Alzheimer's disease than APOE4 homozygotes, this level of APOE4 suppression is potentially therapeutic.

1123. Enhancing Accessibility to Cell and Gene Therapy for Rare Diseases

Cecilia Marinova[1], Miriam Kolnikova[2], Eva Vlkova[3], Veronika Sladkova[4], Martina Losova[1], Sandra Skvarlova[4], Monika Horackova[5], Magdalena Netukova[6], Pavlina Skalicka[7]

[1]Medasol sro, Praha, Czech Republic,[2]Department of Pediatric Neurology, Comenius University in Bratislva, Faculty of Medicine, NUDCH, Bratislava, Slovakia,[3]Department of Ophthalmology, 3Faculty Hospital Brno, Brno, Czech Republic,[4]Medasol sro, Prague, Czech Republic,[5]Faculty Hospital Brno, Brno, Czech Republic,[6]Faculty Hospital of Královské Vinohrady, Praha, Czech Republic,[7]General University Hospital in Prague, Praha, Czech Republic

Introduction: In rare diseases for which cell or gene (C&G) therapy is approved, according to available data, only 30% of patients are diagnosed, and only half of them receive treatment. While the awareness of many rare disorders is usually high, the awareness about the approved treatments and their accessibility is low, with few diagnosed patients spread around the country. Another limitation of innovative therapies as a standard treatment is the administrative and logistical burden associated with the evaluation and management of each patient for whom C&G therapy (CGT) is indicated. Since a significant number of CGT is being developed in the neuro-muscular area, we wanted to assess whether we can learn from previous implementations of CGT and evaluate the role of processes in accelerating the access. Methods: The administrative processes for 6 different gene therapies in 20 diagnostic and treatment centers in Europe, from diagnosis to treatment, were mapped and evaluated. A total of 52 different processes were required from diagnosis to local treatment or cross-border treatment. Results: 133 patients were evaluated, and 29 patients received approved C&G therapy, since it has strict eligibility criteria. Between 6 and 18 months elapsed between diagnosis and initiation of therapy. A total of 52 different processes were required from diagnosis to initiation of therapy including management of cross-border treatment; 29 logistic processes were specific to CGT. 21 processes (40%) required more than 100 additional hours to establish a treatment center, 31 processes

(60%) related to the cross-border treatment (diagnosis assessment, treatment proposal, review, and implementation). Conclusions: A specific aspect to be recognized in the case of CGT is the additional procedures and administration that all stakeholders have to take care of, which limits scaling up if it is not effective.The process is complex and requires significant planning and resources. CGT is realized only in qualified centers and requires medical and treatment coordination patient transfer, often across borders. We observed that it significantly increases already high administrative and procedure demands on treatment centers as well as on diagnostic centers mostly medical expert team.Preparing documentation, and arranging additional tests and logistics require considerable time and a dedicated coordination team to contact stakeholders, from patients to payers. In cases where a diagnostic or referral center does not have its own CGT team, an experienced external team, like Medasol, can handle the coordination. The only way to successfully treat patients with CGT is by managing the CGT processes and administrative burden associated with it. Once a patient is diagnosed and eligible for CGT, the effective management of processes permits a higher number of patients that can access CGT.

1124. Patient Stratification Using iPSC-Derived Organoids as a Pre-Clinical Drug Testing Platform in Alzheimer's Disease

Cristina Zivko, Ram Sagar, Ariadni Xydia, Constantine G. Lyketsos, Vasiliki Machairaki

Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD

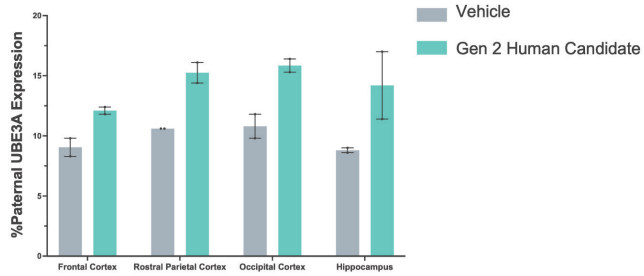
By 2050 the number of Alzheimer's Disease (AD) patients is projected to exceed 150 million worldwide. AD is an incurable, insufficiently understood, and devastating neurodegenerative disease, with high patient heterogeneity in terms of progression, clinical manifestation (including neuropsychiatric symptoms, NPS) and, importantly, responsiveness to treatment options.[1] In the last 20 years, 98% of clinical trials for AD have failed, highlighting the urgent need to drastically change pre-clinical research to develop better predictors of drug safety and effectiveness.[2] This is where our precision medicine approach of organoid in vitro models can be a game changer. Human peripheral blood mononuclear cells from healthy volunteers (n=6) and AD patient with (n=12) and without NPS (n=12) were reprogrammed into induced pluripotent stem cells (iPSCs)[3] and subsequently differentiated into hindbrain organoids, containing serotonin (5-HT) producing neurons. Extensive characterization and validation experiments were conducted to assess the quality of the iPSCs and of the 5-HT-organoids. These include karyotyping, fluorescent microscopy and flow cytometry to reveal the presence of pluripotency (OCT4, NANOG, TRA-1-60) and neuronal markers (TUJ1, TPH2, 5-HT), real time qPCR, ELISA measurement of 5-HT levels, size and circularity over 6 weeks of differentiation, for which we developed an in-house algorithm in Python. The 5-HT-organoids were treated with a selective serotonin reuptake inhibitor acutely (1 h) and for 1 week, upon which we could stratify the 30 individuals according to drug responsiveness. Shotgun proteomics analysis was performed on the treated and non-treated 5-HT-organoids from all 30 individuals by LC/MS to reveal distinctive relative abundance profiles. Experiments were run in biologically independent triplicates for 6 cell lines (3 healthy, 3 AD). The results from that training set were used to classify analytical results from the other 24 lines for AD biomarker discovery, as well as for drug responsiveness and effect. Highly reproducible, people-specific 5-HT-organoids were thus used to study inter-patient phenotypical variability and drug response diversity in a powerful, novel pre-clinical tool. References: [1] J Alzheimers Dis. 2022; 87(1):83-100. doi: 10.3233/JAD-215699. [2] J. Alzheimers Assoc. 2011; 7(5):532-539. doi: 10.1016/j.jalz.2011.05.2410 [3] Cells. 2023; 12(15):1990. doi: 10.3390/cells12151990.

1125. A Vectorized miRNA-Based Approach to Unsilence UBE3A in Angelman Syndrome

Sirika Pillay, Veda Tatavarty, Winnie Lin, Peter Neumann, Pingping Jia, Charles Cao, Sawyer Randles, Maria Abbasi, Szu-Ying Chen, Aurora Horta-Martinez, Cody McDermott, Ron Chen, Greg Lucey, Liz Hallam, Nathan Moerke, Sheila Sears, Serena Liu, Alex Weber, David Rodriguez, Amy Kim, Xiang Zhu, Adam Miller, John McLaughlin, Raghu Hosur, Archana Belle, Suresh Poda, Stephanie Tagliatela

Encoded Therapeutics, South San Francisco, CA

Rationale and Methods: Angelman syndrome (AS) is a neurodevelopmental disorder caused by the loss of function of the maternally inherited allele of ubiquitin-protein ligase E3A (*UBE3A*). Current treatment approaches are symptomatic as no approved disease-modifying therapies exist. Targeted knockdown of the long non-coding RNA, UBE3A-ATS, has the potential to upregulate paternal UBE3A expression in neurons and provide therapeutic benefit. We investigated an AAV-based vectorized miRNA approach targeting UBE3A-ATS to determine the therapeutic value of a microRNA (miRNA) knockdown strategy. **Results:** A miRNA tool candidate targeting the mouse UBE3A-ATS was capable of upregulating Ube3a RNA (11%) and protein (17%) in the brain within the null CNS background of AS mice. This candidate also rescued multiple phenotypes including body weight, hypoactivity and seizure sensitivity. Demonstrating further proof-of-concept, a human miRNA candidate targeting UBE3A-ATS knockdown was tested in non-human primates (NHPs), and showed UBE3A RNA and protein upregulation following intraparenchymal dosing. Subsequent optimization yielded multiple human candidates with more than a 100-fold increase in potency in human iPSC-derived neurons and showed promising off-target profiles *in vitro*. Finally, the top optimized human candidate resulted in paternal UBE3A RNA upregulation in the disease relevant cortex and hippocampus of NHPs following intracerebroventricular (ICV) dosing, a clinically relevant route of administration. **Conclusion:** These data establish the first *in vivo* proof-of-concept for miRNA-based UBE3A upregulation, identify promising human therapeutic candidates, and support the potential of a one-time, AAV-based approach for disease modification in the treatment of AS. **Figure 1.** Paternal UBE3A RNA upregulation in the cortex and hippocampus of wild-type non-human primates 60 days following ICV-dosing of miRNA treatment.



1126. Whole CNS Human Tau Knockdown for the Potential Treatment of Alzheimer's Disease and Other Tauopathies

Bryan J. Zeitler[1], Kimberly Marlen[1], David Ojala[1], Annemarie Ledeboer[1], Qi Yu[1], Yonghua Pan[1], Giulia Cisbani[2], Finn Peters[2], Chiara Melis[2], Matthew R. Tiffany[1], Sarah Mueller[1], Kenneth Kennard[1], Patrick Dunn[1], Hoang-Oanh Nguyen[1], Hung Tran[1], Kathleen Meyer[1], Amy Pooler[1]

[1]Sangamo Therapeutics, Inc., Richmond, CA,[2]Evotec SE, Hamburg, Germany

Alzheimer's disease (AD) affects millions of people and is currently the seventh leading cause of death in the United States. The neuronal accumulation of misfolded, neurotoxic species of Microtubule Associated Protein Tau (MAPT) closely tracks with neurodegeneration, disease progression, and clinical symptoms in AD. Reducing *MAPT* expression is well-tolerated and efficacious in tauopathy animal models, and a recent Phase 1/2 study in early AD evaluating repeated intrathecal injections of an *MAPT*-targeted ASO reported potential reversal of tau tangles in the brain and improvements in clinical symptoms. We are developing an IV-administered, epigenetic regulation approach to reduce tau expression using an engineered zinc finger transcriptional repressor (ZFR). Potential advantages include a single, non-invasive administration, the ability to selectively target and lower tau in neurons, and the use of an engineered BBB-penetrant AAV capsid that affords access to the whole brain, which could enable a potential universal therapy for all tauopathies, including AD. In early proof of concept studies, we previously demonstrated that ZFRs targeting mouse *Mapt* stably reduced tau in the brain. Here, we report the development of a lead ZFR that targets the human *MAPT* locus and achieves >95% knockdown of tau mRNA and protein in human iPSC-derived neurons with no detectable off-targets. The ZFR represses human and nonhuman primate *MAPT* equally due to full conservation of its target sequence, but is inactive in mouse neurons due to a single mismatch between the primate and mouse sequences. We tested the lead ZFR under the control of the Synapsin1 promoter in humanized htau mice using multiple routes of administration. Stereotaxic intrahippocampal AAV9 ZFR injections resulted in stable, dose-dependent mRNA and protein repression of up to 90% at 3 and 6 months after delivery. Single-cell analysis using multiplexed ISH and IHC revealed potent tau repression in neurons was achieved over >100x range of ZFR expression levels. In addition, htau mice dosed with IV AAV.PHP.B ZF-R at 10 months of age showed stable knockdown of tau mRNA and protein across the brain when examined at 18 months. Tau mRNA was reduced >90% in ZFR positive cells. Phosphorylated tau (ptau) at S199 and S202 was also reduced at the bulk and single-cell level, indicating ZFRs can remove a key pathogenic tau species from the brain. Evaluation of the lead ZFR in nonhuman primates using an IV-administered AAV capsid with the ability to cross the BBB is currently ongoing, with results expected shortly. Collectively these data support the development of a ZFR-based genomic medicine for the treatment of AD and other tauopathies.

1127. Focused Ultrasound-Mediated Blood Brain Barrier Opening for Targeted Delivery of Adeno-Associated Virus to the Mouse Striatum

Colleen Curley[1], Sambuddha Basu[1], Brittany Wicks[1], Tiffany Smith[1], Eric Kostuk[1], Brian Kelly[1], Andrew Nelson[1], Drew Peterson[1], Graciela Rivera-Pena[1], Charlie Li[1], Ashley Carter[1], Mohamad Nayal[1], Renee Gentzel[1], Joan Wicks[1], Lihua Shi[1], Matthew Wier[1], Junhua Wu[1], Alec Batts[2], Elisa Konofagou[2], Esteban A. Engel[1], Elizabeth Ramsburg[1]

[1]Spark Therapeutics, Philadelphia, PA,[2]Columbia University, New York, NY

Introduction: Focused ultrasound applied in the presence of intravenously administered microbubbles (FUS + MB) is a method for noninvasive and reversible blood brain barrier opening (BBBO) in targeted brain regions. Here we utilize FUS + MB BBBO applied to the mouse striatum to evaluate safety and efficacy of AAV9 delivery with this technique. **Methods:** AAV9 ($1e^{11}$ or $5e^{11}$ vgs/mouse) and microbubbles ($8e^{8}$ MBs/mL) were injected into mouse tail veins and focused ultrasound was applied bilaterally to striata at either a low 450 kPa or high 650 kPa peak-negative pressure (PNP) over a sonication duration of 2 min. BBBO was confirmed using contrast-enhanced T_1-weighted MR imaging following treatment. T_2-weighted MR imaging was performed on a subset of animals to assess safety. After two months, brain and peripheral organs were collected to assess vector biodistribution. Histopathological analysis was performed on a portion of brain samples from FUS-treated groups. We also plan to assess efficacy of this approach in a Huntington's disease animal model. **Results:** FUS+ MB BBBO yielded gadolinium extravasation into targeted brain tissue, confirmed via quantification of contrast enhancement on T_1-weighted MRI. Additionally, this approach resulted in increased biodistribution of AAV9 in targeted brain tissue compared to animals receiving vector without FUS treatment. AAV9 also exhibited biodistribution in peripheral organs due to systemic administration of the vector. Assessment of additional safety and efficacy readouts are currently in progress. **Conclusion:** Consistent with previous reports, focused ultrasound and microbubble treatment can open the blood brain barrier and enhance AAV9 delivery into the targeted brain region. Here we investigate the effect of vector dose and FUS pressure on AAV9 biodistribution in the brain and peripheral organs. These results inform ongoing work assessing therapeutic potential of focused ultrasound-mediated AAV delivery in neurodegenerative disease models.

1128. AAV-RNAi Mediated Reduction of *MAPT* for the Treatment of Alzheimer's Disease

Jessica Hogestyn[1], Yao Chen[2], Jie Bu[1], Erik Wischhof[1], Jordan Bresse[1], Tiffany Chan[1], Robert Jackson[1], Brenda Richards[1], Catherine O'Riordan[1], Martin Goulet[1], Christian Mueller[1], Shyam Ramachandran[1], Bradford Elmer[1]

[1]Genomic Medicine Unit, Sanofi, Waltham, MA,[2]Precision Medicine and Computational Biology, Sanofi, Cambridge, MA

The microtubule-associated protein tau (*MAPT*) plays a pathogenic role in a spectrum of devastating and incurable neurodegenerative diseases known as tauopathies. Of these, Alzheimer's disease (AD) is the most prevalent, with 6.7 million patients in the United States alone. AD is characterized by neuronal inclusions of phosphorylated tau, extracellular amyloid beta (Aβ) plaques, cortical atrophy, and progressive cognitive and executive dysfunction. Although recent advancements have been made to reduce Aβ pathology, the efficacy of these treatments are limited. Given that tau aggregates correlate more strongly with AD symptoms, targeting tau pathology can address a significant unmet need in these patients. RNA interference (RNAi) has shown increasing promise as a therapeutic modality to reduce levels of target mRNA and protein. Adeno-associated viruses (AAVs) are well-validated gene delivery vectors with the ability to express therapeutic constructs for years in the brain. Here, we describe the use of an AAV-RNAi vector for total *MAPT* lowering for the treatment of AD. *In silico* design tools and *in vitro* screening were used to identify artificial miRNA (amiRNA) sequences with low off-targeting potential that reduce expression of human *MAPT*. Candidates were cloned into AAV vectors to evaluate *MAPT* knockdown in the Tau22 mouse model, which overexpresses aggregate-prone mutant human *MAPT*. Not only did the lead amiRNA candidate Tau01 significantly reduce human *MAPT* mRNA and tau protein expression, but it also reduced pathological forms of phosphorylated tau in the cortex, hindbrain, and spinal cord. Levels of the axonal damage biomarker neurofilament light were also significantly reduced in cerebrospinal fluid and plasma, suggesting that total tau reduction by RNAi can significantly impact neurodegeneration. We further evaluated off-target mRNA reduction in human cells using paired RNA and CLIP sequencing to identify mRNA sequences that were significantly downregulated in Tau01-treated cells with evidence of physical Tau01 targeting within the RNA-induced silencing complex. In a dose-response validation experiment, we identified doses that maintained clinically-relevant *MAPT* reduction without significantly impacting off-target genes. We also conducted a pharmacology study in nonhuman primates (NHPs), using novel capsid AAV.GMU1 and an intrathecal lumbar catheter threaded to cisterna magna to deliver Tau01. While significant *MAPT* reduction was not achieved in bulk tissue RT-dPCR, we did demonstrate proof-of-concept for *MAPT* lowering in transduced neurons by dual RNAscope/Tau01 miRscope. Given the low number of transduced cells observed in this assay, we are now evaluating novel AAV capsids for improved biodistribution in the NHP brain. Improved AAV delivery, combined with the robust performance of the Tau01 amiRNA observed in the Tau22 mouse model, may support AAV-RNAi as a transformative treatment for patients with devastating neurodegenerative tauopathies.

1129. Preclinical Studies in Canines and Nonhuman Primates for IND- and IDE-Enabling Projects

Zhiming Zhang[1], Francois Pomerleau[1], Pete Huettl[1], Jorge E. Quintero[2], Richard Grondin[1], Greg Gerhardt[1]

[1]Neuroscience, University of Kentucky, Lexington, KY,[2]Neurosurgery, University of Kentucky, Lexington, KY

We have established a university-based research organization to facilitate a broad range of studies related to neurology and neurosurgery, from initial discovery to GLP-compliant studies. Our research group has gained considerable experience in the use of canine and non-human primate models for IND- and IDE-enabling studies using protocol-driven procedures. Both non-human primates and canines are excellent large animal models for drug and device development. Canines have been found to be a cost-effective alternative to non-human primates in preclinical research. Our laboratory has developed protocols that use MRI-guided stereotactic procedures to precisely implant devices into the CNS to deliver investigational biologics and drugs directly to the brain parenchyma or lateral ventricles and to investigate the safety and tolerability of intracranial ports and catheters. Both non-human primates and canines exhibit complex behaviors that allow for precise neurological examinations to determine the safety and tolerability of biologics, drugs and devices. In this presentation, we will discuss several examples of IND- and IDE-enabling studies related to Parkinson's disease, epilepsy, Huntington's disease, neuro-oncology and stroke that we have carried out through numerous sponsors at the academic and industrial level.

1130. Ependyma-Derived ApoE2 Protects Against Alzheimer's Disease Phenotypes in the Presence of ApoE4

Rosemary J. Jackson[1,2], Ellie Carrell[3], Megan S. Keiser[3], Jonah C. Meltzer[1,2], Dustin P. Fykstra[1,2], Steven E. Dierksmeier[1,2,4], Soroush Hajizadeh[5,6,7], Johannes Kreuzer[5,8], Robert Morris[5], Alexandra Melloni[1], Tsuneo Nakajima[1,2], Luis Tecedor[9], Paul T. Ranum[9], Yonghong Chen[9], David M. Holtzman[10], Wilhelm Haas[5,8], Beverly L. Davidson[3,11], Bradley T. Hyman[1,2]

[1]Alzheimer Research Unit, The Massachusetts General Hospital Institute for Neurodegenerative Disease, Charlestown, MA,[2]Department of Neurology, Harvard Medical School, Boston, MA,[3]The Raymond G. Perelman Center for Cellular and Molecular Therapeutics, Children's Hospital of Philadelphia, Philadelphia, PA,[4]Medical Sciences Division, University of Oxford, Oxford, United Kingdom,[5]Krantz Family Center for Cancer Research, Massachusetts General Hospital, Boston, MA,[6]Broad Institute of MIT and Harvard, Cambridge, MA,[7]Institute of Molecular Biosciences, University of Graz, Graz, Austria,[8]Department of Medicine, Harvard Medical School, Boston, MA,[9]Children's Hospital of Philadelphia, Philadelphia, PA,[10]Department of Neurology, Hope Center for Neurological Disorders, Knight Alzheimer's Disease Research, Washington University, St. Louis, MO,[11]Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Dementia and more specifically Alzheimer's Disease (AD) currently pose a global health crisis with more than 3 million cases of AD in

the US alone. Inheritance of the ε4 allele of apolipoprotein E (ApoE) is the strongest genetic risk factor associated with the sporadic form of AD, whereas the rare ApoE ε2 allele has the opposite effect. However, the mechanisms whereby ApoE confers risk and protection remain uncertain. ApoEε4 genotype has been associated with worsening plaque deposition, increased speed of cognitive decline and neurodegeneration as well as increased neuroinflammatory phenotype in microglia. We have previously shown that the introduction of ApoE4 or ApoE2 into the ependymal lining via AAV in amyloid plaque-bearing transgenic mice (expressing wild type murine apoE) exacerbated or reduced amyloid plaque burden, respectively (Hudry, et al. 2013 Sci Trans Med). Here a novel, capsid-modified AAV with restricted ependyma expression was used to evaluate ApoE2 replacement in plaque-bearing mice expressing human ApoE4 (mouse *apoe* negative; APP/PS1/ApoE4). Following intracerebroventricular (ICV) delivery, ependyma-derived ApoE2 expression accounted for ~20-30% of total ApoE protein in the CSF, a level sufficient to reduce the size and density of cortical amyloid plaques, the concentration of oligomeric Aβ in brains and notably, levels of activated microglia near plaques. Finally, we found that ApoE2 can prevent or lessen synapse loss near Aβ plaques. Together these data support the therapeutic efficacy of ependyma-derived ApoE2 in ApoE4 carriers by improving the classical lesions of AD (eg plaque deposition and neurodegeneration) as well as the increased neuroinflammation and synapse loss observed in sporadic AD. Studies to address translatability of our approach in non-human primates are ongoing.

1131. Enhancement of a Vectorized Tau Reduction Therapy with Low Intensity Focused Ultrasound Delivery

Irvin T. Garza[1], Sydni K. Holmes[1], Ibrahim Youssef[2], Krishanna Knight[1], Rajiv Chopra[2], Bhavya R. Shah[2], Rachel M. Bailey[3]

[1]UT Southwestern Medical Center, Dallas, TX,[2]Department of Radiology, UT Southwestern Medical Center, Dallas, TX,[3]Center for Alzheimers' and Neurodegenerative Diseases, UT Southwestern Medical Center, Dallas, TX

Microtubule associated protein tau is a protein that can misfold and accumulates in the brain driving neurodegeneration, e.g. Alzheimers' Disease. There are no approved treatments to reduce tau aggregation, but an antisense oligonucleotide therapy targeting tau in AD is currently in clinical trials suggesting that reducing pathological tau is well tolerated. To improve on this approach, we developed a recombinant adeno-associated virus (rAAV) encoding an artificial microRNA (hTaui) that selectively targets tau mRNA transcripts for degradation. Previously, we showed the therapeutic benefits of our vectorized tau gene therapy in a mouse model of tauopathy (PS19). PS19 mice express P301S mutant human tau, which results in tau aggregation, neurodegeneration, and early death. We found that intracisterna magna (ICM) administration of our AAV9/hTaui gene therapy significantly extended survival, improved quality of life, reduced human tau mRNA and protein levels, and reduced pathogenic tau formation. In agreement with the reported distribution of ICM injected AAV9, we found that vector distribution and tau reduction was highest in the hindbrain and low in the hippocampus and frontal cortex. To improve delivery of our gene therapy, we propose to use transcranial low-intensity focused ultrasound (FUS) to enhance delivery of AAV9/hTaui to the brain. Use of FUS to temporarily open the BBB can significantly reduce systemic vector doses and target rAAV vectors to select brain regions. We first tested the safety of hippocampal FUS BBB opening in PS19 mice by using either FUS alone, FUS and microbubbles to open the BBB, or FUS+BBB opening and intravenous (IV) injected AVV9/GFP. Three weeks post injection, immunohistochemical analysis of brains showed equivalent levels of staining for reactive astrocytes (GFAP), activated microglia (Iba1), and phosphorylated tau (AT8) in the treated and untreated hemispheres. These results support that AAV delivery with FUS was well tolerated short-term within PS19 mice. We then performed proof-of-concept studies where FUS was used to target IV injected AAV9/hTaui vector that also expressed a GFP reporter protein to the hippocampus of 6-month-old PS19 mice. Three points in the hippocampus of the right hemisphere were targeted, and BBB opening was confirmed using gadolinium-enhanced MRI. One-month post-treatment, brains were collected for IHC and biochemical analysis. GFP staining showed that AAV9/hTaui was robustly delivered to the hippocampus of the FUS treated side. qPCR analysis of hippocampal human tau showed that tau was significantly reduced in the treated hemisphere versus the untreated hemisphere. While FUS efficiently targeted IV injected AAV9/hTaui to the hippocampus, as expected following IV delivery in a middle-aged adult mouse, transduction efficiency of the remaining brain was quite low. To further improve FUS-mediated delivery of AAV9/hTaui, we propose to use FUS BBB opening in combination with intrathecal (IT) injection of AAV9. Studies by our group have demonstrated that combination IT and FUS delivery of AAV9 vectors results in greater hind brain and hippocampal transduction as compared to combination IV and FUS delivery. To test this, adult PS19 mice were treated with AAV9/hTaui or AAV9/scrambled control that was delivered via IT alone, combination IT and bilateral FUS targeting of hippocampi, or bilateral direct hippocampal injections. IT+FUS delivery of AAV9/hTaui improved survival over IT alone or direct hippocampal injections. Tissue analysis is ongoing and will be reported. Overall, our results support the use of FUS-mediated BBB opening to improve delivery of AAV9/hTaui in adult tauopathy mice.

1132. Endovascular Transdural Cerebello-Pontine Cistern Access Enables Intracisternal Microcatheter Navigation for Selective Intrathecal CNS Drug Delivery

Adel M. Malek[1,2], Aaron J. Levangie[2], Carl B. Heilman[1,2], Brandon M. Beneduce[2]

[1]Tufts Medical Center, Boston, MA,[2]CereVasc Inc., Charlestown, MA

Introduction: Intrathecal administration of cell-based, large molecular/gene therapy viral vectors represents an effective strategy to circumvent the blood-brain barrier and target central nervous system (CNS) tissue. Conventional intrathecal routes of administration are invasive or achieve inconsistent biodistribution to the brain. We previously described a minimally invasive, percutaneous, endovascular transdural catheter approach to access the lateral edge of the cerebrospinal fluid (CSF)-filled cerebellopontine angle (CPA) cistern for intrathecal administration. Here we describe a second-generation transdural access system serving as a platform for co-axial transdural microcatheter delivery and cisternal navigation to contralateral, cranial and caudal adjacent subarachnoid space for superselective

localized intrathecal administration. **Methods:** A 3D-printed model, derived from patient-based MRI segmentation of human anatomy including the CPA cistern and adjacent basilar subarachnoid space surrounding the anterior brainstem was filled with a low-density fibrillar mesh to simulate arachnoid fibers (**Figure 1**). Fluorographic cone-beam CT imaging of the anatomy model was performed to acquire baseline imaging (**Figure 1**) and navigate under roadmap guidance, the second-generation endovascular Polaris™ Cisternal Access System (CereVasc Inc., Boston, MA, USA) through the inferior petrosal sinus in a transdural fashion to serve as a CNS access platform. A neurovascular microcatheter (Echelon™ 10, Medtronic Inc.) was successfully navigated through the lumen of the Polaris catheter in selective fashion in all quadrants of the pontine, basilar and medullary cisterns at distances of >4 cm with the assistance of a laser-cut shapeable 0.014" microwire (Synchro Microwire, Stryker Inc.). **Results:** The neurovascular microcatheter was successfully navigated into the CPA cistern and adjacent subarachnoid spaces (**Figure 2**), directed towards the midline and into the contralateral CPA, as well as superiorly into the upper basilar cistern and inferiorly in front of the medulla. The 0.017" neurovascular microcatheter had a deadspace of 0.22 cc, through which a therapy could be administered. Use of a 0.014" or 0.010" microwire to assist navigation decreased the deadspace to 0.14 cc or 0.07 cc, respectively, to mitigate therapeutic volume-loss during administration. **Conclusions:** Endovascular CPA cistern access facilitates navigation to adjacent subarachnoid spaces via low-profile neurovascular microcatheter, through which AAV or other CNS-bound therapeutics could be administered. This approach enables close proximity to brain tissue and other locations near the base of the brain and upper brainstem that may be difficult to reach using other CNS routes of administration. These data warrant further investigation of the resulting transduction into target brain tissue from AAV administration into various subarachnoid spaces accessible through this technique.

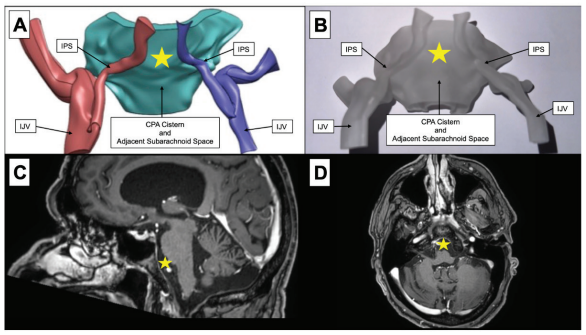


Figure 1: A) Computer visual of the anatomy model showing bilateral internal jugular veins (IJV) as they enter the skull, inferior petrosal sinuses (IPS) and adjacent CSF space. B) The physical 3D-printed model. C) Brain MRI sagittal view. D) Brain MRI transverse view. Yellow star consistently placed in the same CSF space for spatial orientation.

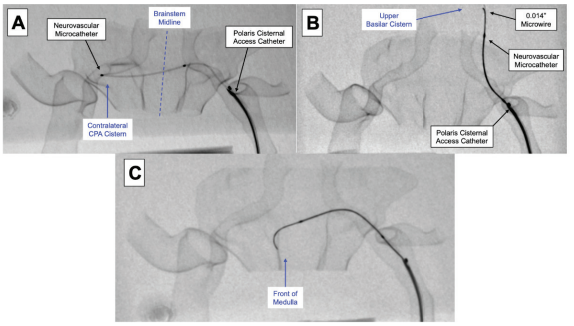


Figure 2: A) Polaris Catheter was navigated through the IPS vein wall for transdural access to the CPA cistern and allow coaxial microcatheter navigation through the subarachnoid space to the contralateral CPA. B) Microcatheter navigation superiorly into the upper basilar cistern. C) Microcatheter navigation inferiorly in front of the medulla.

1133. Metabolic and Neuropathological Profiling of SLC13A5 Epilepsy Gene Therapy

Raegan Adams, Morgan Schackmuth, Lauren E. Bailey, Krishanna Knight, Irvin T. Garza, Meghan Eller, Rachel M. Bailey

Center for Alzheimer's and Neurodegenerative Diseases, UT Southwestern Medical Center, Dallas, TX

SLC13A5 Epilepsy is a rare form of epileptic encephalopathy. This disorder is caused by loss of function variants in the SLC13A5 gene, which encodes for the plasma membrane sodium citrate co-transporter (NaCT) that is highly expressed in the brain and liver. These variants cause a decrease or complete loss of citrate transport, which consistently causes increased extracellular citrate. Studies in a small number of patients and from KO mice also show variable metabolic perturbations in blood, CSF, and brain tissues (mice only). Currently, while most patients are on several anti-seizure medications with varying amounts of control, there are no therapeutic options that target the underlying cause of the disorder. Gene therapy offers a promising therapeutic option for SLC13A5 Epilepsy patients. Our group has developed a recombinant adeno-associated virus (AAV) vector encoding a codon optimized human SLC13A5 gene (scAAV9/SLC13A5). In previous studies, we showed scAAV9/SLC13A5 is efficacious in Slc13a5 KO mice. While KO mice don't have an overt phenotype, like patients, they do have increased epileptic activity and increases in plasma citrate concentrations, which were ameliorated with gene therapy. In this study, we investigated the effects of scAAV9/SLC13A5 on metabolic and neuropathological phenotypes in KO mice. Slc13a5 KO mice were treated with scAAV9/SLC13A5 at post-natal day 10 via intrathecal lumbar puncture along with vehicle treated WT and KO littermate control mice. At ~6 months post-injection, cerebral spinal fluid (CSF) was withdrawn via the cisterna magna and brains were harvested for histological analysis. CSF was submitted for LC-MS analysis for a targeted panel of 17 metabolites, including citrate, aspartate, glutamate, malonate, and lactate, which were previously reported to be altered in patients and/or KO mice. Compared to WT mice, vehicle treated Slc13a5 KO mice had elevated citrate levels in the CSF, while scAAV9/SLC13A5 treated KO mice had levels similar to WT mice. Treatment effects of CSF citrate levels corresponded with changes in plasma citrate levels that were previously assessed. This suggests that the SLC13A5

transgene restored NaCT citrate transport function in the central nervous system and peripheral tissues. In contrast to other reports, at baseline we did not detect significant differences in the other tested metabolites in CSF of WT and KO mice. Interestingly, in treated KO mice, malonate was significantly increased as compared to KO and WT vehicle treated mice, which could indicate increased intracellular citrate. White matter abnormalities have been shown in some patient MRIs, and more generally, seizures are thought to induce reactive astrogliosis and interneuron loss. Histological changes in SLC13A5 patients and KO mice, however, have not been reported. To determine if loss of Slc13a5 function results in brain pathology, WT, KO and KO treated mice were stained for markers likely to be associated with the disease phenotype: parvalbumin (interneurons), myelin basic protein (myelination), and GFAP (astrocytes). At baseline, there were no overt differences in staining between groups, but quantification is ongoing and will be presented. These studies suggest that in addition to being effective in alleviating behavioral disease symptoms in KO mice, scAAV9/SLC13A5 treatment is effective in correcting citrate concentrations, a putative disease biomarker, in both plasma and CSF of KO mice.

1134. Optimization of AAV Vectors Packaged hfCas13Y or hfCas12Max to Treat Angelman Syndrome

Jinhui Li[1], Dong Yang[1], Shaoran Wang[1], Tong Li[1], Xuan Yao[1], Alvin Luk[1], Hui Yang[1,2], Linyu Shi[1]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: Angelman Syndrome (AS) is a rare neurodevelopmental disorder caused by mutation or deletion of the maternally-inherited ubiquitin protein ligase E3A (*UBE3A*) allele, which mediates the targeted degradation of several protein substrates. In neurons, only the maternally-inherited *UBE3A* allele is actively expressed because paternally-inherited *UBE3A* is selectively silenced in *cis* by its antisense transcript, *UBE3A-ATS*. In our previous study, AAV-hfCas13X has shown to restore the expression of paternal *Ube3a* in a mouse model of AS. The treated mice showed improvements in body weight and motor function, but not in the marble burying test or brain weight. Therefore, we optimized our gene-editing tools for the treatment of AS. **Methods:** To enhance the efficiency of AAV vector packaged high-fidelity Cas13Y (hfCas13Y) or Cas12Max (hfCas12Max) to facilitate the expression of *Ube3a*, we constructed a series of AAV transfer plasmids by changing the promoters, engineering gRNA, and adding the *cis*-regulatory motifs. The optimized AAV vectors were then administered to the AS mice on postnatal day 1 via intracerebroventricular (ICV) injection to assess the efficiency of *Ube3a-ATS* silencing *in vivo*. The RNA and protein of reinstated Ube3a in the dissected brain regions was evaluated by RT-qPCR or western blot (WB). **Results:** We delivered four versions of optimized AAV-hfCas13Y (V1, V2, V3, and V4) into the neonatal AS mice and observed that the paternal Ube3a protein expression in the cortex and hippocampus of AS mice surpassed 50% of that found in wild-type (WT) mice at 4 weeks post-infection with AAV-hfCas13Y V4 (Fig. 1A, C). The RNA level of *Ube3a-ATS* was also dramatically decreased by AAV-hfCas13Y V4 (Fig. 1B, D). In addition, we optimized the AAV-hfCas12Max system and assessed the efficacy of four AAV transfer plasmids. RT-qPCR results showed that AS mice injected with AAV-hfCas12Max V2 exhibited the highest *Ube3a* expression in the cortex and hippocampus, with restoration of the *Ube3a* mRNA expression to a level almost similar to that found in WT mice (Fig. 1E, G). WB results showed that AAV-hfCas12Max V2 significantly increased the UBE3A expression (Fig. 1I). **Conclusions:** The optimized AAV vectors, which encapsulated both the hfCas13Y and the hfCas12Max systems, were capable of significantly enhancing the expression of UBE3A, with AAV-hfCas12Max showing particularly robust results. Additional studies, including body weight and behavior tests, are currently being performed. Consequently, the knockdown of *Ube3a-ATS* RNA mediated by AAV-hfCas13Y or hfCas12Max may represent a more favorable approach for AS treatment.

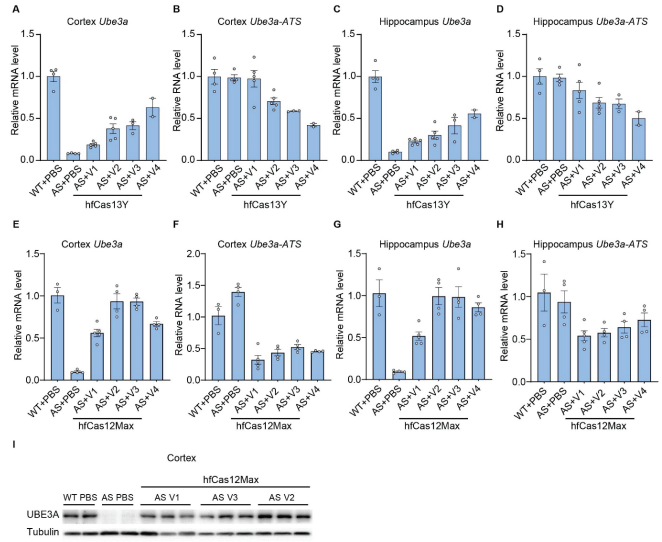


Figure 1. Optimization of AAV vectors encapsulating hfCas13Y or hfCas12Max for the treatment of AS. A-D, The mRNA expression levels of *Ube3a* and *Ube3a-ATS* in the cerebral cortex and hippocampus were analyzed using RT-qPCR with the indicated treatments of AAV-hfCas13Y or AAV-hfCas12Max. **I**, Western blot analysis of protein expression in the cortex was performed with the indicated treatments of AAV-hfCas12Max.

1135. RNA Targeting of *Bace1* by CRISPR-Cas13 Improves Memory Deficits in Alzheimer's Diseases

Yinan Yao[1], Hanbo Ma[2], Dong Yang[3], Yuanhua Liu[1], Hui Yang[1,3]

[1]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China,[2]International Peace Maternity and Child Health Hospital, Shanghai, China,[3]HuidaGene Therapeutics Co., Ltd., Shanghai, China

Background: Alzheimer's disease(AD) is a progressive neurological disease and the most common form of neurodegenerative dementia with no specific disease-modifying treatment, which brings great burden to the family and society. The approved drugs for AD could alleviate the symptoms of AD, but cannot reverse the progression of AD. β-secretase BACE1 (β-site amyloid precursor protein cleaving enzyme) is considered the potential and rationale target because it is involved in the rate-limiting step, which produces toxic amyloid β (Aβ) peptides that leads to deposits in the form of amyloid plaques extracellularly, resulting in AD. Here, we developed CRISPR/high-fidelity Cas13Y (hfCas13Y) RAN-targeting therapy for AD. **Methods:** In this study, we used hfCas13Y system which could specifically and efficiently target the

mRNA of *BACE1* without causing permanent changes at the genomic level. First, we screened the gRNAs targeting *BACE1* (g*BACE1*) by fluorescence-activated cell sorting(FACS) in 293, N2a, and Cos7 cells. Then, we administrated doses of AAV-hfCas13Y-g*BACE1* into the hippocampus of wild-type C57/B6 mice through stereotactic injection and assessed the knock down efficiency through qPCR, Western Blot (WB), and immunostaining at 2- and 8-week post-injection. To evaluate the therapeutic efficacy, we administrated the AAV-hfCas13Y-g*BACE1* into both hippocampal regions of 4-month-old 5xFAD mice, a mouse model of AD, via stereotactic injection. Following a period of 3 months and 6 months post-injection, behavioral assessments, including fear conditioning, Barnes maze, and water maze tests were conducted to evaluate the learning and memory capabilities of the mice. qPCR, WB, immunofluorescence (IF) staining, and enzyme-linked immunosorbent assay (ELISA) analysis were performed to measure Bace1 and Abeta levels. Electron microscopy (EM) was utilized to analyze the synapse number. Moreover, we systematically delivered AAV-hfCas13Y-g*BACE1* to 5xFAD mice via retro-orbital injection and evaluated the treatment's efficacy through the aforementioned behavior tests, qPCR, WB, IF staining, ELISA analysis, Golgi staining, and EM. **Results:** For CRISPR/hfCas13Y systems, we identified two gRNAs that could efficiently target the *Bace1* mRNA in human (reduced 99% for 293 cells, P<0.001), monkey (reduced 98% for Cos7 cells,P<0.001), and mouse (reduced 92% for N2a cells, P <0.01) cells. In C57 mice, we tested the *in-vivo* knockdown efficiency of the two AAV-hfCas13Y-*BACE1*-gRNAs at varying doses and observed that AAV-hfCas13Y-*BACE1*-gRNA2 demonstrated superior performance, reducing *Bace1* mRNA levels by 50% (P<0.01). Upon injection of AAV-hfCas13Y-*BACE1*-gRNA2 into the hippocampus of 5xFAD mice, we observed a reduction in Bace1 expression(Fig. 1A). Furthermore, the results from fear conditioning (Fig. 1B-C) and water maze (Fig. 1D-E) tests indicated that AAV-hfCas13Y-*BACE1*-gRNA2 treated 5xFAD mice via retro-oral injection enhanced the learning and memory capabilities when compared to AAV-hfCas13Y treated 5xFAD mice. **Conclusions:** In summary, we proved the feasibility of RNA-targeting CRISPR/hfCas13Y system as a therapeutic approach for AD, which efficiently reduced the mRNA of *Bace1*, alleviated Abeta pathologies, improved neuronal functions and refined the learning and memory abilities in the mouse model of AD.

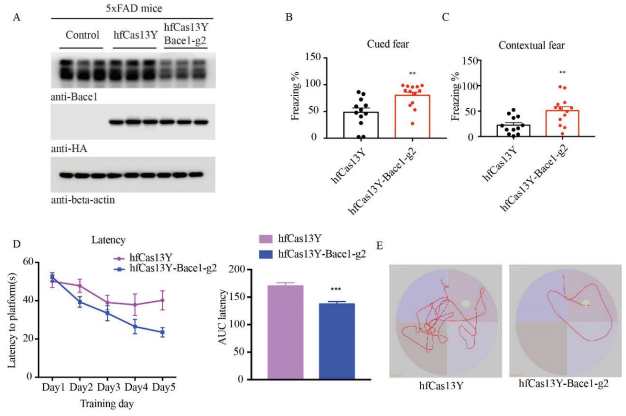


Figure 1. AAV- hfCas13Y-BACE1-gRNA 2 treatment alleviates behavior deficits in 5xFAD mice
(Note: The hfCas13Y-expressing plasmid contains an HA tag element)

1136. Development of an *Ex Vivo* Hematopoietic Stem Cell Gene Therapy for Frontotemporal Dementia (FTD)

Yuri Ciervo[1], Pietro Rigoni[1], Nadia Bologna[1], Massimo Accardo[1], Giulia Santinon[1], Chiara Recchi[2], Rita Milazzo[1], Alessandra Biffi[1]

[1]University of Padua, Padova, Italy,[2]Orchard Therapeutics, London, United Kingdom

Frontotemporal Dementia (FTD, OMIM:607485) is the second most common dementia in people under the age of 65 after Alzheimer Disease and is devoid of any cure or specific approved treatment. The incidence of pathogenic loss-of-function mutations in the progranulin gene (GRN) accounts for approximately 5% of all FTD cases. GRN is a secreted lysosomal protein that functions as neurotrophic factor and regulator of neuroinflammation. Raising GRN levels in the brain of FTD patients, and in particular in microglia, may result in therapeutic benefit. Hematopoietic Stem/Progenitor Cell (HSPC) Gene Therapy (GT) based on the use of lentiviral vectors (LVs) for gene transfer could thus represent a valuable treatment option for GRN-FTD patients as it could generate a long-lasting source of GRN in their brains through its myeloid, microglia-like CNS progeny. To explore this novel treatment option and generate proof of concept preclinical data, we developed and tested two therapeutic LVs with a strong constitutive (human PGK) *versus* a novel inducible synthetic promoter (derived from HLA-DRA) able to safely deliver multiple copies of the human *GRN* cDNA in GRN knock-out (ko) cell models, inducing over-expression of the therapeutic protein which is then correctly secreted and taken up by target GRN knock-out cells. Following *in vivo* and *in vitro* validation, the two vectors were employed in the setting of a proof of concept of efficacy study of HSC-GT for FTD in $Grn^{-/-}$ mice where the gene corrected cells were administered intravenously or in the brain lateral ventricles (ICV) in optimized transplant conditions enabling CNS-specific and selective engraftment of the transplanted cells. Independently from the promoter or delivery route, we showed that this approach efficiently delivers human GRN to the brain of transplanted GRN-ko mice, and that overall, the strategy is feasible, well tolerated, and capable of correcting disease associated phenotypic abnormalities at biochemical, clinical/behavioral and histopathological levels. In our settings, restoration of GRN at the 25-to-40% of wild-type levels in the brain is sufficient for long term phenotypic correction as demonstrated by normalization of well-known soluble disease biomarkers (BMP and GluSph) in plasma and brain, resolution of brain lipofuscinosis and reduction of both microgliosis and astrogliosis at 12 months after transplant. On-going analysis will provide additional conclusion related to the delivery route and/or promoter.

1137. Multi-Targeting Zinc Finger Nucleases as a Gene Therapy Vector for Angelman Syndrome

Hannah O. Bazick, Lucas M. James, Bonnie Taylor-Blake, Mark J. Zylka

UNC Chapel Hill, Chapel Hill, NC

Angelman syndrome (AS) is a severe neurodevelopmental disorder characterized by sleep dysfunction, seizure disorders, impaired cognition and speech, motor deficits, and microcephaly. Due to

epigenetic silencing of the paternally inherited allele of *UBE3A* (*patUBE3A*) by a long non-coding antisense RNA (*UBE3A-ATS*) in neurons, mutation or deletion of maternal *UBE3A* results in near complete loss of *UBE3A* in the brain. However, the intact patUBE3A can be unsilenced with genome editing nucleases, such as zinc finger nucleases (ZFNs), by prematurely truncating *Ube3a-ATS*, rescuing some AS-associated phenotypes. In our present work, we screened 8 ZFNs multi-targeted to repetitive *Snord115* snoRNAs located within *Ube3a-ATS* and evaluated their ability to unsilence *patUbe3a* after transfection onto *Ube3a*$^{mat+patYFP}$ mouse neurons. ZFN 17/18 yielded 1.9-times higher *patYFP* intensity than a multi-target Cas9-guide RNA (gRNA) targeted to a similar region within *Ube3a-ATS*. We then packaged ZFN 17/18 and NmCas9 gRNA9 into AAV2 and transduced *Ube3a*$^{mat+patYFP}$ mouse neuron cultures. At the highest titer tested (50,000 viral particles per cell), NmCas9 gRNA9 and ZFN 17/18 similarly reduced expression of *Ube3a-ATS* (25% and 34% to that of untreated levels, respectively), downregulated *Snord115* snoRNAs (26%, 35%), and upregulated *patUbe3a* (711%, 737% upregulation from baseline). Neither treatment affected expression of *Snord116*, downregulation of which can lead to Prader-Willi syndrome. Bilateral intracerebroventricular (i.c.v.) injection of 1-day-old Angelman syndrome model mice led to widespread unsilencing of UBE3A in the cortex by 90-days-old (34% to that of wild-type untreated), with UBE3A levels in some individual neurons reaching that of untreated wild-type levels. Overall, our study demonstrates a novel potential gene therapy for Angelman syndrome.

1138. Multifunctional Antibody Engineering for Tauopathy Immunotherapeutics

Lianna D'Brant[1], Michael Miller[1], Su Kyoung Na[1], Natasha Rugenstein[1], Anne Messer[1], Sally Temple[2], David C. Butler[1]

[1]Neural Stem Cell Institute, Rensselaer, NY,[2]New York Neural Stem Cell Institute, Rensselaer, NY

Tauopathies, encompassing conditions like Frontotemporal Dementia and Alzheimer's Disease (AD), manifest through the accumulation of insoluble tau proteins in neurons and glia, with the presence of ubiquitin-positive tau in neurofibrillary tangles correlating with the severity of AD-related dementia. We hypothesize that reducing monomeric tau through normal protein clearance pathways will mitigate abnormal tau accumulation, thus alleviating cell toxicity and neurodegeneration. Our approach utilizes bifunctional intrabodies that bind tau and prevent tau aggregation (a chaperone-like function) and target soluble monomer directly to the proteasome for degradation. Fusion with the ornithine decarboxylase (ODC) PEST degron enables ubiquitin-independent proteolysis of intrabody-bound tau by directly interacting with the proteasome. By leveraging this direct molecular interaction with the proteasome, we control the degradation process through programmable target antigen proteolysis technology (PTAP). We engineered 17 human anti-tau-PEST bifunctional intrabodies, selecting candidates based on their efficacy in reducing GFP-labeled 0N4R-tau in an immortalized rat neuronal progenitor cell line (ST14A). Ten intrabodies demonstrated a minimum 25% reduction in eGFP-tau (0N4R) levels compared to the empty-vector control, with three achieving reductions (** $p < 0.01$) of approximately 78%, 70%, and 74% after 72 hours of treatment. When the proteasome was inhibited using epoxomicin or MG132, tau levels significantly increased compared to vehicle-treated controls across all groups. Treatment of iPSC-derived 2-month-old cortical organoids over 21 days showed significant reductions in endogenous tau, with V-PEST, N-PEST, and F-PEST exhibiting reductions of 55.3% (** $p < 0.01$), 57.9% (** $p < 0.01$), and 38.82% (** $p < 0.05$), respectively. These findings underscore the potent efficacy of anti-tau-PEST intrabodies in reducing tau levels in cell lines and 3D organoid models. Excitingly, in 2D cortical cultures, these intrabodies were able to alleviate proteasomal impairment in V337M and N279K mutant cells. Furthermore, partial reduction of tau by PTAP is as effective as high reduction of tau to counteract cell death in the V337M cultures. In vivo efficacy of anti-tau PTAP intrabodies is being evaluated using the hTau.P301S transgenic mouse model, building on our demonstrated success with bifunctional anti-synuclein-PEST intrabodies.

1139. Transcriptomic Profiling of Glutamatergic Neurons Derived from Human Induced Pluripotent Stem Cells of Healthy Controls and Alzheimer's Patients

Ram Sagar[1,2], Ioannis Azoidis[1,2], Cristina Zivko[1,2], Ariadni Xydia[1,2], Esther S. Oh[2,3,4], Paul B. Rosenberg[2,4], Constantine G. Lyketsos[2,4], Vasiliki Mahairaki[1,2], Dimitrios Avramopoulos[1,2]

[1]Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD,[2]The Richman Family Precision Medicine Center of Excellence in Alzheimer's Disease, Johns Hopkins University School of Medicine, Baltimore, MD,[3]Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, MD,[4]Department of Psychiatry and Behavioral Sciences, Johns Hopkins University School of Medicine, Baltimore, MD

Alzheimer's disease (AD) is a gradually progressive, irreversible form of dementia with millions positive cases worldwide and the mechanisms of disease progression are not yet fully elucidated, to date there are no reliable biomarkers for early detection of pathogenic components of AD. Recent advances in the generation of induced pluripotent stem cells (iPSCs) from peripheral blood mononuclear cells (PBMCs) and differentiating them into a variety of cell types is allowing us to study them without the caveats associated with disease-related changes. We generated induced Pluripotent Stem Cells (iPSCs) from Alzheimer's disease (n=8) patients and healthy controls (n=6) and used lentiviral delivery mechanism to differentiate the iPSCs into glutamatergic neurons. The aim of this study was to generate a comparative transcriptomic profile between the diseased and controls to study the gene expression differences. A total of 621 genes were seen to be differentially expressed at adjusted $p<0.05$ between the case and control derived neurons. These genes show significant overlap and directional concordance with genes reported from a single-cell transcriptome study of AD patients; including five genes implicated in AD from genome-wide association studies and they appear to be part of a larger functional network as indicated by an excess of interactions between them observed in the protein-protein interaction database STRING. Exploratory analysis with Uniform Manifold Approximation and Projection (UMAP) suggests distinct clusters of patients, based on gene expression, who may be clinically different. Our research outcomes will

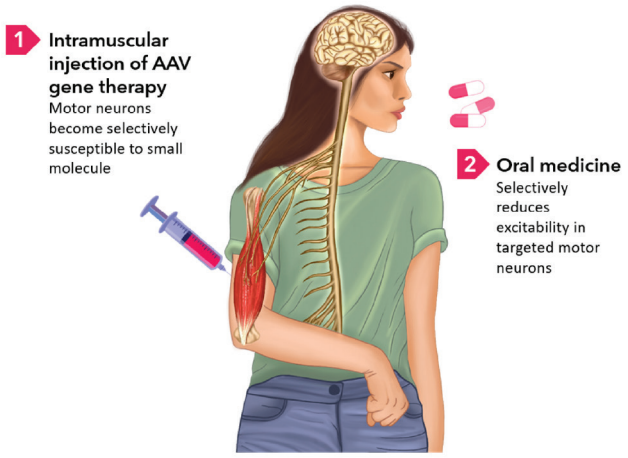
enable the precise identification of distinct biological subtypes among individuals with Alzheimer's disease, facilitating the implementation of tailored precision medicine strategies.

1140. A Low Dose, Targeted, and Controllable Gene Therapy for the Treatment of Spasticity

Miranda A. Mathews, Alba Guijarro Belmar, Calvin Chad Smith, Sylvain Gigout, Panayiota Demosthenous, Riccardo Privolizzi, Hanna Luniak, Brandon Roberts, Marta Zinicola, Devan Parmar, Raj S. Dattani, Robert M. Brownstone, Andrew J. Murray

Sania Therapeutics, London, United Kingdom

Gene therapies could potentially change healthcare by making previously intractable disorders amenable to treatment. These new treatments could improve and/or extend the lives of patients, and thus open substantial new healthcare markets. However, in order for gene therapies to reach widespread adoption, several challenges must be overcome. Many of these challenges relate to the delivery mechanism for genetic material which should achieve sufficient transduction of target cell types whilst minimising vector distribution to non-target tissues and limiting toxicity and immune response. Ideally these goals would be achieved with a low-dose viral vector to further increase safety and reduce overall treatment costs. We are aiming to overcome these challenges by developing low dose, highly targeted gene therapies that have been developed using our human-centric approach. Our lead therapeutic program is a gene therapy for spasticity - a prevalent and debilitating condition associated with several neurological diseases. Spasticity results from hyperexcitability of motor neurons in the spinal cord and is characterised by severe, involuntary muscle spasms, joint stiffness, and pain. Although approximately 2.5 million people in the US and Europe suffer from severe spasticity, they often receive a poor standard of care as current treatments are largely ineffective, invasive, and have non-specific mechanisms of action which leads to significant side effects. Sania's gene therapy for spasticity (SRx-T001) combines an engineered AAV vector and peripheral delivery to target gene delivery only to hyperactive motor neurons. Using our human-centric AAV evolution platform, R-Scan, we have identified multiple novel AAV capsids that show superior transduction of motor neurons. We demonstrate this superior transduction in human motor neurons in culture, human neuromuscular organoids and mouse motor neurons in vivo following intramuscular injection. We have also shown that the intramuscular route of injection prevents biodistribution to non-target cell and tissue types. This precision delivery system enables a targeted form of genetic neuromodulation (chemogenetics) to alleviate spasticity symptoms. Sania uses this novel AAV vector to drive overexpression of a human ion channel selectively in spastic motor neurons. This overexpression means that these motor neurons are selectively susceptible to low doses of a small molecule, taken orally. Binding of this small molecule to this ion channel reduces neuronal excitability. We have demonstrated that expression of our ion channel (SRx-490) in human motor neurons has little effect on the excitability of these neurons alone. However, application of low doses of the small molecule activator significantly reduces the electrical activity of these neurons. This chemogenetic system will allow patients and clinicians titratable control over motor neuron excitability and potentially allow the restoration of muscle function. By combining our unique vectors engineered in human motor neurons, targeted administration, and a novel chemogenetic system, we are developing a gene therapy with a high safety profile, high specificity, that can be delivered at relatively low cost. This combination will facilitate adoption for this prevalent movement disorder and provide a blueprint for future therapeutic approaches.



1141. BIUXX

Meysam Ganjibakhsh[1], Julieth A. Sierra Delgado[1], Babatunde Ogunlade[1], Yanina Tkachenko[1], Abuzar Kaleem[1], Kyle Faulconer[1], Pipasha Biswas[1,2], Krizelle Alcantara[1], Xiaojin Zhang[1], Shibi Likhite[1], Kathrin Meyer[1,2]

[1]The Research Institute at Nationwide Children's Hospital, Columbus, OH,[2]Department of Pediatrics, The Ohio State University, College of Medicine Columbus, Columbus, OH

Objective: GMPPA-Congenital Disorder of Glycosylation is a rare genetic disease characterized by alacrima, achalasia, and intellectual disability. This study aims to develop novel patient specific *in vitro* disease model systems to investigate and screen potential therapeutic avenues including gene therapy for patients with GMPPA. **Methods:** Patient-derived fibroblasts were directly converted into induced neurons (iNs) using a specialized neuronal induction medium with specific small molecules, generating disease-specific neuronal cell models. The iNs underwent characterization through comprehensive cell biology assays to assess disease-specific pathological contributions, including evaluations of cellular morphology and functional properties. In addition, Neural Progenitor Cells (iNPCs) were generated from GMPPA patient fibroblasts using a well-established direct reprogramming method developed by the Meyer lab and differentiated into induced astrocytes (iAs). iA function was evaluated via multiple assays including mitochondrial stress test using the Seahorse Bioscience Extracellular Flux Analyzer and co-culture assays with healthy mouse GFP+ neurons. These assays are currently used to evaluate the efficacy of small molecule drugs as well as an AAV gene therapy construct we recently developed. **Results:** Neurons derived from patients with GMPPA mutations displayed distinct disease phenotypes, such as reduced conversion rates and altered neuronal morphology. Moreover, we found significant changes in basal and ATP-linked respiration compared with healthy controls. In the co-culture system, iAs derived

from patients caused death of the co-cultured neurons, indicating that astrocytes might play a crucial role in the disease manifestation. We applied functionalized nanoparticles to iAs, and the results demonstrated the rescue of neuronal support and improvement of the metabolic phenotype. **Conclusion:** We developed novel *in vitro* model systems to study GMPPA disease mechanisms and test novel therapeutics in a rapid and patient-specific manner. Several therapeutic avenues are currently under evaluation including a AAV based gene therapy approach.

1142. How to Develop Treatments for Ultra Rare Disease + Applications for SCA3

Richard Horgan
Cure Rare Disease, Woodbridge, CT

There are currently over 7,000+ rare diseases, and 50% of patients affected are children. And while 1 in 10 people suffer from a rare or ultra rare disease, tragically only 5% of these diseases have effective treatments or a cure. There is a bias of traditional drug development towards diseases with large populations, leaving ultra rare disease patients with no traditional path forward for identifying new treatments or cures. However, new frameworks are allowing for the development of treatments on a smaller scale, offering hope for these patients. CRD-002 is an ASO drug being developed for the treatment of spinocerebellar ataxia type 3 (SCA3) also known as Machado-Joseph Disease. SCA3 is an ultra-rare genetic disease triggered by triplet expansion in the *ATXN3* gene. It is a debilitating and life-threatening disease, with low prevalence and with no available treatments. There are currently no therapeutic interventions and very few interventional clinical trials for this disease. CRD-002 targets exon 10 of the unprocessed, or pre-mRNA. This exon 10 contains the expanded CAG repeat. Through steric blocking, the ASO binds with the pre-mRNA such that the cellular machinery involved in processing does not recognize exon 10 and doesn't include it in the final transcript. The resulting protein will be slightly shorter, does not contain the toxic mutation but still contains most of its essential functional domains. Preclinical in vitro and in vivo experiments have demonstrated production of a functional ATXN3 protein and reduces disease phenotype in the brain. All SCA3 patients have an expansion of CAG repeats in the ATXN3 gene. Therefore, this treatment is applicable to all SCA3 patients because it targets the ataxin-3 RNA. As clinical research on genetic diseases advances, more traditional pharma companies will have the ability to follow this emerging pathway and explore future applications for advanced modalities, including in rare and ultra rare diseases. Before embarking on this journey, it's important that they learn from those who have established a framework - both in terms of "how to" steps but also lessons learned throughout the process. Moreover, it's important to understand the perspective of regulators and how clinical trials can be designed in light of lower prevalence numbers. This presentation will cover: - How to apply the ultra-rare drug development framework - Identifying disease targets and patients for treatment - The importance of focusing on mutation-specific therapies - How to collaborate with the FDA - Alternative funding pathways - How this approach is currently being taken to develop a therapy for SCA3

Thursday Posters: Musculo-Skeletal Diseases

1143. AAV-Mediated Exon51 Skipping Results in Durable Dystrophin Protein Restoration and Correction of Histopathology in a Mouse Model for Duchenne Muscular Dystrophy

Lan Jin, Randolph Qian, Steven Foltz, Ting Chen, Kirk W. Elliott, Jason Yang, Olivier F. Danos, Ye Liu
REGENXBIO Inc. - Rockville, Rockville, MD

Duchenne muscular dystrophy is caused by loss of function mutations in the *DMD* gene that encodes Dystrophin. Around 80% of these mutations disrupt the translation reading frame but can be reversed by excluding a specific exon of the *DMD* mRNA, which results in a slightly shorter but functional protein. Exon skipping using synthetic antisense oligonucleotides is an approved therapy for eligible DMD patients. Using a self-complementary (sc) AAV vector expressing antisense sequences fused to a modified U7 small-nuclear RNA (AS.snRNA), we have previously reported effective dystrophin exon 53 skipping in the *hDMDdel52/mdx* (Del52) mouse model which harbors a defective human *DMD* gene rescuable by skipping either exon 53 (Ex53) or exon 51 (Ex51). Dystrophin restoration, correction of histopathology, and muscle function improvement were demonstrated at 3 months after AAV.Ex53 treatment in mice. To expand the AAV-mediated exon skipping platform and potentially reach additional DMD patients, we have designed a similar approach for Ex51 skipping. Three antisense sequences were designed to target Ex51, the Ex51/Intron50 junction, and the Ex51/Intron51 junction. Each antisense sequence was fused to the 5' end of a modified U7 snRNA driven by a mouse U7 promoter. Three such U7 expression cassettes were cloned into a scAAV expression plasmid in tandem (AAV.Ex51.3AS), packaged into either AAVhu32 or AAV8 capsids, and tested in immortalized human myoblasts harboring the Del52 mutation. Up to 90% Ex51 skipping and 70% Dystrophin protein restoration were achieved in this system. The skipping efficiency was positively correlated with both AAV multiplicity of infection and the level of each AS.snRNA, indicating that the altered mRNA splicing is a specific result of the treatment. Both AAVhu32 and AAV8 vectors performed similarly in the Del52 human myoblast.Next, we treated Del52 mice with AAVhu32. Ex51.3AS vector at three doses of 5E13, 1E14, and 2E14 gc/kg, delivered intravenously. Necropsies were performed at 2- or 6-months post-dosing. At 2 months, Ex51 skipping efficiency reached up to 80% across skeletal muscles and the heart. This led to 17%, 25%, and 60% of wild-type level dystrophin protein restoration in the diaphragm, gastrocnemius, and heart, respectively. Dose-dependent AS.snRNA expression, *DMD* mRNA Ex.51 skipping, and Dystrophin protein restoration were observed in the diaphragm and gastrocnemius. At the low dose of 5E13 gc/kg, a notable 60% of Dystrophin protein was restored in the heart and did not further increase with a higher dose. In gastrocnemius, immunohistochemical analysis demonstrated up to 80% Dystrophin-positive fiber with proper sarcolemma localization, while Sirius Red staining demonstrated a reduction of fibrosis. At 6 months post-dosing, the Ex51 skipping efficiency remained stable, while Dystrophin protein levels increased further to 42% and 35% in the diaphragm and gastrocnemius, respectively. Dystrophin levels in

the heart remained at nearly 60%, regardless of dose levels, indicating highly efficient AAV transduction and exon skipping in the Del52 mouse heart. These findings collectively highlight the therapeutic and translational potential of the AAV.Ex51.3AS vector, which has the potential to treat up to 13% of all eligible DMD patients.

1144. Development of hfCas12Max-Based Single-Cut Gene Editing Therapy for Duchenne Muscular Dystrophy

Guoling Li[1], Jiajia Lin[2], Ming Jin[2], Zhifang Li[3], Tong Li[1], Shaoran Wang[1], Xuan Yao[1], Alvin Luk[1], Hui Yang[1,4], Linyu Shi[1]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Department of Neurology, First Affiliated Hospital, Fujian Medical University, Fuzhou, China,[3]Lingang Laboratory, Shanghai, China,[4]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: Duchenne muscular dystrophy (DMD) is a rare, fatal, X-linked neuromuscular disease caused by mutations in the *DMD* gene, disrupting the translation of the dystrophin protein which leads to muscle degeneration, progressive muscle weakness, and premature death. Among thousands of *DMD*-causing mutations, the skipping and reframing of exon 45 and exon 51 is estimated to therapeutically benefit at least 8% and 14% of all DMD cases worldwide, respectively. Thus far, the antisense oligonucleotide (ASO) medicine, known as eteplirsen or casimersen, has been approved to treat DMD patients with particular mutations in the exon 45-55 hotspot regions. However, it has been shown that ASO medicines just restore dystrophin protein expression to less than 1% of the normal level after one year of continuous weekly intravenous administration. Therefore, exploring innovative and efficient therapeutic approaches to correct genetic mutations and restore dystrophin expression and muscle functions in DMD individuals is urgently necessary. **Methods:** Two humanized DMD mouse models were generated, one replacing mouse exon 51 with human exon 51 and deleting mouse exon 52 (DMD$^{\Delta mE51E52,hE51KI}$) while the other replacing mouse exon 45 with human exon 45 and deleting mouse exon 46, thereby disrupting the open reading frame of the dystrophin protein. We explored the efficiency of high-fidelity Cas12Max (hfCas12Max)-mediated single-cut genome editing in human *DMD* exon 51 splice donor (SD) site (Figure 1B) and exon 45 *in vitro*. An all-in-one AAV vector system was chosen for *in vivo* delivery of hfCas12Max and a guide RNA (gRNA) targeting the SD site of human exon 51 or exon 45 to manifest the *in-vivo* single-cut efficiency in DMD mice (Figure 1A) and wild-type non-human primates (NHPs) (Figure 1G). **Results:** Both humanized DMD mouse models exhibited phenotypes highly similar to those individuals with DMD, making them suitable for analyzing potential intervention strategies. We subsequently showed that hfCas12Max-mediated single-cut genome editing efficiently targeted the splice donor (SD) sites of human *DMD* exons 51 and 45 *in vitro*. Additionally, systemic delivery of AAV-hfCas12Max effectively restored dystrophin protein expression in both humanized DMD mouse models, and it improved the pathological features of DMD, including the histopathology and grip strength of these mouse models (Figure 1C-F). Moreover, the all-in-one single AAV vector induced therapeutically acceptable editing efficiency in exon 51 SD in healthy wild-type NHP with low dosage, and no hfCas12max-related toxicity was observed, indicating that the AAV-hfCas12Max therapy is relatively safe (Figure 1H,I). **Conclusions:** Our innovative CRISPR-based therapeutic, utilizing hfCas12Max for a precise single-cut modification of genomic DNA, presents a captivating new treatment paradigm, which offers a promising, long-lasting, and "one-and-done" new treatment modality that targets the underlying cause of DMD.

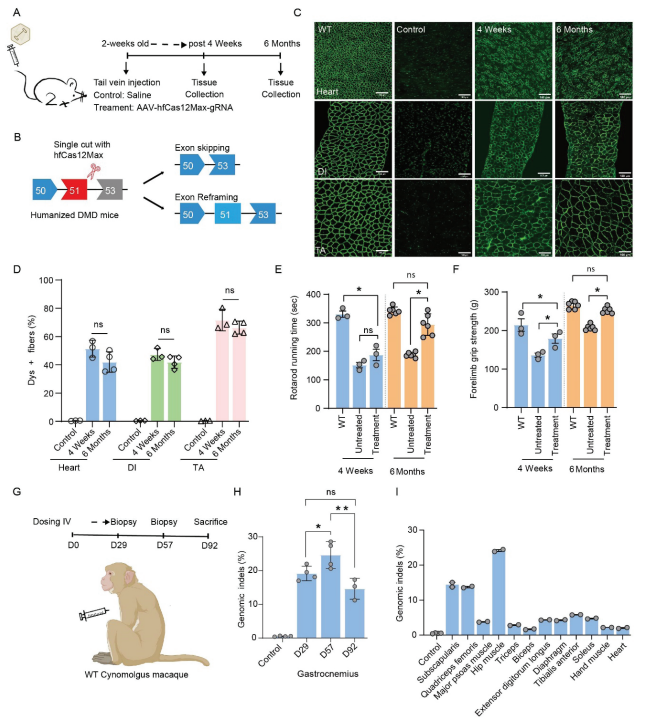


Figure 1. Intravenous delivery of hfCas12Max system efficiently rescues dystrophin expression and muscle function in humanized DMD mice. (A) Schematic of intraperitoneal injection of AAV particles. hfCas12Max was injected intraperitoneally into 2-week-old DMD^{mE51E52,hE51KI} mice. (B) Schematic of the therapeutic vectors and single-cut strategy. (C) Immunohistochemistry for dystrophin in TA, DI, and heart of DMD^{mE51E52,hE51KI} mice were performed at indicated time points. Dystrophin is shown in green. (D) Quantification of Dys+ in cross sections of TA, DI, and heart muscles (n=3-6). Forelimb grip strength (E) and rotarod rod (F) were measured in WT, DMD^{mE51E52,hE51KI} mice, and DMD^{mE51E52,hE51KI} mice treated with all-in-one AAV particles (n=3-6). (G) Systemic delivery of low dose hfCas12Max on 1-year-old wild-type cynomolgus macaque. Black nodes indicate time points for tissue collection after intravenous injection. (H) Gastrocnemius muscle was processed D29, D57and D92 later by biopsy for editing efficiency analysis. D, Day. (I) Editing efficiency analysis (D92) in different tissues of wild-type cynomolgus macaque.

1145. Adenine Base Editing Mediated Exon Skipping Restores Dystrophin Expression in a Humanized Mouse Model of Duchenne Muscular Dystrophy

Guoling Li[1], Jiajia Lin[2], Ming Jin[2], Zhifang Li[3], Qingquan Xiao[1], Tong Li[1], Shaoran Wang[1], Xuan Yao[1], Alvin Luk[1], Ning Wang[2], Wanjin Chen[2], Chunlong Xu[3], Hui Yang[1,4], Linyu Shi[1]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Department of Neurology, First Affiliated Hospital, Fujian Medical University, Fuzhou, China,[3]Lingang Laboratory, Shanghai, China,[4]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: Duchenne muscular dystrophy (DMD) is the most common X-linked neuromuscular disease, caused by mutations in the *DMD* gene. Despite the existence of a multitude of clinically documented mutations, the majority of those responsible for DMD are concentrated within a "hotspot" region, which spans from exons 43 to 55 of the DMD gene. This specific genomic segment encodes the central rod domain of the dystrophin protein. Correction of the *DMD*

exon 51 deletion mutation by skipping exon 50 can therapeutically benefit ~3.8% of DMD patients. Here, we developed CRISPR adenine base-editing therapy for the treatment of DMD. **Methods:** We generated a humanized DMD mouse model by replacing mouse exon 50 with human exon 50, followed by the deletion of mouse exon 51 ($DMD^{\Delta mE50,51,\ hE50KI}$), which disrupted the dystrophin open reading frames (Figure 1A). Then, we explored the potential of a single adeno-associated virus (AAV) vector packaged with CRISPR adenine base editor (ABE) named NG-ABE in modifying splice donor sites of the *DMD* gene by intramuscular injection and systemic delivery, causing skipping of a common *DMD* deletion mutation of exon 51. The outcomes were analyzed at 6 weeks and 10 months post-treatment to evaluate the extent of exon skipping, dystrophin restoration, and phenotypic improvements of cardiac and skeletal muscles (Figure 1B). **Results:** Humanized DMD mice models exhibited numerous features characteristic of DMD pathology, such as the lack of dystrophin, inflammation infiltration, reduced muscle strength, and elevated creatine kinase (CK) levels. Intramuscular administration of AAV vectors encoding ABE components, utilizing a split-intein trans-splicing system, led to more than 90% targeted exon skipping in *DMD* mRNA and restoration of up to 60% dystrophin protein in the affected tissues. Systemic delivery of these AAV-ABE vectors effectively restored dystrophin expression and mitigated the pathologic hallmarks of DMD, encompassing histological changes, serum CK levels, and grip strength (Figure 1C-F). The therapeutic benefits were maintained for over 10 months. **Conclusions:** This distinctive DMD mouse model, which harbors the human genomic sequence, enables the *in vivo* evaluation of clinically relevant ABE gene editing strategies and other therapeutic interventions, signifying a substantial advancement towards the clinical application of adenine base editing for the correction of DMD in patients.

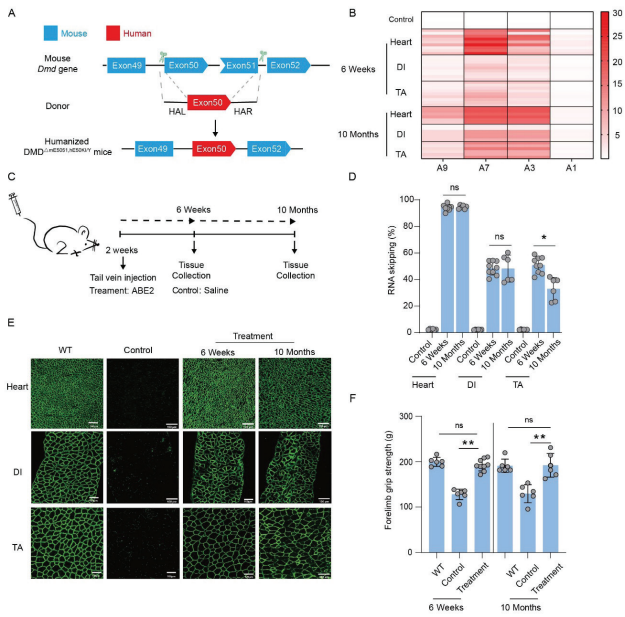


Figure 1. Intravenous delivery of the NG-ABE system efficiently rescues dystrophin expression and muscle function in humanized DMD mice. (A) Strategy for generating humanized DMD mouse model ($DMD^{\Delta mE50,51,\ hE50KI}$ mice). (B) Schematic of intravenous administration of NG-ABE particles. Measurement by deep sequencing of splicing site mutation (C) and exon skipping (D) in tibialis anterior, diaphragm, and heart after systemic delivery (n=6-9). (E) Immunohistochemistry analysis shows restoration of dystrophin expression in multiple muscle groups of $DMD^{\Delta mE50,51,\ hE50KI}$ mice 6 weeks and 10 months after injection of NG-ABE. Dystrophin was shown in green. (F) Forelimb grip strength was measured in WT, $DMD^{\Delta mE50,51,\ hE50KI}$ mice, and $DMD^{\Delta mE50,51,\ hE50KI}$ mice treated with NG-ABE particles (n=6-9).

1146. Precise Correction of Duchenne Muscular Dystrophy by Compact Cytosine Base Editor-Induced Exon Skipping

Guoling Li[1], Qingquan Xiao[1,2], Dingyi Han[2], Haoqiang Wang[1], Tingting Ma[1], Jingxing Zhou[1], Yu Zhang[1], Xiumei Zhang[1], Tong Li[1], Shaoran Wang[1], Yuan Yuan[1], Xuan Yao[1], Linyu Shi[1], Yingsi Zhou[1], Hainan Zhang[1], Hui Yang[1,2]

[1]HuidaGene Therapeutics Co., Ltd., Shanghai, China,[2]Institute of Neuroscience, Center for Excellence in Brain Science and Intelligence Technology, Chinese Academy of Sciences, Shanghai, China

Background: Duchenne muscular dystrophy (DMD) is a fatal, X-linked recessive neuromuscular disorder, with an incidence rate of approximately 1:3500-5000 in live male births. Although the CRISPR base-editor-mediated exon skipping is highly effective for most DMD cases, the length of the coding sequence is more than 4.7 kb, rendering its ability to be packaged into a single adeno-associated viral (AAV) vector. The use of dual AAV vectors to deliver the CRISPR/Cas9 system not only necessitates the increased clinical dosages, poising greater AAV-related safety risks but also faces the drawback of low *in-vivo* recombination efficiency of the split CRISPR system, thus compromising editing efficiency and limiting its clinical application. As an evolutionary ancestor of the Cas9 nuclease, the IscB protein serves as a compact RNA-guided DNA endonuclease, positioning it as a potent candidate for DMD treatment. **Methods:** We employed a computational pipeline to annotate IscB orthologs,CRISPR array, ωRNA, and target adjacent motif (TAM) preferences by data mining assembled bacterial genomes (Figure 1A). An EGFP-activation reporter system was designed and used to screen functional CRISPR-IscB systems in human cells. Protein engineering combined with ωRNA optimization was used to generate enhanced IscB variants with high editing efficiency and broad TAMs. Subsequently, we explored the potential of a compact cytosine base editor (CBE) derived from IscB variants to modify the splice acceptor (SA) sites of the *DMD* gene, to induce skipping of a common *DMD* deletion mutation of exon 51 via intramuscular injection of AAV-CBE (Figure 1D). This evaluation aimed to assess the extent of compact CBE-mediated exon skipping, dystrophin restoration, and phenotypic improvements of cardiac and skeletal muscles in a humanized DMD mouse model, in which the exon 50 and 51 of mouse *Dmd* gene were replaced with human exon 50 sequence (Figure 1E). **Results:** We have identified 10 out of 19 uncharacterized IscB proteins from uncultured microorganisms, which are active in mammalian cells (Figure A). Through protein and ωRNA engineering, we have further enhanced the activity of the IscB ortholog IscB.m16 and expanded its TAM range from MRNRAA to NNNGNA, producing a variant known as IscB.m16*. By fusing the deaminase domain with the IscB.m16* nickase, we have created a derived compact CBE termed IscB.m16*-CBE (Figure 1B,C). Subsequent delivery of the IscB.m16*-CBE via a single AAV vector to the humanized DMD mice demonstrated effective exon 50 skipping by modifying the SA site of the targeted exon and restoration of dystrophin protein expression in the humanized DMD mice (Figure 1F-H). **Conclusions:** The engineered compact IscB-derived base editors have demonstrated to be a platform capable of highly efficient, specific, and broad-TAM-scope DNA base

editing in mammalian cells and in mouse models of diseases, such as DMD, which facilitates the subsequent drug development of single AAV-delivered base editor for gene therapy.

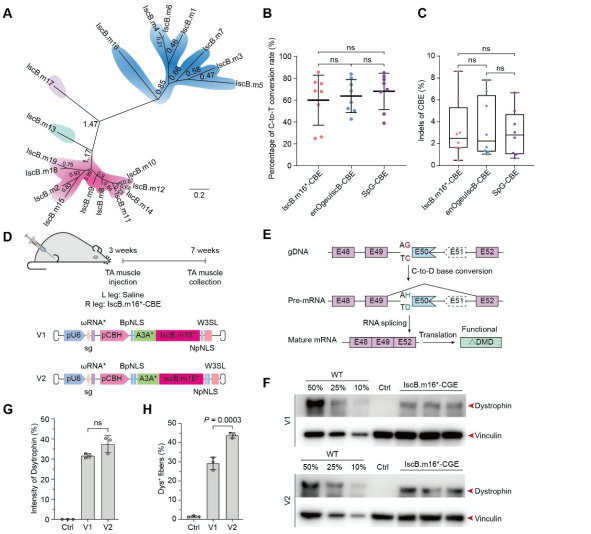


Figure 1. IscB.m16*-based cytosine base editor (CBE) mediates effective base editing and restores dystrophin expression in humanized DMD mice. (A) Phylogenetic tree of 19 uncharacterized IscB orthologs. (B) Comparison of the C-to-T conversion efficiency of IscB.m16-CBE, IscB.m16*-CBE, enOgueIscB-CBE and SpG-CBE at 8 target sites. (C) Indel efficiency of IscB- and SpG-derived CBEs at 8 endogenous loci. (D) Schematics of a single AAV carrying two different versions of IscB.m16*-CBE delivered to the muscles in mice via TA muscle injection. (E) Schematics of the strategy of IscB.m16*-CBE treating DMD. IscB.m16*-CBE disrupts the conserved guanine within the splice acceptor site for programmable exon 50 skipping, leading to the restoration of dystrophin expression. (F) Western blot analysis of dystrophin and vinculin expression in TA muscles 4 weeks after injection with AAV-IscB.m16*-CBE or saline. Quantification of dystrophin expression (G) and Dys+ fibers (H) in TA muscles.

1147. Therapeutic Exon Skipping Through Cytidine Base Editing *In Vivo*

Chunyan He, Xiao Li, Kaixuan Qiao, Pei Tao, Jinting Zhang, Kai Jiang, Qiuyu Cao, Guangyuan Xiao, Li Chen, Fengpeng Li

SuZhou GenAssist Therapeutics Co., Ltd, Suzhou, China

Background Loss of dystrophin protein causes Duchenne muscular dystrophy (DMD), characterized by progressive degeneration of cardiac and skeletal muscles. GEN60mE4 is a mouse DMD exon 4 skipping base editing product containing two AAV9 vectors, ss.AAV9.oTAM (optimized Targeted AID-mediated Mutagenesis) and ss.AAV9.mE4-sgRNA. GEN60mE4 target the IVS4 5'SS in the mouse Dmd gene, leading to interruption of the recognition of exon 4 during splicing process, resulted in exon 4 skipping. *Dmd*^{E4*} is a DMD mouse model has 4-bp deletion leading to dystrophin protein disruption. GEN60mE4 induced exon 4 skipping resulted in truncated yet in-framed dystrophin protein (**Fig.1**). **Method** PND21 *Dmd*^{E4*} /BL10 mice received a single IV administration of formulation buffer or different dose of GEN60mE4. Age-matched WT mice were included as controls. Interim and terminal sacrifices occurred at the end of 4-, 8-, and 28-weeks post-dose, respectively. **Results** The transcripts of oTAM were highly distributed in heart and skeletal muscles confirmed strong muscle specificity of SYN promoter. The sgRNA has been reported to be stabilized by forming a complex with Cas9 protein, similar expression pattern of mE4-sgRNA as oTAM was observed. Dose-dependent exon 4 skipping was observed in both heart and other tested muscular tissues. Especially in heart of 3E14 vg/kg GEN60mE4 group, exon skipping efficiency is close to 80%, the edited Dmd mRNA level was comparable to that of WT controls. The dystrophin protein reached approximately 20% or 7 % of the WT level in the heart or TA of the animals in 3E14 vg/kg group, respectively (**Fig.2**). Serum CK-MB exhibited a 2-fold decrease in 3E14 vg/kg group compared to untreated *Dmd*^{E4*} mice (P=0.0181). Deep RNA sequencing of heart tissue was performed to analyze the gene expression after treatment. The results showed that the genes that were differentially expressed in WT versus *Dmd*^{E4*} groups were highly rescued with GEN60mE4 treatment. Most importantly, TAM-based cytosine base editor induced non-detectable RNA off-target at the transcriptome level (**Fig.3**). Vector copies of oTAM and sgRNA gradually decreased over time over 28-week observation. Interestingly, the restored dystrophin protein dramatically decreased at 8-week and further catching up over 28-week in heart. The Dystrophin protein restored to 25.58% and 35.59% of WT level in 5E13 and 1E14vg/kg group at 28-week, respectively. Consistently, the editing efficiency decreased at 8-week in the heart of treated mice. In comparison, no decreased editing was observed in TA. The minimal editing efficiency in TA was observed (3%) at 4-week. At week 28, the editing efficiency accumulated to 12.4% in 5E13 or 11.28% in 1E14vg/kg treated mice (**Fig.4**). Immune response against transgene or AAV was evaluated at 8- and 28-week. Strong humoral and cellular immune response against AAV9 or cas9 but not dystrophin protein was observed (**Fig.5**), which might be a reason for the rapid reduction of editing efficiency at week 8 since the successfully transduced cells were easy to be attacked by immune cells. **Conclusion** These studies demonstrate that TAM BE can restore the dystrophin protein and provide some therapeutic potential with long term observation. Immune response against AAV and transgene should be considered before human therapeutic application of base editors.

Figure1. Schematic of GEN60mE4 induced exon 4 skipping in *Dmd*^{E4*} mice model

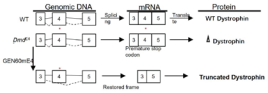
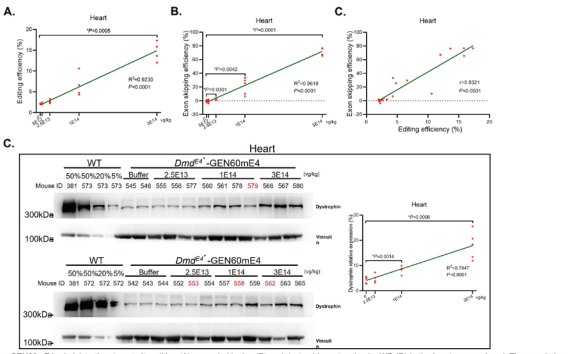
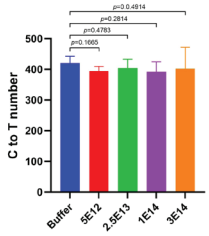


Figure2. GEN60mE4 induced target site editing, exon 4 skipping and dystrophin protein restoration in *Dmd*^{E4*} mice model



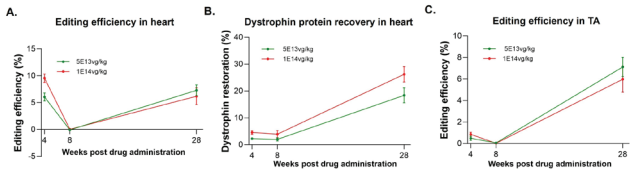
Four weeks after GEN60mE4 administration, target site editing (A), exon 4 skipping (B) and dystrophin restoration by WB (D) in the heart was analyzed. The correlation of target site editing and exon 4 skipping was analyzed (C). Statistical analysis was performed using simple linear regression model, two-tailed t-test was used for two groups comparison, *P*<0.05 was considered as statistically significant. N>=5 mice in each group.

Figure3. Non overall RNA off-target was observed



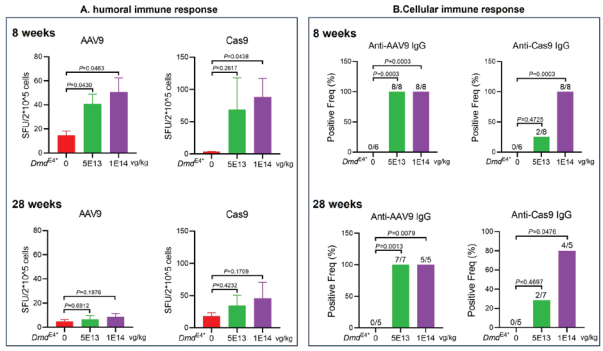
Four weeks after GEN60mE4 administration, RNAseq was performed in heart tissues, C to T conversion was calculated. Two-tailed t- test was used for two groups comparison, N=3 mice were included in each group.

Figure4. Target site editing and dystrophin protein restoration over 28-week observation



Target site editing (A and C), and exon 4 skipping was analyzed at 4-, 8- and 28-week after drug administration. N>=5 mice in each group.

Figure5. Immunogenicity analysis over 28-week observation



A. T cells that are stimulated by expsoure to AAV9 or SaCas9 to produce IFNγ as shown by enzyme-linked immune absorbent spot (ELISpot). SFU, spot-forming unit. B. Antibodies against AAV9 or SaCas9 are detected after 8- or 28-weeks. Two-tailed t-test was used for two groups comparisons, P < 0.05 was considered as statically significant, n numbers as indicated).

1148. Assessing the Burden of Duchenne Muscular Dystrophy and Willingness to Take Gene Therapy in China

Chunyan He[1], Guangyuan Xiao[1], Yi Dai[2]

[1]SuZhou GenAssist Therapeutics Co., Ltd, Suzhou, China,[2]Peking Union Medical Hospital, Beijing, China

Introduction: Duchenne muscular dystrophy (DMD) is a devastating genetic disorder characterized by progressive muscle weakness. Individuals with DMD experience muscle weakness, which often leads to the use of wheelchairs during adolescence and finally resulting in cardiac and respiratory failure in their 20-30s. While the natural history of the disease is relatively well understood, knowledge about the overall costs of managing DMD patients in China remains largely unknown. **Objective:** The aim of this study was to assess the comprehensive cost of illness and economic impact associated with Duchenne muscular dystrophy (DMD) in China. In addition, the willingness to gene therapy were evaluated. **Methods**: The questionnaire was designed, distributed, and collected utilizing the online survey function of WeChat app, which is shared within patient organization groups from October 17, 2023, to November 3, 2023. Identification of patients with DMD is through their WeChat ID, telephone number, date of birth, and self-reported mutation type. Information on healthcare utilization, quality of life, employment status, and household expenses is gathered to calculate the costs associated with DMD from both the societal perspective and the perspective of caregiver households. Additionally, the willingness of patients to participate in clinical trials and how much they are willing to pay for gene therapy were collected. **Results:** The questionnaire was completed by a total of 2287 patient-caregiver pairs from 31 provinces in China. Among those patients, 13.55% were under 3 years old, 39.92% were in the early ambulatory stage, 34.85% were in the late ambulatory, and the remaining 11.67% were in the non-ambulatory stage (**table 1**). The total mean per-Patient annual cost of illness was estimated 128629 ￥. Mean per-Patient annual direct cost of illness was estimated 67749 ￥, the median is 42500￥(**table 2**). Indirect costs were substantial, constituting 34% of total costs (**Figure 1A**). There is an increasing trend in hospital expenses, travel for medical, rehabilitation, and wheelchair costs as the disease progresses to the early non-ambulatory stage, but a decrease is observed in the late non-ambulatory stage (**Figure 1B**). Most of patients express a strong willingness to participate in gene therapy clinical trials, with 52.92% patients preferring for enrollment in Phase III clinical trial (**Figure 2A**). Although with substantial economic burden of DMD patients, most of the patients are willing to pay for gene therapy. Notably, 80% of patients choose 30% down payment (**Figure 2B**) and more than 40% patients are willing to pay above 1million￥as down payment for gene therapy(**Figure 2C**). **Conclusion**: We show that DMD is associated with a substantial economic burden. Most of the DMD patients are willing to participate in gene therapy, with more than 40% are willing to pay around three million￥. We first described the economic context of a rare disease DMD, serves as important intelligence input to health policy evaluations of intervention programs and novel therapies, financial support schemes for patients and their families, and the design of future cost studies. **Limitation**: The questionnaire is exclusively obtained from WeChat app, in which the patients' identity cannot be fully confirmed. Most questionnaire providers are in the ambulatory stage (88.33%), which has a strong bias to underestimate the annual cost for all stage of DMD patients.

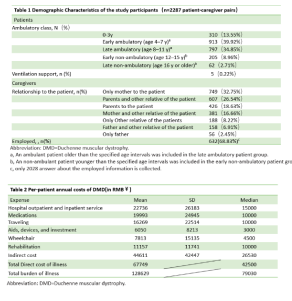


Table 1 Demographic Characteristics of the study participants (n=2287 patient-caregiver pairs)

Patients			
Ambulatory class, N (%)			
	0-3y	310	(13.55%)
	Early ambulatory (age 4-7 y)[a]	913	(39.92%)
	Late ambulatory (age 8-13 y)[a]	797	(34.85%)
	Early non-ambulatory (age 12- 15 y)[b]	205	(8.96%)
	Late non-ambulatory (age 16 y or older)[b]	62	(2.71%)
Ventilation support, n (%)		5	(0.22%)
Caregivers			
Relationship to the patient, n(%)	Only mother to the patient	749	(32.75%)
	Parents and other relative of the patient	607	(26.54%)
	Parents to the patient	426	(18.64%)
	Mother and other relative of the patient	381	(16.66%)
	Only Other relative of the patients	188	(8.22%)
	Father and other relative of the patient	158	(6.91%)
	Only father	56	(2.45%)
Employed, , n(%)		69268	8.97%

Abbreviation: DMD=Duchenne muscular dystrophy.
a, An ambulant patient older than the specified age intervals was included in the late ambulatory patient group.
b, An non-ambulant patient younger than the specified age intervals was included in the early non-ambulatory patient group.
c, only 2036 answer about the employed information is collected.

Table 2 Per-patient annual costs of DMD(in RMB￥)

Expense	Mean	SD	Median
Hospital outpatient and inpatient service	22756	26183	15000
Medications	19960	24845	10000
Traveling	16269	22514	10000
Aids, devices, and investment	6030	8213	3000
Wheelchair	7813	15155	4500
Rehabilitation	11157	13743	10000
Indirect cost	44611	42441	26540
Total Direct cost of illness	67749		42500
Total burden of illness	128629		79030

Abbreviation: DMD=Duchenne muscular dystrophy.



Figure 1. Components of annual cost of Duchenne muscular dystrophy and Mean per-patient annual cost of illness in different ambulatory stages. A) Percentage of each components of annual cost for DMD. Traveling including fees for transportation and hotels. Aids, devices, and investments include investments to and reconstructions of the home (e.g., fees for using stair board, corrective shoes.) B) The annual cost for each components in different ambulatory stage of DMD patients.

Figure 2. A) The distribution of patients willingness to participate into Phase I, Phase II, Phase III clinical trials. B) The percentage of patients' willingness to make various down payments, including 30%, 50%, 70%, and full payment. C) The percentage for the maximum down payment for patients can afford for gene editing therapy, including 1million,1.5 million, 2million, 3million, 4million and less than 1 million(all in RMB￥)

1149. Single Cell Analysis of Duchenne Muscular Dystrophy Skeletal Muscle Post Micro-Dystrophin Gene Therapy

Stanley Nelson[1], Kevin Chesmore[1], Deirdre Scripture-Adams[2], Florian Barthelemy[2], Richard Wang[1], Emilie Douine[1], Isaiah Little[2], Shirley Nieves-Rodriguez[1], Artemis Yip[2], Nicole Steady[2], Rosario Corona[1], M Carrie Miceli[3]

[1]Human Genetics, UCLA, Los Angeles, CA,[2]MIMG, UCLA, Los Angeles, CA,[3]CDMD, UCLA, Los Angeles, CA

Skeletal muscle is a cellularly complex and plastic tissue that responds to injury throughout life by recruiting and expanding immune cells and fibroblasts to guide muscle stem cell mediated repair of damaged multinucleated myofibers. Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy accounting for 80% of deaths from muscular dystrophy before the age of 30 years. The lack of dystrophin, caused by mutations in *DMD*, leads to chronic and synchronous myofiber damage, inflammation, immune infiltration, dysregulation of repair, fibro-fatty replacement, and progressive loss of muscle function. Recently, micro-dystrophin gene therapy (GT) and mRNA exon skipping demonstrate replacement/repair of the missing dystrophin protein in myofibers helps to slow the disease progression. We have created a dataset of single nuclei/cell data from dozens of DMD individuals throughout the disease course and from some individuals post gene therapy from an amalgamated dataset of over 400,000 transcriptomes to reveal changes in the multi-cellular environment and cell-cell communications within dystrophic muscle using single cell and nuclei transcriptomics and infer muscle stem cell defects, mechanisms of fibroblast-mediated inflammation/fibrosis, and immune activation to reveal novel disease biomarkers and therapeutic targets.

1150. Fibrosis Restricts rAAV Gene Transfer and Can be Relieved by FAP-Specific CAR T Cells in a Murine Model of Duchenne Muscular Dystrophy

Maxime Ferrand[1], Sonia Albini[1], Céline Rocca[1], Valentina Buffa[1], Isabelle Richard[1], Anne Galy[1,2]

[1]UMR_S951 Integrare Research Unit, Genethon, Evry-Courcouronnes, France,[2]Art-tg, Inserm, Corbeil-Essonnes, France

Duchenne muscular dystrophy (DMD) is a muscle disorder that progressively weakens skeletal and cardiac muscles and causes fibrosis in muscle tissues. In the DBA2mdx mouse model of severe DMD, rapid and intense fibrosis was observed, characterized by dense collagen deposits in skeletal muscles, with over-expression of the FAP and Col3 genes in the TA, GA, EDL muscles at 2, 3, and 4 months of age compared to their aged-matched DBA2 littermates. It is hypothesized that fibrosis may limit the effectiveness of gene transfer therapy in DMD but direct evidence is lacking and strategies to reduce skeletal muscle fibrosis are limited. Because FAP-specific CAR-T cells were shown to reduce drug-induced cardiac fibrosis in mice, we tested whether FAP-specific CAR-T cells could also reduce the broad and spontaneously occurring skeletal muscle fibrosis in DBA2mdx mice. A FAP-CAR lentiviral vector (LV) encoding a third-generation CAR with an mFAP-specific ScFv was generated and validated on FAP-overexpressing 3T3 cells. The FAP-CAR LV was used to generate FAP-specific cytotoxic T cells from DBA2 spleen cells. Two consecutive administrations of FAP-CAR T cells to DBA2mdx mice reduced the histological and molecular biomarkers of fibrosis, as measured 2 weeks post-cell injection. When FAP-CAR T cells were injected into DBA2mdx mice before the intravenous administration of rAAV9 encoding a microdystrophin (5xE12 vg/Kg), there was an increased gene transfer efficacy as demonstrated by higher viral genome and transgene expression in TA and EDL muscles compared to controls. These results demonstrate for the first time that fibrosis acts as a restriction to rAAV gene transfer and provide a novel therapeutic option to treat muscular dystrophies by combining gene therapy and immunotherapy. *MF and SA contributed equally. IR and AG are co-senior authors of this study.*

1151. Utilizing Gene Therapy in Duchenne Muscular Dystrophy - Impact of Continuing Medical Education on Participant Knowledge and Behavior

Vandana Gupta[1], Carole Drexel[1], Crystal Proud[2]

[1]PlatformQ Health Educaton, Needham, MA,[2]Children's Hospital of The King's Daughters, Norfolk, VA

Background: In June 2023, the US Food and Drug Administration (FDA) approved the first gene therapy (GT) for the treatment of patients with Duchenne muscular dystrophy (DMD). We sought to characterize the impact of continuing medical education (CME) on healthcare professionals' (HCPs') awareness of the rationale for and data related to GT for DMD, as well as their ability to recognize and counsel patients who are candidates for GT. **Methods:** On September 15, 2023, we collaborated with the American Society of Gene & Cell Therapy (ASGCT) to launch a live, online, 1-hour, CME-certified activity featuring key opinion leaders experienced with newly available GT for DMD. The activity will remain on-demand for 1 year. Topics included: 1) rationale for GT in DMD, 2) clinical trial findings, and 3) pre-, peri-, and post-GT practical considerations, such as patient preparation, adverse-event management, and multidisciplinary care delivery. Session components included downloadable resources, live polling, and an audience real-time question-and-answer session. Educational impact was evaluated with knowledge and competence questions administered pre- and immediately post-activity (pre vs post). Chi-squared tests were used for statistical analysis. **Results:** As of January 2023, 1,003 HCPs from a variety of specialties (79% neurologists) had participated in the activity. 46% self-identified as prescribers seeing an average of 7 DMD patients per month. At baseline, only 30% of neurologists and 19% of all other learners were confident in their ability to counsel patients with DMD and their caregivers about GT. After the CME activity, there were marked improvements in knowledge and competence in each of the topics covered. Overall scores demonstrated learning gains on pre- vs post-education assessments (neurologists 72% pre vs 83% post; other learners 43% pre vs 58% post). Post activity, 42% of learners indicated that they intended to implement a practice change related to treatment, such as referral to specialty care. Learners also reported a need for greater understanding of the role of genetic testing in treatment decision-making (28%), and for strategies to address diagnostic challenges (16%). **Conclusions:** CME was successful in improving HCPs' ability to discuss the topic of GT with their patients by enhancing their knowledge and competence in critical areas related to the implementation of GT. Data also highlight a continued need for education related to DMD diagnosis, including genetic testing, which is an important issue based on the number of GTs on the horizon for DMD and other neuromuscular diseases.

1152. Immunomodulatory Effects of Amnion-Derived Mesenchymal Stromal Cells Preserve Muscle Function in a Mouse Model of Duchenne Muscular Dystrophy

Yuko N. Kasahara[1], Soya Nakayama[2], Koichi Kimura[1], Sho Yamaguchi[2], Yuko Kakiuchi[1], Chikako Nito[3], Masahiro Hayashi[2], Tomoyuki Nakaishi[2], Yasuyoshi Ueda[2], Takashi Okada[1]

[1]The Institute of Medical Science, The University of Tokyo, Tokyo, Japan,[2]Kaneka Corporation, Kobe, Japan,[3]Nippon Medical School, Tokyo, Japan

[Background] Duchenne muscular dystrophy (DMD) is an incurable genetic disease characterized by degeneration and necrosis of myofibers, chronic inflammation, and progressive muscle weakness resulting in premature mortality. Immunosuppressive multipotent mesenchymal stromal cell (MSC) therapy could be an option for DMD patients. We focused on human amnion-derived mesenchymal stromal cells (hAMSCs), a clinically viable cell source owing to their unique characteristics, such as non-invasive isolation, mitotic stability, ethical acceptability, and minimal risk of immune reaction and cancer. We aimed to identify novel immunomodulatory effects of AMSCs on macrophage polarization and their transplantation strategies for the functional recovery of skeletal and cardiac muscles. **[Methods]** We used flow cytometry to analyze the expression of anti-inflammatory M2 macrophage markers on human peripheral blood mononuclear cells (hPBMCs) co-cultured with hAMSCs. hAMSCs were intravenously injected into DMD model mice (*mdx* mice) to assess the safety and efficacy of therapeutic interventions. hAMSC-treated *mdx* mice were monitored using blood tests, histological examinations, spontaneous wheel-running activities, grip strength, and echocardiography. **[Results]** hAMSCs induced M2 macrophage polarization in hPBMCs via prostaglandin E_2 production. After repeated systemic hAMSC injections, *mdx* mice exhibited a transient downregulation of serum creatin kinase. Limited mononuclear cell infiltration and a decreased number of centrally nucleated fibers were indicative of regenerated myofibers following degeneration, suggesting an improved histological appearance of the skeletal muscle of hAMSC-treated *mdx* mice. Upregulated M2 macrophages and altered cytokine/chemokine expressions were observed in the muscles of hAMSC-treated *mdx* mice. During long-term experiments, a significant decrease in the grip strength in untreated *mdx* mice significantly improved in the hAMSC-treated *mdx* mice. hAMSC-treated *mdx* mice maintained running activity and enhanced daily running distance. Notably, the treated mice could run longer distances per minute, indicating high running endurance. Left ventricular function in DMD mice improved in hAMSC-treated *mdx* mice. **[Conclusions]** Early systemic hAMSCs administration in *mdx* mice ameliorated progressive phenotypes, including pathological inflammation and motor dysfunction, resulting in the long-term improvement of the skeletal and cardiac muscle function. The therapeutic effects might be associated with the immunosuppressive properties of hAMSCs via M2 macrophage polarization. Although gene therapy and exon skipping are already approved for DMD drugs, hAMSCs treatment strategy could provide therapeutic benefits to DMD patients in the cases where these therapies are not indicated.

1153. Characterization of rAAV Genomic Integrity by Long-Read Sequencing of AAV9-MyoDys$^{45\text{-}55}$, a Gene Editing Therapy for Duchenne Muscular Dystrophy

Elizabeth Gibbs[1], William Dorst[1], Michael R. Emami[1], Laura Hagerty[1], Niclas E. Bengtsson[2], April D. Pyle[3], Melissa J. Spencer[3], Courtney Young[1]

[1]MyoGene Bio, San Diego, CA,[2]University of Washington, Seattle, WA,[3]UCLA, Los Angeles, CA

Duchenne muscular dystrophy is a fatal muscle wasting disease typically caused by out-of-frame mutations in the *DMD* gene resulting in a loss of the dystrophin protein. Thus, restoration of the reading frame offers a therapeutic approach which would turn Duchenne into a mild form of the allelic disease, Becker muscular dystrophy. Gene editing offers a reframing approach that would permanently change the patient's own DNA and retain more of the protein coding sequence than AAV-based gene replacement therapies. We have developed a gene editing therapy, MyoDys$^{45\text{-}55}$, that generates an in-frame deletion of exons 45-55, mimicking one of the mildest versions of Becker where some patients have been asymptomatic into their 60s. Importantly, deletion of this hotspot region would be applicable for 50% of Duchenne patients. We are currently developing a dual vector adeno-associated virus (AAV) delivery approach for MyoDys$^{45\text{-}55}$ where one vector carries Cas9 and one carries the guide RNAs. Here we describe long-read PacBio sequencing of the AAV cargos. Long-read sequencing is useful for understanding the packaging of AAV by sequencing the cargo in a single read. Since our Cas9 vector is slightly oversized and the guide RNA vector contains secondary structures, fully understanding the packaged genomes is critically important. For each vector, we sequenced AAV produced by three different commercial vendors and compared genomic integrity and contamination. These data demonstrate we do observe complete packaging of both vectors although some preps of the Cas9 vector show higher levels of truncation. This work supports the comprehensive assessment of AAV9-MyoDys$^{45\text{-}55}$ for Duchenne, which aims to permanently restore the reading frame for a large proportion of patients.

1154. MyoAAV-Mediated Systematic Delivery of Full-Length Dystrophin Improved Histopathology and Function in DMD Mice

Chen Zhang, Yuan Zhou, Renzhi Han

Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN

Recently, the US Food and Drug Administration (FDA) granted accelerated approval to Sarepta's Elevidys for Duchenne muscular dystrophy (DMD) patients at 4 to 5 years of age. Elevidys and other similar gene therapy approaches for DMD utilize adeno-associated virus (AAV) to deliver miniaturized dystrophin, also known as micro-dystrophin (μDys). However, due to the lack of 2/3 of the dystrophin coding sequence containing critical rod and hinge domains of dystrophin needed to connect with other proteins in the dystrophin complex, these μDys gene therapies are unable to provide full protection of skeletal and heart muscle integrity and function. Here we developed a novel triple vector system to address this limitation, aiming

to deliver full-length dystrophin (FL-dystrophin) into striated muscles. The FL-dystrophin is strategically split into three fragments (N, M, and C), fused with two orthogonal pairs of split intein. This design enabled the efficient and unidirectional assembly of FL-dystrophin. These three fragments were packaged into a recently engineered myotropic AAV capsid (MyoAAV4A). Systemic delivery of MyoAAV4A leads to the restoration of FL-dystrophin and the dystrophin-glycoprotein complex components at the sarcolemma of both skeletal and cardiac muscles in a mouse model of DMD. Notably, muscle histopathology, contractility, and overall strength were significantly improved following MyoAAV4A delivery. In summary, the FL-dystrophin gene therapy promises to offer maximum protection for DMD, addressing the limitations of the current μDys-based gene therapy.

1155. Targeting Muscle Stem Cells for Permanent Correction of Dystrophin Expression in Duchenne Muscular Dystrophy

Stephen D. Hauschka, Jeffrey S. Chamberlain, Niclas E. Bengtsson

University of Washington, Seattle, WA

Our longtime research efforts have been focused on correcting dystrophin expression and pathophysiology in Duchenne muscular dystrophy (DMD). An integral part of these efforts has involved development of miniaturized muscle-specific gene regulatory cassettes (RCs) to express high levels of therapeutic proteins in striated muscle following Adeno-associated viral (AAV) vector-mediated delivery. To date, these have been used to restore expression of dystrophin in cardiac and post-mitotic skeletal muscles via AAV-mediated gene transfer of ectopically expressed dystrophins, or via editing of the endogenous dystrophin gene using CRISPR/Cas9. However, the lifelong workloads of skeletal muscles may cause therapeutic efficacy to decrease over time due to muscle remodeling in response to growth, maintenance, exercise, or trauma. Thus, sustained therapeutic outcomes could benefit greatly from co-treatment of muscle stem cells (satellite cells, or SCs), in which successful gene editing of dystrophin mutations could provide newly regenerated myofibers with a lifelong supply of myonuclei that express functional dystrophins. Studies have suggested that at least some SCs and/or their replicating progeny can be transduced by AAV vectors to express delivered cargo genes using constitutively active promoters. However, non-specific Cas9 expression raises potential safety issues due to elevated immunogenicity and unintended editing effects in non-muscle cells. We are thus developing AAV packaging size-compatible RCs derived from highly conserved gene regulatory regions of *PAX7* and *MYOD1* (genes expressed in SCs, and myoblasts). SC RCs containing single as well as multiple *PAX7* and *MYOD1* regulatory regions or mosaic combinations (400 to 1800 base pairs), exhibit a range of activities in primary SC-derived myoblasts *in vitro* as well as in FACS-isolated SCs from wildtype versus dystrophic mice following AAV transduction. Promising RC candidates are currently being used to optimize dystrophin gene editing in SCs of DMD mice. Further tests also involve screening for AAV capsids that allow for the most efficient SC transduction, as well as exploring SC RC design enhancements via miniaturization and multimerization of the most active sequences to boost activities. Safe and effective correction of dystrophin mutations in SCs using promising RC candidates from this study in conjunction with dystrophin replacement or gene editing of postmitotic myonuclei, constitutes an instrumental element for addressing current limitations of skeletal muscle gene therapies aiming to treat DMD and potentially many other genetic muscle diseases.

Thursday Posters: Genetic Disorders of the Blood and Immune System

1156. Efficient rAAV6-Mediated HDR with Low Toxicity as a Therapy for GATA2 Deficiency Through Allele-Specific Gene Correction in Hematopoietic Stem Cells

Thomas W. Skov[1], Jonas H. Wolff[1], Didde Haslund[2], Anne L. S. Revenfeld[2], Bjarne K. Møller[2], Trine H. Mogensen[1], Rasmus O. Bak[1], Jacob G. Mikkelsen[1]

[1]Department of Biomedicine, Aarhus University, Aarhus C, Denmark,[2]Department of Clinical Immunology, Aarhus University Hospital, Aarhus N, Denmark

GATA2 deficiency is an autosomal dominant disorder caused by mutations in the gene encoding the transcription factor GATA2, leading to hematopoietic stem cell (HSC) dysfunction. This condition is associated with a reduction in immune cells, known as cytopenia, and a substantially increased risk of developing malignant blood diseases. Our study focuses on developing a novel therapy for GATA2 deficiency through ex vivo allele-specific correction of the *GATA2* variant c.956_962delGTGGCCT based on Cas9/sgRNA RNP delivery via nucleofection, coupled with DNA donor delivery for homology-directed repair (HDR) using rAAV6. Through the screening of multiple sgRNAs, we identified a sgRNA that exhibited no activity against the WT *GATA2* sequence and efficiently facilitated indel formation in the c.956_962delGTGGCCT allele in K562 cells, peripheral blood mononuclear cells (PBMCs), and hematopoietic stem and progenitor cells (HSPCs). In K562 cells engineered to carry c.956_962delGTGGCCT, co-delivery of Cas9/sgRNA RNPs and a rAAV6 vector carrying a DNA donor designed to correct the variant resulted in complete correction (>99% HDR) and fully restored GATA2 protein levels. Off-target analyses using DISCOVER-seq and subsequent high-throughput amplicon sequencing revealed a single bona fide off-target site in the lncRNA gene *AATBC*, which was targeted by HiFi Cas9 RNPs with 5.25% indel frequency in patient-derived PBMCs. Initially, editing of HSPCs using RNP nucleofection and rAAV6 donor delivery at an MOI of 2500, yielding an HDR rate of 71%, resulted in high toxicity and a severe reduction in colony-forming units (CFUs). Modification of medium composition, codelivery of multiple different mRNA transcripts to mitigate toxicity and increase HDR (GSE56, Ad5-E4orf6/7, and i53), and a 10-fold reduction in rAAV6 MOI alleviated toxicity while maintaining an HDR efficiency of 35% with no reduction in viability and CFUs. We evaluated the impact of *GATA2* editing in HSPCs by examining their differentiation through CFU assays, comparing normal HSPCs to those with a *GATA2* knockout (KO). *GATA2* KO increased the proportion of macrophage colony-forming units (CFU-M) at the expense of granulocyte-macrophage (CFU-GM) and erythroid colonies, along with a marked reduction in cell proliferation. Rescue of the KO through HDR partially

recovered differentiation and proliferation in the HSPCs to a level corresponding to the HDR rate. To investigate the potential selective advantage of *GATA2*-corrected HSCs *in vivo*, immunodeficient mice were engrafted with mixed populations of HSPCs containing cells engineered to carry either (i) the GATA2 c.956_962delGTGGCCT variant or (ii) silent mutations in *GATA2*. We found a significant enrichment of non-disruptive mutations after engraftment, suggesting that HSCs with functional *GATA2* outcompeted HSCs carrying defective *GATA2* alleles. Notably, using the optimized editing protocol in patient-derived HSPCs, we achieved 82% and 73% correction of the c.956_962delGTGGCCT allele at MOIs of 500 and 250, respectively. Ongoing studies focus on engraftment of corrected patient-derived HSPCs in immune-deficient mice, exploration of potential unintended large on-target modifications, and further investigation of potential off-target sites in patient-derived HSPCs using DISCOVER-seq.

1157. Expansion of CD33/HBG-Promoter Edited HSPCs with Mylotarg for *In Vivo* HSC Gene Therapy of Hemoglobinopathies

Aphrodite Georgakopoulou[1], Chang Li[1], Hans-Peter Kiem[2], Andre Lieber[1]

[1]University of Washington, Seattle, WA,[2]Fred Hutch, Seattle, WA

We developed an *in vivo* HSC gene therapy approach that consists of HSC mobilization and intravenous injection of HSC-tropic HDAd vectors. To achieve therapeutically relevant numbers of corrected cells, we incorporated *in vivo* expansion of transduced cells. We used an HDAd vector for a multiplex adenine base editing approach to *i)* remove the region within CD33 that is recognized by gemtuzumab ozogamicin (GO) (Mylotarg™), and *ii*) create therapeutic edits within the HBG1/2 promoters to reactivate γ-globin/HbF. *In vitro* studies with HDAd-transduced human CD34$^+$ cells showed editing of both targeted sites and a 2- to 3-fold GO-mediated expansion of edited erythroid/myeloid progenitors. After erythroid *in vitro* differentiation, up to 40% of erythrocytes were HbF positive. For *in vivo* studies, mice were transplanted with human CD34$^+$ cells. After engraftment, HSCs were mobilized with G-CSF/AMD3100 followed by an intravenous HDAd injection and GO-mediated *in vivo* selection. Two months later, editing in human cells within the bone marrow was significantly higher in GO-treated mice. The percentage of HbF$^+$ human erythroid cells was 2.5-fold greater compared to untreated mice. These data indicate that *in vivo* GO selection can increase edited erythroid cells to levels that are clinically relevant for treatment of hemoglobinopathies. These findings should also have important implications beyond hemoglobinopathies since the ability to use nongenotoxic in vivo selection / enrichment approaches of genetically modified cells can also be applied to other genetic diseases.

1158. A Comparison of Red Blood Cell (RBC) Functional Biomarkers in Successful Hematopoietic Stem Cell Transplantation (HSCT) versus Graft Failure

Aliya U. Zaidi[1], Rasa Borhan[1], Robert Goodrich[1], Marta Ferranti[1], Xiufeng Gao[1], Cynthia Joseph[2], Emily Limerick[2], Courtney D. Fitzhugh[2], Patrick C. Hines[1,3,4]

[1]Functional Fluidics Inc., Detroit, MI,[2]NHLBI/NIH, Bethesda, MD,[3]Division of Pediatric Critical Care Medicine, Wayne State University, Detroit, MI,[4]Division of Pediatric Critical Care Medicine, Wayne Pediatrics, Detroit, MI

Introduction: Sickle cell disease (SCD) is characterized by a mutation in the hemoglobin subunit β (HBB). Allogeneic HSCT is the most widely available cure, and the use of haploidentical donors has expanded the donor pool. Successful HSCT (S-HSCT) is associated with absence of SCD manifestations, including pain, normalized hemoglobin (Hb), and reversal of hemolysis. Critical aspects of red blood cell health (RBCH), such as adhesion and Hb polymerization, can predict future SCD severity and may provide earlier and more precise insight into HSCT efficacy. We assessed changes in flow adhesion of whole blood to vascular cell adhesion molecule (FA-WB-VCAM) and P-selectin (FA-WB-Psel), and RBC sickling with the dynamic sickling assay (DSA) before and after HSCT. Graft failure (GF), pain, opioid use, and clinical labs were captured. **Methods:** Samples were collected from 4 SCD subjects and 4 donors enrolled in NHLBI HSCT protocol 17-H-0069 or 000539-H. FA-WB-VCAM and FA-WB-Psel utilized a microfluidic well-plate system and were reported as cells/mm^2. DSA samples were exposed to a proprietary enzymatically-induced hypoxic environment, time-lapse photomicroscopy and AI-based SICKLE image analysis software quantifying RBC sickling. Parameters measured include: time to 5% and 50% morphological point of sickling (mPoS@5%, mPoS@50%), maximum rate of sickling (% /min), maximum sickling (%), and AUC10 (%*min). All values presented are mean ± standard error; $p<0.05$ considered statistically significant. **Results:** 3 subjects had S-HSCT, 1 experienced GF. In S-HSCT, Hb increased from 9.2±0.3 to 11±2 g/dL, bilirubin was reduced significantly from 1.7±0.3 at BL to 0.4±0.06 mg/dL at 6M ($p<0.01$). FA-WB-VCAM and FA-WB-Psel BLs in S-HSCT were 369±63 and 36±18 cells/mm^2, respectively. FA-WB-VCAM decreased significantly (26±9 cells/mm^2, $p<0.01$) by 3M and sustained at 6M (59±8 cells/mm^2, $p<0.01$). Although FA-WB-Psel decreased p-HSCT, it was not statistically significant. In GF, FA-WB-VCAM increased p-HSCT and FA-WB-Psel adhesion doubled from BL to 6M. Max rate of sickling in S-HSCT significantly decreased from 38±7 to 1.1±0.7 %/min ($p<0.01$) at 3M, and further to 0.4±0.4 %/min ($p<0.01$) at 6M. In GF, an initial reduction in sickling rate from BL to 3M was followed by an increase at 6M p-HSCT. Max sickling in S-HSCT significantly decreased from 0.68±0.16 at BL to 0.1±0.01 ($p=0.02$) and 0.06±0.01 ($p=0.02$) at 3M and 6M, respectively. In contrast, max sickling increased in GF from a BL of 0.50 to 0.75 at 6M (Fig.1). **Conclusions:** RBC health normalizes following S-HSCT as evidenced by statistically significant reductions in adhesive properties, reduced sickling rate and amplitude within 3 months. The subject experiencing GF demonstrated a worsening of adhesive and sickling parameters of RBC health consistent with sickle RBCs. A longer post HSCT follow-up period is needed to establish clinical improvement following S-HSCT, but reduced pain and lower

opioid usage in patients is encouraging. These findings suggest that FA-WB-VCAM, FA-WB-Psel and DSA could serve as objective indicators of RBC health normalization p-HSCT. Future studies will determine if these biomarkers could be surrogates for long-term clinical efficacy.

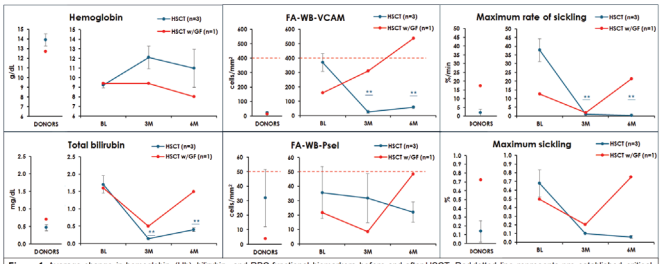


Figure 1. Average change in hemoglobin (Hb), bilirubin, and RBC functional biomarkers before and after HSCT. Red dotted line represents pre-established critical threshold. * *p*-values <0.05 ** *p*-value<0.01

1159. Comparative Interrater Analysis of Regulatory Challenges in Recently Approves Gene Therapies for Sickle Cell Disease: Towards a Semi-Quantitative Method for Assessing Gene Therapy Regulatory Development Complexity

Angela N. Johnson[1,2], Limin Wang[3], Johanna Nurbo[1], Arthur Fulgoni[4], Andreia Domingues[5], Sandra Racordon-Pape[5]

[1]Global Regulatory, Cytiva, Boston, MA,[2]School of Professional Studies, Regulatory Sciences, Northeastern University, Boston, MA,[3]Regulatory Sciences, Cytiva, Marlborough, MA,[4]Biopharma Product Regulatory Compliance, Cytiva, Boston, MA,[5]Regulatory Sciences, Cytiva, Boston, MA

Background: Standardized methods are needed in regulatory science to enable objective evaluation of regulatory complexities for gene therapies. In December 2023, two landmark gene therapies came to market with United States Food and Drug Administration (FDA) approval for treating sickle cell disease (SCD), using a lentiviral vector platform (*lovotibeglogene autotemcel*, LYFGENIA®, Bluebird Bio) and first-in-class CRISPR (*exagamglogene autotemce*l, CASGEVY®, Vertex). The CRISPR product functions by editing (or "cutting") a specific gene that enables reactivation of fetal hemoglobin production to dilute faulty red blood cells, while the lentiviral therapy employs a viral envelope to deliver a healthy hemoglobin-producing gene *in vivo*. This research aims to develop and employ a standard multi-rater instrument to examine relative complexity of regulatory domains of these products. **Methods:** US FDA approved labeling and summary basis for approval documents (FDA.gov) for *lovotibeglogene autotemcel* (Bluebird Bio) and *exagamglogene autotemce*l (Vertex) were analyzed by 3 independent expert regulatory science raters with 5Y+ experience. Domain areas of regulatory complexity were agreed prospectively between raters, and the resultant instrument was used by each rater independently to record differences in regulatory development effort/complexity level between the 2 products on a 5-point Likert scale (1 = no significant difference, 5 = highly differentiated). Interrater reliability (IRR) was assessed, and results were summarized with descriptive statistics using SAS® 9.4 TS1M8. **Results:** Regulatory expert raters assessed that the most substantive developmental differences in the two commercial products were severe reaction warnings (mean 4.7±0.6), limitations of use (3.7±1.2), and labelled adverse reactions with the potential to trigger need long-term follow-up (4.3±0.6). All other differences were ranked <2.7, with minor differences in mobilization and apheresis in product manufacture (2.0±1.0), cryopreservation manufacturing practices (2.0±0), and long-term follow-up study design and complexity (2.3±0.6) observed, along with notes on variations in product positioning domains. IRR was high (>0.6), except in rater evaluation of cryopreservation complexity (i.e. suspension methods, use of bags vs vials), importance of hypersensitivity reactions and relative importance of addressing differences in preparation for infusion (centrally specificity around number of days prep, differing between the two products), which may reflect variable manufacturing vs clinical experience between raters. **Conclusions:** Modern regulatory science has often been critiqued for deploying subjective, anecdotal methods with variable and non-replicable outcomes. Using this proof of concept data, we propose that standard assessment tools help us move the needle towards highly replicable regulatory assessments, potentially useful in policy making, drug development strategy, assessing efficacy of regulatory programs, and fostering drug developer-supplier-regulatory discourse on regulatory outcomes.

Regulatory Domain	3-rater Mean±SD	IRR
MATURITY OF SAFETY DATA		
Labelled Warnings	4.7±0.6	0.7
Hypersensitivity, *including raw materials and animal products constituents*	1.0±0	0.2
Clinical trial reported safety data	4.3±0.6	0.6
Nonclinical toxicology program	1.0±0	1
PRODUCT POSITIONING		
Scope and Specificity of Indication language	1.3±0.6	0.7
Specificity of Indications for Use	1.7±1.2	0.7
Labelled Limitations of Use	3.7±1.2	0.7
Description of drug product	2.7±2.1	0.2
Description of cell line and vectors	3.5±0.7	0.6
Description of Mechanism of Action	3.0±0	1
DOSE & ADMINISTRATION		
Dosage form	1.0±0	1
Preparation: Mobilization	1.0±0	1
Preparation: Apheresis	2.0±1.0	0.6
Myeloablative Conditioning	1.7±1.2	0.6
Clinical Administration Protocol	1.3±0.6	0.6
Clinical infusion preparation and process	1.7±0.6	0.4
MANUFACTURING & SUPPLY CHAIN		
Cryopreservation approach	2.0±0	1
Media content risks	1.7±1.2	0.7
Supply chain / cold chain risks	1.0±0	1
CERTAINTY OF EFFICACY AND DURABILITY		
Clinical trial size and scope	2.3±0.6	0.6
Clinical trial primary measures	2.0±0.0	0.5

1160. The Novel Animal Models to Evaluate Preclinical Studies of Thalassemia

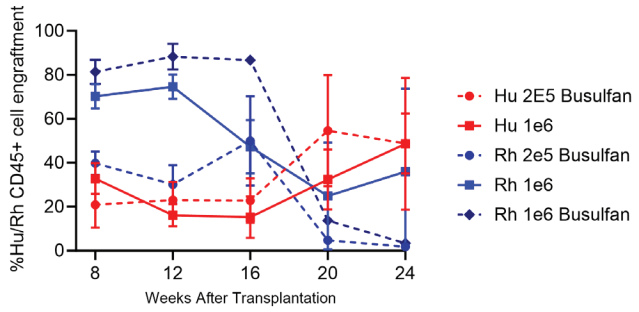
Tingting Gu, Shiying Guo, Jianming Xu, Hongyu Wang, Jing Zhao, Xiang Gao
GemPharmatech Co. Ltd, Nanjing, China

Thalassemia is a diverse group of genetic disorders with a worldwide distribution affecting globin chain synthesis the pathogenesis lies in the unbalanced globin chain production, leading to ineffective erythropoiesis, increased hemolysis, and deranged iron homeostasis. α thalassemia and β thalassemia are the main types of thalassemia, while α thalassemia is caused by reduced or absent synthesis of alpha globin chains, and β thalassemia is caused by reduced or absent synthesis of beta-globin chains. Traditional clinical therapy, requiring lifelong transfusion support and iron chelation is mainly used with a prolonged life expectancy, however, this therapy results in co-morbidities and substantial disease burden. Curative therapy with edited hematopoietic stem cell (HSC) transplantation, a kind of cell gene therapy, is developed for thalassemia patients, which has shown bright efficacy in improving anemia and transfusion burden, making it a promising approach. However, we lack animal models to evaluate the ability of edited HSC in thalassemia-relative preclinical studies. GemPharmatech developed 2 types of mouse models to assist CGT therapy of thalassemia in preclinical studies. One type is NCG-X mouse, a novel mouse model with W41 mutation in proto-oncogene c-KIT in NCG immunodeficient mice. The mutated mice have spontaneous anemia and are the only immunodeficient mice that can support the erythroid reconstitution in bone marrow, which could help to evaluate erythroid relative indicators. The other type is β-Thalassemia mouse with the knockout of Hbb-bs and Hbb-bt and α-Thalassemia mouse with the knockout of Hba-x, Hba-a1, and Hba-a2 in C57BL/6JGpt mice. The knockout mice have the thalassemia-like phenotype, including homozygous lethal, lower hemoglobin level (~105 g/L), and so on. Benefiting from the advantage of being immunodeficient, NCG-X mice are widely used to evaluate the human-origin HSC, while the α and β-Thalassemia mice are usually applied to evaluate the mouse-origin HSC. For CGT preclinical studies, the edited HSCs are injected into the mice. Body weight, survival, and clinical signs were used to evaluate the health status. Blood routine and FACS analysis of blood and bone marrow are used to evaluate the reconstitution level. DNA sequencing, qPCR of mRNA, and protein HPLC are used to evaluate the hemoglobin repair ability. All in all, NCG-X, β-Thalassemia, and α-Thalassemia mice are ideal mouse models for thalassemia preclinical studies.

1161. Establishment of a Rhesusized Mouse Model for Predictive Rhesus Hematopoietic Studies

Selami Demirci[1], Henna Butt[1], Evan London[1], Naoya Uchida[1], Matthew M. Hsieh[1], Chuanfeng Wu[2], Taha B. Hayal[2], N. Seth Linde[2], Aylin C. Bonifacino[2], Cynthia E. Dunbar[2], Robert E. Donahue[1], John F. Tisdale[1]
[1]CMTB, NHLBI/NIH, Bethesda, MD,[2]TSCB, NHLBI/NIH, Bethesda, MD

While mouse models are common for *in vivo* investigations for gene therapies, their physiological and immunological differences limit their predictability in humans (HU). Conversely, large animal models such as rhesus macaques (RM) offer greater fidelity due to phylogenetic proximity, similar hematopoietic system, longer lifespan, and immunological relevance. However, RM models are constrained by high costs, limited availability, slower breeding and life cycle, and the need for specialized facilities and expertise. As such, we have long sought to develop a "rhesusized" xenograft mouse model to allow parallel studies in the RM that would permit comparisons with results obtained *in vivo*. Prior work spanning many years in a series of xenograft mouse models were unsuccessful. Further improvements in the xenograft model have led to the availability of the highly immunodeficient NBSGW model which carries a *c-Kit* mutation allowing engraftment without conditioning. Thus, the NBSGW may provide a novel platform for evaluating gene therapy approaches if the results match those in the RM. The humanized NBSGW mouse model requires 1 - 2 × 10e6 HU CD34+ HSPCs per mouse. We previously showed that busulfan (BU) conditioning significantly reduces the cell doses required (Leonard et al., 2019). Accordingly, we infused HU or RM peripheral blood CD34+ HSPCs after one-day pre-stimulation into unconditioned or BU-conditioned (10 mg/kg) NBSGW mice at cell doses of 2 × 10e5 or 1 × 10e6 per mouse (n=5/group). Peripheral blood (PB) analysis at 16 weeks revealed a range of 47 - 86% RM and 15 - 22% HU CD45+ cells. Strikingly, BU conditioning facilitated superior engraftment in mice receiving RM cells (Figure). However, RM cell frequency gradually declined (3.4 - 36.0%) by week 24, in contrast to HU cell frequency of 46.8 - 48.8%. Examination of PB cell lineage demonstrated CD20+ B-cell dominance at 12 - 16 weeks, followed by a reduction in CD20+ B and CD33+ myeloid cells and an increase in CD3+ T-cell frequency (74.1 - 95.7%) at 24 weeks across all groups. Notably, in the mice receiving RM cells, 80% of high-cell dose BU-conditioned mice and 60% of high-cell dose non-conditioned mice in whom high levels of engraftment were obtained expired by week 16 (presumably from GVHD), accounting for the drop in engraftment for the groups as a whole. Analysis of bone marrow (BM) from deceased animals revealed robust engraftment in both HU and RM groups (84.8% to 98.8%). Pathology results indicated lymphocytic and/or histiocytic infiltration in the lungs, liver, BM, and spleen. However, BM engraftment in the surviving mice from the RM groups diminished to 0.92% - 6.5% at week 24, aligning with the observed reduction in PB cell frequency. In contrast, HU cell engraftment remained consistently high at 92.8% - 94.0%. The prevalence of BM CD3+ T-cells was notably elevated in RM groups (47.2% - 92.5%), contrasting with the relatively lower frequency observed in HU groups (8.7% - 12.4%). Additionally, the frequency of Band3+ red blood cells ranged from 46.1% to 50.4% in HU groups, while remaining low in RM groups (0.2% - 0.45%). In summary, our "rhesusized" mouse model efficiently supports RM cell engraftment, providing a valuable platform for assessing HSPC gene therapy approaches. These findings hold the potential for further advancements in preclinical animal models.



1162. Alpha-Globin Lentiviral Vectors for Hematopoietic Stem Cell Gene Therapy of Alpha Thalassemia

Eva Segura Gensler[1], Kevin Tam[1], Beatriz Campo Fernandez[1], Karen Li[1], Carol Mulumba[1], Andrea Gutierrez[1], Roger Hollis[1], Tippi MacKenzie[2], Donald Kohn[1]

[1]Microbiology, Immunology, & Molecular Genetics, University of California, Los Angeles (UCLA), Los Angeles, CA,[2]School of Medicine, University of California, San Francisco (UCSF), San Francisco, CA

INTRODUCTION Alpha thalassemia is an inherited blood disorder caused by mutations in α-globin genes (*HBA1* and *HBA2*) resulting in the reduction of the α-globin subunit, which together with β-globin chain, compose adult hemoglobin (HbA). Severe cases of alpha thalassemia appear when α-globin expression drops to 30% or less, which is often caused by the absence of three or all four α-globin genes (Hemoglobin H or Alpha Thalassemia Major (ATM), respectively). Because most patients suffering from severe anemia do not have a suitable donor, they depend on blood transfusions every 2-5 weeks and daily iron chelation therapy. While enabling patients to live into mid- to late-adulthood, these frequently required therapies drastically lower patients' quality of life and can cause serious clinical manifestations. **METHODS** Our laboratory has developed a stem cell gene addition therapy in which functional copies of α-globin genes integrate into the genome of patient's hematopoietic and progenitor stem cells (HSPCs) by lentiviral vectors (LVs) to normalize the globin chain imbalance and restore hemoglobin function. A series of α-globin LVs have been developed and are originally based on *GLOBE* β-globin LV utilized in a clinical trial for β-thalassemia (NCT02453477), which successfully led to transfusion independence in patients with transfusion-dependent β-thalassemia major. To target infective erythropoiesis and hemoglobin dysregulation, we have constructed a series of vectors optimized for titer production, high CD34+ HSPC infectivity, and high levels of α-globin expression. Vectors were first characterized in bone marrow HSPCs from healthy donors (HD) following transduction and differentiation into red blood cells (RBCs). The top two candidate vectors, A2-UV and A2-Globe, were then assessed in differentiated RBCs derived from HSPCs from bone marrow, fetal liver, and cord-blood of three ATM donors. **RESULTS** A2-UV and A2-Globe are two highly efficient vectors resulting in similar total α-globin chains and complete hemoglobin restoration, which they achieved through different modes: A2-UV is a small-proviral length vector that transduces in a higher percentage of CD34+, and yields a higher average vector copy number (VCN) when compared to A2-Globe, which in turns possesses a larger β-globin enhancer and higher α-globin gene expression per VCN. A2-Globe yielded up to 88% transduction efficiency in HD CD34+ HSPCs while maintaining a steady and well-distributed VCN ranging from 1 - 3 in 81% of transduced HSPCs and averaging 46% α-globin gene expression per VCN. At half a log lower concentration, A2-UV similarly transduced up to 88% HD CD34+ HSPCs, with a VCN ranging from 1 - 4 in 66% of transduced HSPCs and averaged 33% α-globin gene expression per VCN. (These studies are now being conducted in ATM HSPCs). Both vectors led to ~92% of endogenous α-globin chain production and successfully achieved a complete restoration of HbA levels in bone marrow-derived ATM patient RBCs while retaining a VCN of 5 or below. Similar results have been observed in two other ATM donors. **FUTURE DIRECTIONS** *In vivo* studies, conducted in NSG mice, are underway to assess engraftment, gene transfer distribution, and vector integration site analysis. Furthermore, single cell RNA-sequencing on transduced and erythroid differentiated bone marrow HSPCs from ATM patient cells is ongoing to assess the upregulation of genes involved in oxygen, iron transport and hemostasis, as well as hydrogen peroxide processes that are downregulated in patient RBCs. **CONCLUSION** Overall, this project describes successful pre-clinical data and offers a promising one-time autologous stem cell gene therapy for alpha thalassemia.

1163. Sickle Cell Mouse Diet (59M3) Strengthens Animals for *In Vivo* HSC Gene Therapy with HDAd5/35++ Vectors

Sucheol Gil, Theo Koob, Chang Li, Andre Lieber

University of Washington, Seattle, WA

Townes mice are an adequate mouse model for Sickle Cell Disease (SCD). In this mouse model, the murine α-globin genes were replaced with the human α-globin and the murine adult β-globin genes were replaced with human sickle β^S and fetal γ-globin genes linked together. To make the Townes model suitable for HDAd5/35++ transduction, which requires human CD46 as a receptor, we bred Townes mice with human CD46 transgenic mice (CD46/Townes). After three rounds of backcrossing, mice homozygous for human CD46 and the two human (α, β^S/γ) globin genes were used for experiments. Triple homozygous CD46/Townes mice displayed sickle-like erythrocytes, severe anemia, ~40% reticulocytes in the peripheral blood as well as leukocytosis and thrombocytosis. This pathological phenotype of SCD caused high mortality, both before and during in vivo studies, which involve HSC mobilization with G-CSF/AMD3100, intravenous injection of HDAd5/35++ vectors and in vivo selection with O^6BG/BCNU. We therefore used an individual diet (59M3 from TestDiet, St. Louis, MO) with higher amounts of protein, fat, carbohydrates, specific amino acids, and vitamins. While the mortality rate of triple homozygous mice before initiation of the diet was greater than 50%, only ~25% of mice on 59M3-diet died over an 8-month period. The effect of the 59M3-diet was visible in a ~20% body weight gain within one month after initiation. Long-term, body weight of animals on diet were comparable with those of control CD46-transgenic mice. These improvements in the animal model allowed us to perform *in vivo* HSC prime editing studies with the goal of correcting the SCD mutation in CD46/Townes mice. The treated mice demonstrated phenotypic correction, reflected by normalized blood parameters and spleen size.

1164. *In Vivo* HSC Base Editing to Install the Hemoglobin G-Makassar Variant for Sickle Cell Disease Gene Therapy in a Mouse Model

Chang Li[1], Aphrodite Georgakopoulou[1], Sucheol Gil[1], Kiriaki Paschoudi[2], Evangelia Yannaki[2], Hans-Peter Kiem[3], Andre Lieber[1]

[1]University of Washington, Seattle, WA,[2]G. Papanicolaou Hospital, Thessaloniki, Greece,[3]Fred Hutch, Seattle, WA

Sickle Cell Disease (SCD) results from a single nucleotide transversion (A to T) in the sixth codon of the β-globin gene. Precise genome engineering in hematopoietic stem cells (HSCs) represents a potential curative option for SCD patients. We aimed to correct the SCD phenotype by converting the sickle mutation (GTG) into a benign G-Makassar variant (GCG) using in vivo base editing in HSCs. We have developed a next-generation gene therapy approach involving *in vivo* transduction of mobilized hematopoietic stem cells. The central idea of this *in vivo* approach is to mobilize HSCs from the bone marrow, and while they circulate at high numbers in the periphery, transduce them with an intravenously injected HSC-tropic, helper-dependent adenovirus (HDAd) gene transfer vector system. Transduced cells return to the bone marrow where they persist long-term. For efficient HDAd vector production, we utilized miRNA-regulated gene expression, virus associated RNA (vaRNA), and anti-CRISPR inhibitors to suppress transgene expression in HDAd producer cells. HDAd vectors expressing the latest version of an adenine base editor (ABE8e) linked with a Cas9 variant (NRCH) under a human PGK or EF1α promoter were successfully produced. *Ex vivo* transduction of lineage-minus (Lin^-) cells from SCD mice (Townes/CD46) with HDAd-PGK-Maka or HDAd-EF1α-Maka followed by transplantation into irradiated recipient mice led to 55.2% to 88.2% stable correction of the sickle cell mutation (GTG>GCG; V6>A) in peripheral blood cells. The HDAd-EF1α-Maka was chosen for downstream experiments due to its higher activity. In an *in vivo* HSC transduction setting, involving HSC mobilization by G-CSF/AMD3100, intravenous injection with HDAd-EF1α-Maka, and short-term selection with low doses of O^6BG/BCNU, an average of 25.4% correction of the sickle mutation was achieved. Deep sequencing of week 16 bone marrow samples revealed that most of the edited alleles contained the expected GTG>GCG conversion. Less than 1.5% byproducts (mostly short deletions) around the predicted nicking site were found, consistent with the property of base editors to function independently of double-stranded DNA breaks. Phenotypic correction was observed in treated Townes/CD46 mice, including reticulocyte counts, spleen sizes, hematological and histological parameters. We did not observe significant adverse effects related to *in vivo* transduction/selection. Lin^- cells were isolated from *in vivo* transduced animals and plated for colony-forming unit (CFU) assay as well as transplanted into irradiated C57BL6 recipient mice. The CFU assay showed that 19.2% and 12.5% of colony-forming stem cell progenitors had monoallelic and biallelic correction, respectively. The editing levels remained stable in the secondary recipients. In $CD34^+$ cells from a SCD patient, transduction with HDAd-EF1α-Maka led to 11% GTG>GCG conversion at the sickle mutation site. This is significantly higher than the 4.5% correction level with a prime editing strategy (HDAd-PE5max) reported previously. SCD $CD34^+$ cells from additional donors are being investigated. We are currently evaluating the fidelity of in vivo base editing by measuring off-target events at top-ranked genomic sites. Our *in vivo* approach requires only one non-integrating vector, only intravenous/subcutaneous injections, and minimal *in vivo* selection. This technically simple approach has the potential for application in resource-poor countries where SCD is prevalent.

1165. Further Investigation of HBB Gene Editing with CRISPR/Cas9/rAAV6 in Human HSPCs to Treat Sickle Cell Disease

Liwen Xu[1], Sridhar Selvaraj[2], Matthew H. Porteus[3]

[1]Pediatrics, Stanford Univ. School of Medicine, Stanford, CA,[2]Pediatrics, Stanford University, Stanford, CA,[3]Pediatrics, Stanford University School of Medicine, Stanford, CA

Sickle cell disease (SCD) is the most common serious monogenic disease with 500,000 births annually worldwide. It is an autosomal recessive disease resulting from a single nucleotide variant in codon six of the β-globin (HBB) gene. The current curative treatment of SCD is allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, HLA-matched donors are limited and graft-vs-host disease (GVHD) may occur after allo-HSCT. *Ex vivo* gene correction in autologous hematopoietic stem/progenitor cells (HSPCs) by editing the disease-causing mutation in HBB gene with CRISPR/Cas9 and a recombinant adeno-associated virus serotype 6 (rAAV6) as the correct DNA donor template to treat SCD has now entered early-phase clinical investigation (NCT04819841). Finding the optimal *ex vivo* gene editing (GE) condition in human HSPCs and understating molecular status of some key gene editing reagents are important to ensure the progress and success of this technology. In this work, we demonstrated that multiplicity of infection (MOI) 600 with 12pmol p53-siRNA and 0.5uM AZD7648 (DNA-PKcs inhibitor) results in better cell proliferation, higher HDR% and lower p53 pathway activation. Furthermore, we showed no detectable rAAV6 virus remaining on cell surface or existing in cells at 48-hour post GE, the endpoint of drug manufacture. We also detected approximately 170,000-280,000 Cas9 protein molecules inside each cell at 6-hour post GE, the peak period of Cas9 protein inside the cells. Our results will provide important insights into the development and application of CRISPR/Cas9/rAAV6 genome editing and highlight some of the current safety and efficacy properties of the process.

1166. Development of Adenoviral Vectors Targeted to Human Hematopoietic Stem Cells, Expressing Mutated CXCR4 Variants, for the Sequential Mobilization and Transduction of HSC-Enriched Populations *In Vivo*

Jia Yao[1], Svetlana Atasheva[2], Dmitry M. Shayakhmetov[1]

[1]Emory University, Atlanta, GA,[2]Pedaitrics, Emory University, Atlanta, GA

Safe and efficient gene correction in human hematopoietic stem cells (HSCs) in vivo may provide curative therapeutic options to millions of patients with inherited diseases of blood and immune system. One limitation of current gene editing approaches in HSCs is the necessity to mobilize HSCs from the bone marrow into peripheral blood. Following in vivo or ex vivo editing, corrected HSCs must re-home to their bone marrow niche in sufficient numbers for polyclonal reconstitution of all blood cell lineages throughout the patient's life. Previous studies

have shown that the re-homing of mobilized hematopoietic stem and progenitor cells (HSPCs) to the bone marrow is inefficient. However, the efficacy of HSPC re-homing can be increased through transient ectopic expression of CXCR4, which plays a critical role in guiding and retaining HSPCs within their bone marrow niches. In this study, we explored how primitive human HSPC populations respond to ectopic expression of two CXCR4 splice variants, CXCR4-V1 and CXCR4-V2, both carrying the D262N mutation that abrogates binding to the HSC-mobilizer AMD3100, while preserving functional receptor interaction with the CXCL12 ligand. To deliver CXCR4 variants to human HSPCs, we employed the recently developed AVID-388 adenovirus vector-based platform, which preferentially transduces primitive human HSPC populations in vitro and in vivo after intravenous vector administration. The AVID-CXCR4 vectors expressed CXCR4 under the control of the EF1a promoter, along with a GFP reporter for analyzing CXCR4 expression and function in vector transduced cells. Flow cytometry and single cell RNA-seq analyses revealed that cells of the CD34+CD45RA-CD38+ phenotype expressed the highest levels of CXCR4 among other HSPC populations. In contrast, CD34+CD45RA-CD38- HSPCs expressing CD90, which are highly enriched for LT-HSCs, expressed medium to low CXCR4 levels. Analysis of primary human CD34+ cells infected with CXCR4-expressing and control vectors showed that up to 70% of LT-HSCs expressed GFP 24h post virus infection. Migration analysis of LT-HSCs toward CXCL12 gradient showed that the proportion of GFP-negative LT-HSCs migrating toward CXCL12, was not different between cells infected with all three vectors. However, we observed a 100% increase in the number of GFP+ LT-HSCs migrating toward CXCL12 after cell transduction with the AVID-CXCR4-V1 variant, whereas migration of GFP+ LT-HSCs increased only marginally compared to control after cell infection with the AVID-CXCR4-V2 vector. Next, we will explore whether a strategy of repeated mobilization and intravenous administration of the AVID-CXCR4-V1 vector to humanized huCD34+ NSG mice may increase the efficacy of gene transfer to the LT-HSC compartment in vivo by promoting the re-homing and retention of in vivo transduced LT-HSCs in their bone marrow niche. Because AVID vectors can accommodate all currently available gene editing technologies, the HSC-targeted CXCR4-expressing AVID platform may enable safe and effective therapeutic gene editing of human hematopoietic stem cells in vivo.

1167. Social Media Engagement Post Announcement of the FDA Approval of Two Gene Therapies for Sickle Cell Disease

Christineil Thompson

Mayo Clinic, Rochester, MN

Introduction On December 8th 2023, the United States Food and Drug Association (FDA) approved two gene therapies for the treatment of sickle cell disease. Social media sites have been the preferred news source for most US adults with previous surveys demonstrating that 86% get their news from a digital device often or sometimes and 58% preferring a digital method of news dissemination. X (formerly known as Twitter) has in 2023 been surveyed as the site where the highest percentage of users (53%) regularly receive their news. The ability to share critical medical advances to a large audience in real time can be harnessed by health care providers who can capitalize this social impetus to increase advocacy and patient counselling. Objective To analyze the most followed and popular news accounts on X (formerly known as Twitter) to determine the social media impact of the news of the U.S. FDA approval of two gene therapies for sickle cell disease. Methods: The top 30 most followed news accounts were identified through Intelligence Fusion statistical report. The top 10 accounts were selected then were individually searched for the terms "FDA" "sickle cell" and "gene therapy". The accounts which posted on the FDA approval on December 8th 2023 were selected and the key interactions, likes, comments, retweets and views were recorded. Popular US news media sites Bloomberg, NBC, Fox News and ABC News were also included. Results Of the 13 accounts selected there were 2, 751 likes, 1, 443 retweets, 511 comments and 1, 438,000 views. CNN Breaking News, The U.S. FDA, the Washington Post (WP), CNN and the New York Times were the accounts with the highest interactions generating over a 1.11 million views. Conclusion With nearly 1.5 million individuals viewing the announcement of the U.S. FDA approval of two gene therapies for sickle cell disease there is vast potential for launching this social capital to further conversations with patients and families with sickle cell disease.

X (Twitter) Interaction	U.S. FDA	The New York Times	CNN Breaking News	CNN	Fox News	NBC	Bloomberg	Reuters	WSJ	WP	Time	ABC News	Huff Post	Total
Likes	631	237	746	258	84	123	83	32	78	295	91	52	41	2,751
Retweets	439	119	330	127	26	63	50	13	69	128	43	28	8	1443
Comments	22	33	109	203	56	24	8	2	14	24	8	5	3	511
Views	209 K	217 K	356K	222K	58K	31K	35K	42K	57K	106K	52K	27K	26 K	1,438K

Table 1: X formerly known as Twitter Social Media Engagement Post Announcement of the FDA Approval of Two Gene Therapies for Sickle Cell Disease

1168. The Impact of Social Networks on Participant Engagement in Gene Therapy Community-Based Research

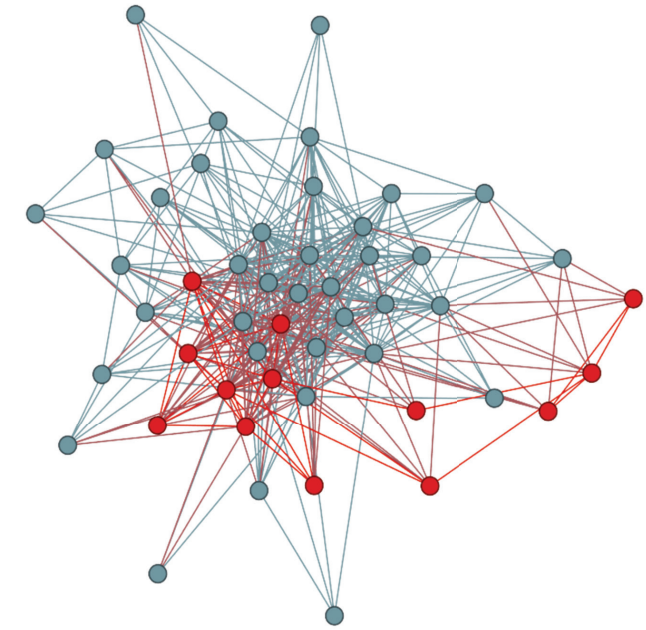
Diba Seddighi, Laura M. Koehly, Vence L. Bonham

Social and Behavioral Research Branch, National Human Genome Research Institute, Bethesda, MD

Background: Community-based participatory research (CBPR) serves as a bridge directly connecting researchers with the communities impacted by their work. Collaboration among diverse contributors from relevant research, industry, academia, patient, and advocacy communities fosters a more inclusive approach for the development and implementation of gene therapies. Given the recent milestone FDA approvals of two gene therapies for the treatment of sickle cell disease (SCD), it is increasingly important to understand the factors that shape the successful integration of research with community partnerships as gene therapy becomes more accessible to the public. Social networks, or the web of interpersonal relationships within a group, are crucial influencers in community engagement. Within collaborative processes, interpersonal networks impact engagement dynamics, communication patterns, decision-making, and collaborative outcomes. *Methods*: Data were acquired from a four-day virtual SCD CBPR meeting, conducted as part of the Democratizing Education for Sickle Cell Disease Gene Therapy project between January and March 2022. After day three, participants completed a social network survey detailing their pre-existing connections with other participants. Survey responses were input into Gephi software (v0.10) for diagram production and

network analyses. Transcripts of the virtual meetings will be analyzed using R (v4.3.1) to assess engagement dynamics through measures of speaking frequency, speaking duration, and statement valence. *Results*: This study utilized a mixed methods approach including social network analysis (SNA) and engagement analytics to assess the interactions among 52 SCD partners across the research community (representatives of industry, research, government, healthcare, and academia) and the patient and advocacy community (individuals with SCD, community based SCD advocacy groups, and parents of children with SCD). The network analysis produced a diagram containing 52 nodes, or participants, and 588 ties, or connections between a pair of participants, out of 1,326 total possible ties (Figure 1). Participants affiliated with the research community exhibited a significantly higher number of connections to other participants and were better situated to act as bridges or intermediates between other nodes, compared to the patient and advocacy community. These network centrality measures emphasize the significant contribution of the research community to enhancing network connectivity, implying a heightened influence of these participants on the dynamics of information flow and interactions within the network. Engagement data from eight break-out group sessions and four whole-group sessions will be analyzed within the framework of each meeting's social network diagram to further evaluate the influence of network structure on participation. *Conclusions*: The findings of this research offer insights into how social networks play a significant role in shaping the active participation and meaningful inclusion of community partners in development of education resources related to gene therapies. As we strive to advance the dynamic field of gene therapy, intentionally considering established network ties when forming community partnerships will promote more equitable engagement, enabling the incorporation of a broader range of voices and perspectives.

Figure 1. Social Network Diagram



Thursday Posters: Epigenetic Editing and RNA Editing

1169. Characterization and Repair of Sodium Channel Models for Autism Spectrum Disorders

Ahmad Al Saneh[1], Lionel Gissot[1], Aislinn Williams[2], Christopher Ahern[1], Karina Kruth[2], Katelin Scott[2], Hsiang Wen[2], Olivia Klein[2]

[1]Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA,[2]Neuroscience, University of Iowa, Iowa City, IA

Autism spectrum disorder (ASD) is estimated to occur in one out of every 36 children. The understanding of the mechanism behind ASD remains limited largely due to the disorder's remarkably complex etiology. Hundreds of genes have been implicated in autism risk, in addition to factors such as parental age, environmental conditions, and even some medications. Nevertheless, a small percentage of ASD cases are monogenic in origin and can be traced to a single causal genetic variation. These ASD examples provide an invaluable, simplified model of ASD that can be used to better correlate molecular dysfunction to phenotype. One such monogenic form of autism is caused by variations in the *Scn2a* gene which lead to premature truncation of the encoded protein, the sodium channel NaV1.2. Nonsense mutations leading to the abortive translation of the *Scn2a* gene result in reduced social interactions and repetitive behaviors, as well as ataxia and cerebellar atrophy, highlighting the important role of *Scn2a* in the cerebellar cortex as well.We have developed two *Scn2a* PTC mice (p.Tyr84X and p.Arg1626X), the first PTC autism rodent models to date. These mice are specific genetic models for ASD-linked *Scn2a* mutations that provide an opportunity to investigate the effects of *Scn2a* haploinsufficiency. In parallel, we have developed an approach using anticodon-edited transfer RNA (tRNA) to repair in-frame termination codons. This strategy is generally agnostic to the position of the variation and has the potential to be disease-modifying, that is, of the primary defect of the disease, not just symptomatic treatment. We have previously developed a platform of anti-codon modified human tRNAs to identify multiple tRNA types for every known human PTC disease variant. Using ribosomal profiling and mass spectrometry we have found that expressed suppressor tRNAs have minimal contact with native stop codons. Additionally, qRT PCR proved no significant upregulation of ER stress and UPR pathway proteins *in vitro*. Further, through quantitative RNA methods, we showed that SCN2a PTC mRNAs display modest nonsense mediated decay. Those suppressor-tRNAs have been packaged into Adenovirus as well as Adeno-Associated virus and transduced into cerebellar granular neurons that have been obtained from *Scn2a* p.Y84X and p.R1626X mice, as well as induced pluripotent stem cells (iPSCs) from patients with *Scn2a* PTC variants. This enables the investigation of human neurons derived from *Scn2a* syndrome patients. Additionally, we will also present data on a new mouse model that stably expresses a suppressor-tRNA targeted to the "safe harbor" *Hipp11* mouse locus. This resource will allow us to investigate, *in vivo,* the possible interactions between suppressor tRNA and native stops, and ER stress/UPR pathway activation, thereby further validating our codon-edited tRNA as a viable therapeutic strategy.The project provides valuable new resources for therapy as we investigate

the engineered tRNA's fidelity, interactions with native stop codons and its potential to activate the ER and UPR stress pathways. Such developments have significant implications for all diseases resulting from PTCs, beyond monogenic autism.

1170. Modeling of a Rare X-Linked Disorder for a CRISPR/dCas9 Based Epigenetic Rescue

Casiana E. Gonzalez, Julian A. N. M. Halmai, Isaac J. Villegas, Nicole B. Halmai, Kyle D. Fink

UC Davis, Sacramento, CA

CASK-related microcephaly with pontine and cerebellar hypoplasia (MICPCH) is a rare X-linked neurodevelopmental disorder caused by mutations in calcium/calmodulin-dependent serine protein kinase (CASK). We aim to create iPSC-based CASK relevant *in vitro* model systems and to rescue CASK expression via an CRISPR/dCas9 based epigenetic therapeutic. As females have two X-chromosomes, disease causing mutations present with a 50/50 expression of mutant and wildtype, due to the mosaicism caused by random X-chromosome inactivation (XCI). To this point, we will utilize a fibroblast cell line with nonpathogenic single nucleotide polymorphisms within *CASK,* which have been clonally selected to express from only one allele. This allows for analysis of expression changes from either allele via RT-qPCR. Additionally, we created a disease relevant model via prime editing on a healthy iPSC background to recapitulate clinically described disease-causing point mutations that result in a loss of function of CASK protein. Reduction of *CASK* expression and CASK protein was confirmed by next-generation sequencing and western blot and these iPSCs will be differentiated into neurons for future therapeutic analysis. Further, this project will adapt an established CRISPR/dCas9 epigenetic approach to rescue expression from the silenced, wild-type CASK allele. We aim to accomplish this through testing different dCas9 orthologues and a guide RNA screen targeting the *CASK* promoter. Constructs will be tested for optimal targeting efficacy *in vitro* and assessed via RT-qPCR. Additionally, epigenetic modifications from our approach will be analyzed through bisulfite sequencing, as we anticipate that following an increase of *CASK* expression, there would be a decrease in region specific promoter methylation. Ultimately, this project aims to show the potential of an epigenetic therapeutic for CASK-related MICPCH, as well as create a disease relevant iPSC-derived neuronal model for measurement a functional rescue. Currently, our results show the ability to target *CASK* and assess gene expression changes with CRISPR/dCas9 paired with an epigenetic modifier and transcriptional activator. This project will demonstrate the first therapeutic avenue for CASK-related MICPCH, as well as demonstrate the potential to utilize targeted X-reactivation as a platform approach for X-linked disorders. Further, investigation of smaller dCas9 orthologues prepares our approach for future translational applications such as packaging into AAV for delivery.

1171. Rescue of Haploinsufficient Expression of *ADNP* in Human Cell Lines Using CRISPR Activation and Gene Replacement Therapy

Katherine L. Lucot[1], Renee E. Napoliello[2], Julian A. N. M. Halmai[3], David J. Segal[4], Jill Silverman[5], Kyle D. Fink[5]

[1]Neurology, UC Davis School of Medicine, Sacramento, CA,[2]Neurology, UC Davis, Davis, CA,[3]Neurology, UC Davis, Sacramento, CA,[4]UC Davis Genome Center, Davis, CA,[5]UC Davis, Sacramento, CA

Introduction: *Activity-dependent neuroprotective protein* (*ADNP*) Syndrome, is a rare neurological disorder caused by a pathogenic mutation in one copy of the *ADNP* gene. These mutations are *de novo* and result in a loss of function of the mutated copy of the gene causing haploinsufficency; with the p.Y719X mutation being the most prevalent. Clinically, patients with *ADNP* Syndrome present with autism, mild to severe intellectual disability, brain abnormalities (e.g., sleep disorders and seizures), organ system complications (e.g., gastrointestial and cardiovascular defects), skeletal abnormalities, and dysmorphic features. *ADNP* plays a critical role in brain development, cognition, and motor function; and while CP201 (NAP) and ketamine have been tested for off-label use and/or are currently in clincial trials, they have not been able to fully restore a healthy phenotype in these children. Herein, we investigate the utility of a CRISPR activation (CRISPRa) system in an AAV-ready construct, paired with a single guide RNA (gRNA), to target and increase expression of endogenous *ADNP* and gene replacement therapy with an AAV- codon optimized *ADNP* casette. Methods: A human gRNA, targeting the promoter of *ADNP*, was triple transfected with intein-containing vectors, each containing one terminus of dCas9 fused to either the activating domains mini-VPR (VP64-p65-Rta) or MS2-P65-HSF1 (MPH) into a neural stem cell (NSC) line harboring the p.Y719X mutation (Y719X) and an isogenic control; or with the same gRNA paired with the same vectors containing GFP or dsRed instead of activating domains as a control. The potency of the gRNA to increase *ADNP* transcription was measured using RT-qPCR. Concurrently, both cell lines were transfected with the gene replacement therapy and the codon optimized transcript was measured using RT-qPCR. Results: When treated with the CRISPRa system, the Y719X line showed a 1.3- fold ($p = 0.0625$) increase in *ADNP* transcripts, and the isogenic line showed a 2.5-fold ($p = 0.1386$) increase, respectively. When treated with the gene replacement therapy, the Y719X line showed 1×10^7-fold ($p = 0.0012$) increase in codon optimized *ADNP* expression compared to endogenous expression, while the isogenic line showed a 7.6×10^6-fold increase, respectively. Conclusion and Future Directions: Both the split dCas9 system with activation domains and the *ADNP* gene replacement therapy are promising tools for altering the epigenetic state and increasing endogenous *ADNP* expression and codon optimized *ADNP* expression, respectively. Experiments using highly sensitive ELISAs are being done to evaluate if ADNP protein levels increase, post treatment, with the therapeutic constructs. Additionally, these therapies are currently being evaluated in NSC lines containing other common patient mutations, to further evaluate rescue of cellular function.

1172. CRISPR Activation of *SYNGAP1* Haploinsufficiency

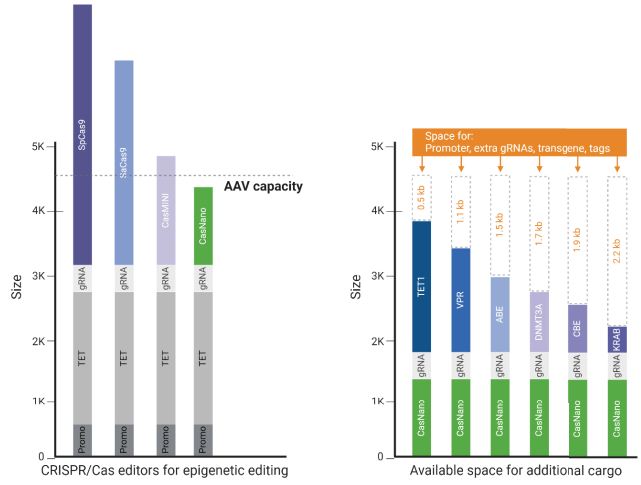
Paula Vij, Jasmine L. Carter, Julian Halmai, Peter Deng, David Cameron, Jill Silverman, Kyle D. Fink
University of California, Davis, Sacramento, CA

SYNGAP1-related intellectual disability (ID) is a genetic disorder that affects brain development and causes intellectual disability, seizures, and other neurological symptoms. *SYNGAP1* encodes for the SynGAP protein which has been shown to play an important role in the modification of synaptic strength by altering membrane trafficking of AMPARs through GTPase regulation. De novo mutations in the *SYNGAP1* gene lead to genetic haploinsufficiency causing a severe to mild ID. A concept of possible therapeutic relevance is the 'reversal of haploinsufficiency,' which is the increase of expression levels of the normal gene copy by targeting the regulatory elements that control the expression of the gene. Therefore, the purpose of our study is to use a genome-modifying mechanism, CRISPR-mediated activation (CRISPRa), which targets a transcriptional activator to the gene's promoter as a viable approach that could potentially correct *SYNGAP1* expression and rescue haploinsufficiency. Preliminary data from our lab has demonstrated increased expression of SYNGAP1 mRNA *in vitro*, specifically using mouse Neuro 2A (N2a) cells and patient-derived Neuronal Stem Cells (NSCs). Furthermore, our *in vivo* testing has demonstrated a significant upregulation in *SYNGAP1* mRNA expression within our experimental system. Given the pivotal role of *SYNGAP1* in neurodevelopment, our study also seeks to investigate its neurobiology deeper, aiming to bridge existing gaps and enhance our comprehension of normal and abnormal development. Thus, we aim to establish a disease model by characterizing a patient-derived iPSC neuronal model and conducting rescue experiments to gain insight into the *SYNGAP1* haploinsufficiency phenotype. Preliminary data underscores significant developmental and morphological differences in neurons derived from patients' cell lines harboring mutations in the *SYNGAP1* gene in comparison to those from control subjects. Our future directions involve the validation of our system in patient-derived neurons, along with comprehensive functional characterizations and rescue experiments with CRISPRa using patient-derived iPSC neurons.

1173. CasNano, an Ultracompact CRISPR/Cas for AAV-Based Therapeutic Applications

Woofung (Yi-Ting) Tsai, Dongkyeong Kim
Epitor Therapeutics, NYC, NY

The development of compact CRISPR-Cas systems is important for therapeutic delivery applications with size constraints such as adeno-associated viruses (AAVs). We report the engineering of a novel class 2 type V Cas nuclease named CasNano for efficient mammalian genome editing. CasNano was identified from a commensal bacterium species and constitutes the smallest known Cas effector to date at only 399 amino acids (1.2kb). Importantly, CasNano recognizes a GC-rich PAM sequence rather than the T-rich PAMs typical of other popular compact effectors such as CasMINI and Cas12a (Cpf1). Wildtype CasNano shows moderate DNA cutting or gene regulation activity in human cells. We therefore developed an iterative protein engineering strategy to optimize CasNano functionality in the context of the human genome. Beginning with bioinformative prediction guided by ESM and AlphaFold, we identified key residues involved in DNA binding for further engineering. After several rounds of directed evolution and screening, the optimized CasNano variants demonstrated robust activation capability when fused to transcriptional activators, greatly improved over wildtype levels. In addition, the gRNA size was reduced via scaffold optimization to improve performance. Importantly, CasNano showed ability to epigenetically regulate gene expression when fused to a TET 5-methylcytosine dioxygenase, a DNA methyltransferase (DNMT), VPR or KRAB. Furthermore, when fused with base editor domain, CasNano mediated efficient C-to-G editing of target DNA sites in human cells. Finally, we show that nuclease-active CasNano induces cuts and frameshift mutations for gene knockout. With its reduced size and non-pathogenic bacterial source, CasNano is well-suited for therapeutic delivery and gene editing applications via AAVs, lipid nanoparticles, or other modalities. Overall, CasNano expands and diversifies the toolkit of programmable Cas effectors available for developing next generation CRISPR-based genetic medicines.

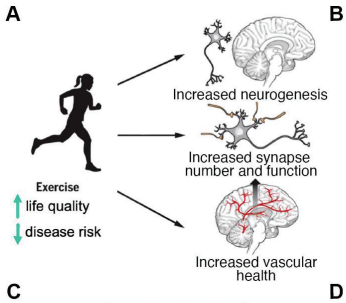


1174. CRISPR Epigenetic Reprogramming of Myokines as Therapy for Alzheimer's Disease

Thyago Leal Calvo[1,2], Viviana Salinas-Rios[1], Ilana Burg[1], Fernanda G. De Felice[3], Fyodor D. Urnov[1]
[1]Innovative Genomics Institute, University of California Berkeley, Berkeley, CA,[2]Pioneer Science Initiative, D'Or Institute for Research and Education, Rio de Janeiro, Brazil,[3]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON, Canada

Background. Alzheimer's disease (AD) is a neurodegenerative condition imposing a significant burden on healthcare systems globally. Despite extensive efforts, it remains incurable, with only moderately effective treatments available for some patients. Gene therapy, particularly using CRISPR-Cas, holds promise as an advanced alternative. The ability to reprogram cell phenotypes by regulating gene expression via epigenetic reprogramming using the CRISPR-dCas9 variant has widened our options for complex disease therapies, like AD. It's known that exercising modulates genes encoding for myokines that regulate other factors in the brain, leading to neurogenesis, reduced neuropathology, and fewer cognitive deficits in AD. e hypothesized that using CRISPR-dCas epigenetic reprogramming to upregulate

exercise-responsive genes could mimic the beneficial effects of exercise in the brain, potentially slowing AD progression. As CRISPR delivery methods advance, there's optimism that this strategy could not only delay the onset of AD and its progression but also reduce cognitive decline in patients. ***Experiments.*** We used the CRISPR-dCas9-VPR-WPRE/BGH containing both viral (VP64, Rta) and human transactivation domains (RELA, p65), known to recruit epigenetic effector proteins. We tested six single-guide RNAs (sgRNAs) designed around 200 bases upstream of our top myokine gene transcription start site (Fig. 1A). We also tested two 3' untranslated sequences (WPRE or BGH) to see if they affect mRNA stability and modulation efficiency. The cells used were iPS-derived neural progenitors. sgRNAs and mRNAs were transfected with cationic lipids. Our in vitro transcriptions incorporated pseudouridine instead of uridine into the mRNAs. Experiments have as readout the mRNA expression measured by TaqMan RT-qPCR (Fig. 1B). ***Results.*** For the six tested sgRNAs and 3'-UTRs, we obtained at least a 3-fold increase in mRNA expression of the target gene. Two-way ANOVA showed no interaction between sgRNA and 3'-UTR, but a slightly higher upregulation with the WPRE ($F = 7.37_{df = 39}$, P = 0.011, Fig. 1C). The fold-change among the six sgRNAs for the WPRE construct ranged from 3.3× up to 31× over the non-targeting control (Fig.1D). As a result, the top three sgRNAs with the largest fold-changes were W0-CB3 (31.2×), C1 (19.1×), and WE3 (10.8×). In summary, the sgRNAs located around -292 bases upstream of the transcription start site showed improved upregulation of the target transcript. ***Conclusions.*** We have shown that all sgRNAs can effectively upregulate the target gene mRNA up to 30-fold. Next experiments will evaluate if protein levels correlate with the mRNA's. We will also try the transcriptional activation of selected sgRNAs on cortical neurons. In the future, in vivo experiments on Alzheimer's disease mouse models will measure the effect of this approach on disease progression, memory, and neuropathology.



1175. Chemical Design of Oligonucleotides That Support Targeted RNA Editing in the CNS of Non-Human Primates

Chikdu Shivalila[1], Genliang Lu[1], Christopher Acker[1], Ian Harding[1], Jigar Desai[1], Jack Godfrey[1], Naoki Iwamoto[1], Pachamuthu Kandasamy[2], Hui Yu[1], Tomomi Kawamoto[1], Jake Metterville[1], Megan Cannon[1], Purcell-Estabrook Erin[1], Faraone Alyse[1], Prashant Monian[1], Stephany Standley[1], Jesse Turner[1], Ryan Yordanoff[1], Fangjun Liu[1], Hailin Yang[1], Michael Byrne[1], Chandra Vargeese[1]

[1]Wave Life Sciences, Cambridge, MA,[2]Wave Life Sciecnes, Cambridge, MA

Using chemically modified oligonucleotides to harness endogenous adenosine deaminase acting on RNA (ADAR) enzymes is an emerging approach to targeted therapeutic RNA editing, and it has been shown to direct editing in the liver of non-human primates (NHPs). The central nervous system (CNS) is another attractive tissue for RNA editing via endogenous ADAR enzymes because ADAR1 and ADAR2 are highly expressed in the CNS, and there is a large unmet need for approaches that can repair causal mutations in CNS disorders. However, published reports of measurable directed RNA editing in the CNS *in vivo* have been limited to murine models and relied on expression of hyperactive ADAR enzymes. Here, we describe high efficiency A-to-I RNA editing in human neuronal cell models and in the CNS of NHPs *in vivo* using a novel chemical design of Wave's AIMers, which are short, chemically modified oligonucleotides that direct RNA editing via endogenous ADAR enzymes. We identify sugar, base, and backbone modifications across the ~30mer AIMer structure that enhance potency *in vitro* and *in vivo* compared to our benchmark AIMers and help mitigate ADAR's natural bias against adenosines positioned within certain sequence contexts. The optimized design resulted in >50% maximal editing in primary mouse hepatocytes for most sequences tested, including sequence combinations that have very low editing efficiencies reported due to ADAR's sequence preferences. A key feature of the design is the introduction of an N-3-uracil (N3U) modified base positioned across from the target adenosine, which is hypothesized to support enhanced editing efficiency by creating RNA-enzyme interactions that mimic a hyperactive ADAR mutant. These AIMer design developments support targeted editing in CNS cell types and tissues. In human iPSC-derived neuron and astrocyte cell models, AIMers direct up to 80% and 70% editing, respectively, against the ubiquitous housekeeping transcript *ACTB*. To assess the ability to edit a clinically relevant target in the CNS, we designed AIMers to edit the *MECP2* R168X mutation, the most frequent nonsense mutation that causes Rett Syndrome (RTT), a rare neurodevelopmental disorder. These AIMers are designed to convert the premature stop codon to tryptophan, promoting translation readthrough and protein restoration. In human and mouse neuronal lines, *MECP2* AIMers direct ~15% to 90% RNA editing, respectively. In a mouse neuronal cell model of RTT, we restore detectable expression of full-length MECP2 protein. To investigate whether AIMers can support editing in the CNS *in vivo*, we delivered AIMers directed against the housekeeping gene *Ugp2* via intracerebroventricular (ICV) administration to transgenic mice expressing wild-type human *ADAR1*. Single-dose injection resulted in 25% to 65% RNA editing of *Ugp2* across the entire CNS, which was durable up to 16 weeks. In NHPs, intrathecal injection of a single dose of AIMers directed

against *ACTB* resulted in around 50% RNA editing in the spinal cord and up to 30% in the brain one-week post-dosing. These preclinical investigations describe the development of an oligonucleotide design that supports directed *in vivo* RNA editing in the CNS of NHPs via harnessing the endogenous ADAR machinery and lay the foundation for the development of AIMers as potential RNA medicines to treat human neurological diseases.

1176. CRISPR-Activation of Syngap1 in Neurodevelopmental Disease Models

Molly Reilly, Elizabeth Heller
University of Pennsylvania, Philadelphia, PA

Almost 400,000 children born each year develop neurodevelopmental disorders whereby a *de novo* genetic mutation arises in one of the two alleles, leading to only one functional copy of the gene. Mutations in one such gene, Syngap1, results in SYNGAP1-related intellectual disability characterized by cognitive impairment, seizures and sleep disturbances. We hypothesize that upregulating the "good" copy of each gene using CRISPR-activation will rescue the disordered phenotypes in mouse disease models and patient cells, similar to therapeutic strategies for Spinal Muscular Atrophy. We have verified that our CRISPR activation strategy increases Syngap1 expression when injected into the hippocampus of adult mice and we are currently testing if this molecular rescue impacts cognitive behavior and sleep deficits. The results of these experiments will elucidate the efficacy of CRISPR activation in treating neurodevelopmental disorders that result from a lack of functional protein.

1177. A Modular CRISPR System for Molecular Therapy of Neurodevelopmental Disorders

Julian Halmai, Jennifer Waldo, Casiana Gonzalez, David Cameron, Isaac Joseph Villegas, Kyle D. Fink
UC Davis, Sacramento, CA

Out of the over 20 000 genes in the human genome, only a small fraction of genes can be currently targeted by the existing pharmacopeia. The advent of gene editing has vastly increased our ability to make the undruggable genome druggable. Here, we developed a novel, modular tool for transcriptional regulation of genes involved in neurodevelopmental disease in the absence of creating double-stranded breaks. Following an intein screen using transfection in HEK293T cells we identified *Rma*DnaE for efficient trans-splicing of amino- and carboxyl-terminal split, catalytically inactive, dCas9 both *in vitro* and *in vivo* following packaging into AAV9. Likewise, direct intracranial injection of two split constructs containing fluorescent proteins fused to dCas9 (N-terminal dsRed and C-terminal turboGFP) resulted in efficient distribution and co-transduction of neurons in the mouse brain. We utilized this novel platform technology to identify sgRNAs that efficiently regulate expression of two intellectual disability genes, *FOXG1* and *SYNGAP1*. Using a modular assembly of CRISPR trans-activators, including single and dual VP64 dCas9 fusion proteins, sgRNA-mediated MS2 p65 HSF (MPH) recruitment or the addition of the VPR tripartite effector (VP64, p65, Rta) can induce dynamic gene regulation. We can further increase mRNA levels of *FOXG1* and *SYNGAP1* using duplexed sgRNAs paired with a combination of N-terminal VPR-dCas9 and C-terminal dCas9-MPH. We demonstrate that *FOXG1* gene upregulation results in a significant increase in FoxG1 protein translation. Following treatment of mutant *FOXG1* neural stem cells with the CRISPR activators, we observed a significant increase in *FOXG1* expression with no-off targets as determined by *in silico* sgRNA binding prediction paired with RNA-sequencing. In addition to transcriptional modulation, we also demonstrated that the modular assembly allows for the fusion of DNA methylation editing effector proteins that when targeted to the X-linked *CDKL5* promoter can result in erasure of CpG island promoter methylation. We demonstrate the clinical utility of the editor in patient-derived organoids, demonstrating reactivation of methylation-silenced *CDKL5* and rescue of phosphorylation substrates of the kinase. In addition, we optimized promoter and polyA configurations to reduce construct size and improve AAV manufacturing to further increase capsid ratios and *in vivo* bioactivity. Ongoing studies are addressing molecular and behavioral rescue in multiple animal models of disease and patient-derived brain organoids. This approach holds great promise for those affected by rare genetic conditions.

1178. Adeno-Associated Viral Vector Resource for the RNA-Targeting Cas13d: A Comparison of High-Fidelity Variants, DjCas13d and hfCas13d

Frank Back[1], Alfredo Sandoval[2], Rachel Barkey[1], Sven Kroener[3], Benedict Kolber[3], Jonathan E. Ploski[1]
[1]Neural and Behavioral Sciences, Penn State College of Medicine, Hershey, PA,[2]University of Texas Medical Branch, Galveston, TX,[3]Neuroscience, UT Dallas, Richardson, TX

RNA-targeting CRISPR-Cas systems have emerged as alternatives to RNA interference technology to knock down specific RNA transcripts. In particular, Cas13d derived from Ruminococcus flavefaciens (CasRx, RfxCas13d) has generated sustained interest due to its superior knockdown efficiencies; however, accumulating evidence indicates that CasRx is prone to inducing significant transcriptome alterations due to its tendency to cleave bystander RNAs. High-fidelity Cas13d (hfCas13d) derived from CasRx and DjCas13d, an ortholog of Cas13d derived from Ruminococcus sp. UBA7013 (gut metagenome), are two recently identified variants that have both been shown to be superior to CasRx. They both show reduced tendency to cleave bystander RNAs. In this study, we created a resource of adeno-associated viral (AAV) vectors designed to deliver Cas13d, including hfCas13d and DjCas13d. We present data that CasRx is prone to causing transcriptome disturbances when targeting highly-expressed genes. Then, we directly compared hfCas13d and DjCas13d for their on- and off-target potential in 293FT cells. We report that while both of these enzymes exhibit generally similar levels of knockdown potential, hfCas13d caused significantly fewer transcriptome alterations when targeting highly-expressed genes compared to DjCas13d.

1180. The Ethics of Human Epigenetic Editing: Just a New Approach or Entirely New Problems?

Rami M. Major[1], R. Jean Cadigan[2]
[1]Curriculum in Genetics and Molecular Biology, The University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Department of Social Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC

Ethical concerns associated with classic gene editing (i.e., the cutting and subsequent repair of target DNA sequences) have been heavily described and debated in the literature. Some of the most pressing concerns invoke questions of how humanity might reckon with permanent changes to something as intrinsic as the human genome, in somatic cells and beyond to germline cells altering the very fabric of future generations. The permanence of these interventions seems to elevate the gravity of them in the eyes of many stakeholders. As gene editing technologies evolve, new approaches have been developed that may skirt this issue, challenging our assessment of the ethical concerns routinely associated with gene editing. One emerging use that raises the prospect of re-examination is epigenetic editing, which alters gene expression rather than genes themselves. Two technological features of epigenetic editing particularly warrant additional ethical analyses beyond those completed for classic gene editing because they potentially alter the risk/benefit ratio of the interventions: 1) epigenetic editing is not permanent, which may be desirable for reversibility attempts but may be undesirable if the goal is to deliver a long-lasting therapeutic, and 2) epigenetic editing has unique features beyond classic gene editing, including its potential to regulate many genes at once, which may be desirable for the treatment of polygenic disorders but undesirable for potentially more significant off-target risks. These two features of epigenetic editing will push the bounds of what the gene editing field has conventionally considered treatable conditions, creating new opportunities to impact humanity and warranting a frank discussion about how to eventually deploy this new technology in the clinic. Drawing on interviews we conducted with scientists who use gene editing technologies in their research, we will highlight conditions that our interviewees proposed for potential epigenetic editing, including neuropsychiatric disorders, pain, viral immunity, and obesity, and offer an ethical analysis of its use in these different contexts. We will also offer insight into how these scientists think about the future of epigenetic editing in comparison to gene editing. Before epigenetic editing extends beyond basic research, it will be important to consider the facets of epigenetic editing that set it apart from classic gene editing to inform a safe and ethical path forward to the clinic.

Thursday Posters: Gene Disruption and Excision

1181. Optimized Homogenization Matrix to Produce High Purity, Tag-Less, 160kDa Nuclease Using Ion Exchange and Size Exclusion Chromatography

Virginia L. Nash, Brittany A. M. Sangiovanni, Eva L. Geygan, Dylan J. Vasconcelles, Dennis O. Concepcion, Carson G. White, Mahajoub B. Bello Roufai
Research and Process Development, Akron Biotech, FL, Sarasota, FL

The discovery of the type II prokaryotic CRISPR "immune system" has allowed the development of an RNA-guided genome editing tool that is simple, easy, and quick to implement. Since then, several approaches to genome editing have emerged. The applications of such nucleases are ever-expanding and were recently validated with the US FDA approval of Casgevy, the first gene editing therapy for the treatment of sickle cell disease. The approval of Casgevy, coupled with the many gene editing programs necessitates versatile, cost-effective enzyme production and purification processes. A well-known strategy to facilitate protein purification is to engineer and express the protein with a Tag (e.g., Histidine Tag). This allows the use of affinity-based purification to achieve high purity. Cleavage of the Tag post-purification may be necessary, as its retention can cause interference with the protein's intended function. In this work, we describe a purification process using Akron's optimized homogenization matrix which allows isolation of the nuclease of interest at a relatively high purity after capture chromatography (Cation Exchange chromatography). A second chromatography step consisting of Size Exclusion Chromatography (SEC) resolves impurities (up to 100kDa) from the 160kDa nuclease using an enhanced buffer system. A 97.4% purity, measured by RP-HPLC, was achieved after two chromatography columns. A final chromatography step, designed solely for the reduction of endotoxin, achieved a reduction from ~69.8 EU/mg to levels as low as <1.0 EU/mg. The efficiency of the first CEX was shown to be scalable from a 10mL column volume (CV) to 5,000mL CV. This newly developed purification process using cGMP compatible resins, chemicals, and supplies is being assessed for scalability at Akron to establish a cost-effective nuclease purification process that does not require affinity chromatography.

1182. Chemically Modified AsCas12a Guide RNAs Improve Lipid Nanoparticle-Mediated *In Vivo* Gene Editing in Different Tissues

Paul J. Wrighton, Judith Newmark, Chrysa Latrick, Vikram Soman, Andrea Pinilla, Mark Jones, Eleanor Quenzer, Alison Miller, Steve Bottega, Alexis Benedis, Bryant Chica, Bridget Moffet, Meng Wu, Eugenio Marco, Swarali Lele, Ramya Viswanathan, He (Harry) Wang, Xiaoqi (Kevin) Sun, John A. Zuris, James Bochicchio, Kayla Delano, Georgia Giannoukos, Kai-Hsin Chang, Stephen Pietrasiewicz, Mariacarmela Allocca, Heather MacLeod, Steven Wolk

Editas Medicine, Cambridge, MA

CRISPR-Cas gene editing is a transformative technology with the potential to enable durable treatments for many diseases with unmet clinical need. Compared to the typically employed SpCas9 nuclease, our engineered AsCas12a nuclease has advantages in potency, specificity, and guide RNA (gRNA) chemical synthesis. To realize the potential of AsCas12a gene editing, methods must be developed to deliver AsCas12a nuclease and gRNA to the target cells *in vivo*. Lipid nanoparticles (LNPs) are capable of packaging AsCas12a mRNA with gRNA and delivering the cargo to various target organs *in vivo*. Yet, once the LNP delivers the payload into the cell, the RNA components can be rapidly degraded, reducing editing efficiency. Much effort has focused on protecting mRNA cargoes with chemical modifications to resist nuclease digestion and increase translation. However, less is known about protecting small AsCas12a gRNAs. Our goal was to develop a set of AsCas12a gRNA modifications that would universally enable high-potency gene editing regardless of target cell type. To this end, we performed three iterative rounds of chemical modification testing. The first round demonstrated several different chemically modified gRNAs increased editing potency compared to unmodified gRNA. The second round built upon the most efficacious initial designs and showed further improvements to editing potency. The third round of modifications consisted of combinations of modifications tested in the first two rounds and resulted in maximum editing potency. Our data showed that top modifications consistently improved gene editing potency across multiple immortalized cell lines and primary cell types *in vitro*, including HEK293T, Hep3B, primary trabecular meshwork (TM) cells, and primary human hepatocytes. Importantly, local administration of LNPs containing AsCas12a mRNA and modified gRNAs into the anterior chamber of the eye enabled *in vivo* editing in mouse TM cells. Furthermore, systemic delivery of LNPs via intravenous injection demonstrated similarly improved editing potency in the liver as a result of gRNA modification optimization. Notably, we demonstrated that improved binding affinity between engineered AsCas12a protein and gRNA correlates with improved gene editing potency following LNP delivery, providing a potential mechanistic explanation. These data demonstrate universal applicability of gRNA modifications to enhance gene editing outcome *in vitro* and *in vivo* and will enable the development of efficacious gene editing medicines.

1183. Improving HDR-Editing of the Sickle Cell Disease Mutation in Human CD34+ Cells via Electroporation

Anh Le[1], Julia Ball[1], Avery Bradley[1], Selami Demirci[1], Xiong Liu[1], Matthew M. Hsieh[1], Megan Embrey[2], Diwash Acharya[2], John F. Tisdale[1], Naoya Uchida[1]

[1]Cellular and Molecular Therapeutics Branch (CMTB), National Heart Lung and Blood Institutes (NHLBI), National Institutes of Health (NIH), Bethesda, MD,[2]MaxCyte®, Inc., Rockville, MD

Gene-editing technologies have advanced over time and applied to correct genetic disorders such as sickle cell disease (SCD). SCD is caused by the 20A>T mutation in the β-globin gene, and gene correction (HDR) of the SCD mutation in hematopoietic stem cells (HSCs) allows for phenotypic correction with adult hemoglobin production, eliminating sickle hemoglobin. In our SCD gene-correction model, CRISPR-edited CD34+ HSCs via electroporation (EP) were demonstrated to achieve ~30% HDR *in vitro* and engraftable post-transplant in xenograft mice and rhesus macaques (Cell Rep Med. 2021). HDR-edited cells were detectable on both DNA and protein levels long term; however, HDR-editing ratios were reduced to ~1% in rhesus. Therefore, here, we further optimized our system to increase editing efficiencies as well as cell recovery for HDR-edited CD34+ cells. Mobilized healthy-donor CD34+ cells were pre-stimulated for 2 days and electroporated by MaxCyte ATX® electroporator with normal β-globin-specific guide RNA, SpCas9 protein, and 100b donor single-strand DNA including the SCD mutation. We evaluated 1-week cell recovery by a semi-automated counter and gene-editing efficiency by reverse-phase HPLC after 2-week erythroid differentiation or digital-droplet PCR (ddPCR) after 1-week culture. At first, we evaluated 1-5x amounts of RNP (guide RNA and Cas9 protein), 0.5-10e7/mL cell densities in the EP cuvette, 1-40e5/mL of pre-stimulation cell culture densities, with HDR analysis by HPLC. The greatest amount of 5x RNP resulted in a lower level of 11% HDR as compared to 47-49% HDR at 1-2.5x RNP, likely due to cell toxicity with 2-fold lower cell recovery. All tested cell densities in the EP cuvette allowed for comparable levels of cell recovery, and HDR ratios were proportionate at 2-10e7/mL when we obtained sufficient amounts of HPLC samples. Up to 4-fold greater cell expansion was observed when CD34+ cells were seeded at lower cell densities, with 2.5e5/mL having the highest cell recovery post-EP ($p<0.05$, compared to 20-40e5/mL). HDR efficiencies gradually increased by reducing pre-stimulation cell density, from 15% HDR at 40e5/mL to 46% HDR at 1e5/mL. These data demonstrate that the low-amount RNP, a wide-range cell density in the EP cuvette at 0.5-10e7/mL, and low cell density in pre-stimulation culture allow for higher HDR editing in CD34+ cells. Next, we evaluated 100-300μg/mL donor DNA amounts, 2-40min resting times post-EP, and EP energy among preset programs HSC1-5, with HDR analysis by ddPCR. We found that a donor DNA addition reduced cell recovery ($p<0.01$), compared to no donor DNA and no cargo. 100μg/mL donor DNA resulted in up to 2-fold higher cell recovery and the highest 34% HDR editing among the tested donor DNA amounts. When resting CD34+ cells post-EP for 10-40min, 10min resting allowed for the highest 4-fold improvement of cell recovery, compared to no resting ($p<0.05$), along with similar 11-25% HDR editing efficiencies. A shorter resting at 2-10min increased cell recovery by 3 fold with similar HDR editing. We observed a trend of lower cell recovery up to a 5-fold range when

using higher energy of EP ($p<0.01$), while similar 19-21% HDR ratios resulted from HSC3-5, suggesting that HSC3 is optimal. These data demonstrate that a low-amount donor DNA, a short resting period post-EP, and program HSC3 improve HDR editing and cell recovery in CD34+ cells. In summary, we optimized HDR editing in human CD34+ cells *in vitro* to achieve higher HDR efficiencies and reduce cell toxicity. These findings should be useful to counter HDR toxicity and improve the engraftment of HDR-edited CD34+ cells.

1184. Designer Recombinase Mediated Reactivation of Fetal Hemoglobin for Treating β-Hemoglobinopathies

Manavi Jain[1], Nadja Schubert[2], Maciej Paszkowski-Rogacz[1,3], Lukas T. Schmitt[1,3], Duran Sürün[1], Frank Buchholz[1]

[1]UCC Medical Systems Biology, Medical Faculty, Technical University Dresden, Dresden, Germany,[2]DKMS Life Sciences Lab gGmbH, Dresden, Germany,[3]Present Address: Seamless Therapeutics GmbH, Dresden, Germany

Hemoglobinopathies are prevalent monogenic disorders with a profound global health impact affecting millions of individuals. Defects in the β-globin gene leads to abnormal structure or production of β-chains causing sickle cell disease (SCD) or β-thalassemia (β-thal), respectively. Allogenic hematopoietic stem cell transplantation (HSCT), the conventional curative option, is severely limited by donor availability and risk of immunological complications. Genetically modified autologous HSCT emerges as a promising alternative for patients lacking compatible donors. In contrast, hereditary persistence of fetal hemoglobin (HPFH) is a naturally occurring benign condition characterized by a substantial elevation of fetal hemoglobin (HbF) in adult red blood cells. Co-inheritance of HPFH with hemoglobinopathies results in milder clinical symptoms. Consequently, the reactivation of HbF has been extensively explored as a favorable approach to alleviate the clinical manifestations of both β-thal and SCD. Endogenous reactivation of HbF expression requires the development of efficient and safe genome editing tools. Tyrosine site-specific recombinases (Y-SSRs) present an attractive tool due to their exceptional ability to mediate DNA excision between two target sites with nucleotide precision. In our work, we leveraged this precise mechanism of Y-SSRs to reactivate HbF by seamlessly editing its transcriptional repressor, BCL11A (B-cell lymphoma 11A) gene. For reprogramming SSRs to predefined sequences in the BCL11A gene, we applied substrate linked directed evolution. High-throughput deep-sequencing-based screening of the resulting recombinase library was then used for rational selection of candidates displaying specific editing of the BCL11A target sites. Furthermore, expression of selected variants via mRNA electroporation resulted in the accurate DNA excision in reporter cell lines with an efficiency of up to 35%. Exact deletion at the endogenous BCL11A locus was confirmed through sequencing. Altogether, our findings highlight the therapeutic potential of designer recombinases as precision genome editing tool for treating β-hemoglobinopathies.

1185. Gene Editing Validation of Engineered Hypercompact OsCas12f Using LoxP-Targeted Reporter Assay in Mammalian Cells

Araba Abaidoo-Myles[1], Xinxu Yuan[1], Abdul Rasheed Baloch[1], Dabbu Jaijyan[1], Fang Li[1], Binhua Ling[2], Wenhui Hu[1]

[1]Virginia Commonwealth University School of Medicine, Richmond, VA,[2]Texas Biomedical Research Institute, San Antonio, TX

Due to size constraints for AAV gene therapy, efforts have been directed towards minimizing CRISPR/Cas editors, resulting in the identification of compact variants like Cas12f (Cas14a), Cas12j, and Cas13x. Compact editors offer advantages in viral packaging and targeting efficiency. Un1Cas12f (529 aa) from an Uncultured archaeon, initially recognized as Cas14a in 2018, originally demonstrated ssDNA cleavage. Subsequent studies revealed detectable nuclease activity (dsDNA) in prokaryotic cells, but not in eukaryotic cells. Recent findings highlighted the enhanced editing efficiency of Un1Cas12f in mammalian cells following protein engineering and sgRNA scaffold modification. AsCas12f, a newly discovered variant from Acidibacillus sulfuroxidans, with only 422 aa (1266 bp), exhibits high editing activity in mammalian cells in vitro and in vivo upon protein and scaffold engineering. Similarly, Oscillibacter sp. Cas12f (OsCas12f, 433 aa) demonstrates significant editing efficiency following protein and sgRNA engineering. This study aims to validate editing efficiency of the enhanced OsCas12f (enOsCas12f) in mammalian cells using LoxP-STOP-LoxP-tdTomato (LSL-tdT) reporter system. The LoxP-targeted sgRNA expression cassette was integrated into both constitutive (AAV-CBs-enOsCas12f and AAV-CBs-EGFP-enOsCas12f) and inducible vectors (AAV-CBs-ecDHFR-enOsCas12f and AAV-CBs-EGFP-ecDHFR-enOsCas12f). High editing efficiencies were observed in transiently transfected LSL-tdT reporter plasmids in HEK293T cells under both constitutive (85-90%) and inducible (50-60%) enOsCas12f expression. However, AAV-DJ8 delivery into neural stem cells from LSL-tdT mice (Ai14) yielded lower editing efficiencies (15-30%). In conclusion, the LoxP-targeted reporter assay validated the high editing efficiency of enOscas12f in mammalian cells, chromatin loci influence editing efficiency. Further evaluation of AAV-mediated in vivo delivery and gene editing with enOsCas12f is warranted.

1186. Targeting a Mutation Agnostic Regulator of *PKD1* Using CRISPRCas9 for Therapeutic Intervention in Autosomal Dominant Polycystic Kidney Disease

Poulami Chaudhuri[1], Neha Gaur[1], Rakesh Kappagantu[1], Yugantak Raj Gupta[1], Sadhana Mutalik[2], Harsha Rohira[1], Naveen Kumar D[2], Rohini Kalvakuntla[1], Anirudh Nishtala[1]

[1]Helex, Inc., New York, NY,[2]Unicas Biotech, Hyderabad, India

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a hereditary condition caused by mutations in the *PKD1* (85% cases) or *PKD2* (13% cases) genes, which encode polycystin-1 and polycystin-2 proteins, respectively. This condition is characterized by the formation of fluid-filled cysts within the kidneys, which arise from renal tubules and can lead to end-stage renal disease (ESRD). *PKD1/PKD2* genes

are significantly repressed in ADPKD and lead to cytogenesis. In various pre-clinical models and humans with ADPKD, *PKD1/PKD2* gene re-expression has displayed attenuation as well as reversal of cyst formation proving this strategy to be of therapeutic potential. As 85% of ADPKD cases are contributed by *PKD1* mutations and there are more than 800 pathogenic mutations in *PKD1*, we are pursuing a mutation-agnostic approach than the conventional single-target therapies for ADPKD caused by *PKD1*. We employ Helex's proprietary Epic-Cure™ platform, which uses 3D genome architecture and epigenetics of ADPKD patients to design the gRNAs in conjunction with the molecular scissor CRISPR-cas9, to edit the mutation agnostic target that regulates *PKD1* gene in the most effective, precise and safe way. One of the most crucial challenges for developing a therapeutic for renal indications is the delivery to the kidney cells. We designed our own proprietary LNP that could facilitate kidney delivery of the gene editing cargo. We confirmed the knockout of the mutation agnostic target region by using the T7E1 assay and Sanger Sequencing in patient derived immortalised cell line, WT9-12 and patient derived cyst cells. Additionally, we observed a significant four-fold increase in *PKD1* mRNA levels, as evidenced by reverse transcription polymerase chain reaction (RT-PCR) post editing. This demonstrates our editing approach, facilitated by Helex's proprietary EPIC Cure platform, effectively eliminates the mutation agnostic target that regulates the *PKD1* gene. We were able to display significant kidney tropism for our proprietary LNP via tail vein injections in BALB/c mice using a DiR dye both in in vivo and ex vivo imaging. This precise intervention of the mutation agnostic target showing a significant increase in *PKD1* mRNA expression and the specificity of the proprietary kidney tropic LNP vector together showcases the potential as a therapeutic modality for ADPKD. Our mutation-agnostic strategy, harnessing Helex's proprietary Epic-Cure™ platform and genome editing (CRISPR-Cas9), holds promise in advancing therapeutic interventions to not just halting the disease but reversing the disease phenotype which goes way beyond current treatments, which focus on managing symptoms and slowing the progression of the disease.

1187. Investigating the Impact of CRISPR-Induced DSB Repair Mechanisms on *In Vivo* Recombinant AAV Integration

Martin Bilbao-Arribas[1,2], Julen Torrens-Baile[1], Laura Torella[1], Nerea Zabaleta[3], Gloria Gonzalez-Aseguinolaza[1]

[1]DNA & RNA Medicine Division, CIMA Universidad de Navarra, Pamplona, Spain,[2]IdISNA, Navarra Institute for Health Research, Pamplona, Spain,[3]Mass Eye and Ear/Harvard Medical School, Boston, MA

The use of paired Cas9 nickases has been proposed as a safer alternative to Cas9 nucleases for therapeutic gene disruption, but this strategy requires further characterization for in vivo applications. The choice of double strand break (DSB) repair pathway can be influenced by different factors such as the sequence context, cell type and cell cycle stage, or the nature of the free cut-ends. In a previous study we used adeno associated viruses (AAVs) to deliver CRISPR-Cas9 systems to hepatocytes for in vivo gene disruption of *Hao1* gene. We showed that paired Cas9 nickase-induced DSBs are mainly repaired through microhomology-dependent repair pathways and exhibit lower rates of vector sequence insertion compared to Cas9 nuclease-induced DSBs. Here, we shed light on the relationship between AAV integration frequency and the choice of DSB repair pathway by analysing the editing outcomes of several in vivo studies. The widely used tools for indel analysis of targeted amplicon sequencing data have not been optimised for the characterization of variants resulting from paired Cas9 nickase-induced DSBs, particularly concerning large insertions like AAV sequence insertions. Our study aimed to benchmark different tools to identify the best performing for paired Cas9 nickases in terms of total indel quantification and AAV insertion detection. Additionally, we conducted an alignment parameter optimisation to further improve AAV insertion quantification. Importantly, these optimised parameters can be applied to commonly used gene editing analysis tools, refining the efficiency of AAV insertion recovery. Seeking to understand the factors contributing to the observed reduction in AAV insertions with paired nicks, we delved into the DNA ends and repair systems involved in AAV integration. A reanalysis of a set of short-read amplicon sequencing data from various publications utilizing different CRISPR-Cas systems alongside AAVs for gene editing revealed a correlation between increased microhomology (MH) involvement and diminished AAV insertions. Interestingly, nucleases generating staggered ends, such as those created by the type V CRISPR family or paired Cas9 nickases, resulted in the highest MH scores and the lowest AAV insertion frequencies. Conversely, in the case of nucleases that create blunt ends, like those generated by Cas9 enzymes, indel outcomes - including AAV insertions - likely depended on the sequence context or other variables. Consequently, we are performing ad hoc experiments in vivo using selected guides having high or low predicted MH scores, as well as on-target long-read sequencing, to elucidate whether the frequency of AAV insertions depend on the staggered-end formation and/or the repair mechanisms involved. These results support the use of paired Cas9 nickases for in vivo gene disruption because on one side, it is known to minimise the risk of off-target modifications, and on the other, reduces the frequency of AAV insertions.

1188. CRISPR/CasX-Mediated Gene Editing as a Therapeutic Approach for HSV-1 Keratitis

Guoxiang Ruan[1], Nadia Amrani[1], Kevin Luk[1], Meltem Isik[1], Sarmistha Bhattacharya[2], Niles P. Donegan[3], Martina Donadoni[4], Anna Bellizzi[4], Ilker K. Sariyer[4], Matthew Hayden[2], Jennifer Gordon[1]

[1]Excision BioTherapeutics, Watertown, MA,[2]Dartmouth Health, Lebanon, NH,[3]White River Junction Veterans Affairs Medical Center, White River Junction, NH,[4]Temple University School of Medicine, Philadelphia, PA

Herpes simplex keratitis (HSK), caused by the infection of herpes simplex virus type 1 (HSV-1) of the cornea, is a major cause of blindness worldwide. After primary infection of the ocular surface, HSV-1 is transported to trigeminal ganglia (TG) and other ganglia and establishes lifelong latency. Periodically reactivated HSV-1 virus travels back to the cornea, causing recurrent HSK. Current anti-HSV-1 therapies, such as acyclovir, valacyclovir, and famiciclovir, interfere with viral DNA replication. However, these antiviral agents do not eliminate HSV-1 reservoirs or prevent recurrence. CRISPR/Cas-mediated gene editing can potentially address the underlying causes of the disease by eliminating the latent HSV-1 reservoirs directly. While efficient gene editing can be achieved by Cas9 nucleases, their large size has hindered delivery by a single adeno-associated virus (AAV)

vector. CasX (type V Cas12e) from *Planctomycetes* (hereafter CasX2) has gained significant attention due to its compact size (<1000 aa). Compared to Cas9, CasX2 is small enough to be delivered by a single AAV, with additional room for multiplexed guide RNAs. This study aims to identify CasX2 guide RNA pairs that effectively excise the DNA fragment between two vital HSV-1 genes, ICP27 and ICP0. We first designed 20 and 22 gRNAs targeting conserved sequences of the ICP27 and ICP0 genes, respectively. To identify the most effective gRNA pairs, we created a LentiCasX2-2xgRNA library comprised of the 440 CasX2 gRNA pairs formed from the gRNAs targeting the two genes. Lentiviral vectors prepared from the LentiCasX2-2xgRNA library were used to infect an HSV-1 gain-of-signal knock-in reporter HEK 293FT cell line containing the ICP27 and ICP0 consensus sequences. Cells with successful ICP27-ICP0 excision were enriched with blasticidin drug selection and harvested with FACS. The region containing the paired CasX2 gRNAs was PCR amplified and sequenced using Nanopore long-range sequencing. Out of the 440 gRNA pairs, 23 pairs showed more than two-fold enrichment with lentiviral screening. The top two CasX2 gRNA pairs from the screen were tested in Vero cells infected with the HSV-1 virus. To slow down the quick replication of the HSV-1 virus, the Vero cells were treated with valacyclovir for 24 hours post-infection, and then cultured for an additional four days to allow the HSV-1 to replicate. Compared to the empty vector control, the two CasX2 gRNA pairs reduced HSV-1 genome DNA and viral titer by over 96%. In conclusion, we successfully used a high-throughput lentiviral screening method to identify effective guide RNA pairs to disrupt the HSV-1 viral genome and inactivate the HSV-1 virus. Our findings suggest that CRISPR/CasX gene editing could be a potential cure, preventing HSV-1 recurrence in HSK and other HSV-1 related diseases.

1189. Allele-Specific CRISPR/Cas9 Editing Inactivates a Single Nucleotide Variant Associated with Collagen VI Muscular Dystrophy

Veronique Bolduc[1], Katherine Sizov[1], Astrid Brull[1], Eric Esposito[1], Grace S. Chen[1], Prech Uapinyoying[1], Apurva Sarathy[1], Kory Johnson[2], Carsten G. Bonnemann[1]

[1]Neuromuscular and Neurogenetic Disorders of Childhood Section, NINDS/NIH, Bethesda, MD,[2]Bioinformatics Core, NINDS, Bethesda, MD

The application of allele-specific gene editing tools can expand the therapeutic options for dominant genetic conditions, either via gene correction or via allelic gene inactivation in situations where haploinsufficiency is tolerated. Here, we used allele-targeted CRISPR/Cas9 guide RNAs (gRNAs) to introduce inactivating frameshifting indels at a single nucleotide variant in the *COL6A1* gene (c.868G>A; G290R), a variant that acts as dominant negative and that is associated with a severe form of congenital muscular dystrophy. We expressed spCas9 along with allele-targeted gRNAs, without providing a repair template, in primary fibroblasts derived from four patients and one control subject. Amplicon deep-sequencing for two gRNAs tested showed that single nucleotide deletions accounted for the majority of indels introduced. While activity of the two gRNAs was greater at the G290R allele, both gRNAs were also active at the wild-type allele. To enhance allele-selectivity, we introduced deliberate additional mismatches to one gRNA. One of these optimized gRNAs showed minimal activity at the WT allele, while generating productive edits and improving collagen VI matrix in cultured patient fibroblasts. This study strengthens the potential of gene editing to treat dominant-negative disorders, but also underscores the challenges in achieving allele selectivity with gRNAs.

1190. Optimization of Multiplex CRISPR-Cas9 Editing of Human Primary T Cells Using Lipid Nanoparticles (Lnp's) and Subsequent Off-Target Evaluation

Erica Warriner[1], Samuel Clarke[1], Meenakshi Swaminathan[1], Bernice Thommandru[2], Reka Geczy[1], Rita Zhao[1], Krystal Tan[1], Kevin Low[1], Aruna Balgi[1], Stella Park[1], Ethan Watt[1], Vaughn Thada[3], Garrett Rettig[2], Angela Zhang[1], Rolf Turk[2]

[1]Cytiva, Vancouver, BC, Canada,[2]Integrated DNA Technologies, Coralville, IA,[3]Integrated DNA Technologies, Coralville, ID

Background and Aims: CRISPR/Cas9 has emerged as a dynamic tool for gene editing of human primary T cells. Previously, we demonstrated a novel lipid nanoparticle (LNP) reagent for engineering of gene-edited CAR T cells with high cell viabilities and potency, even after multiple genetic manipulations. Here, we extend this work by optimizing multi-gene knockouts and assessing potential off-target editing effects in T cells, with RNAs delivered either by electroporation or LNPs. Further, through extensive T cell media evaluation, we show the critical nature of cell culture conditions for efficient LNP-mediated transfections. **Methods:** LNPs encapsulating wild type or high fidelity S.p. Cas9 mRNA and various TRAC and CD52 targeted guide RNAs (sgRNAs) were produced using the scalable NxGenTM mixing platform. Alongside, electroporation was performed to deliver equivalent cargoes. Primary T cells were cultured, activated, and expanded in different commercial media T cell media in static culture. Gene expression and cell viability were measured using flow cytometry. Multi-target performance of CRISPR-Cas9 editing was evaluated through rhAmpSeq-based targeted next-generation sequencing (NGS) and indels analyzed for with CRISPRAltRations. **Results:** TCR or CD52 targeted Cas9 mRNA-LNP addition or electroporation yielded high single and double knockout efficiencies. We tested a range of sgRNA targets, wild-type and high-fidelity Cas9 mRNAs, and determined off-target editing. We achieved 90% ±2.6% double edited T cells (TCR-/CD52-). Similar results were obtained when comparing different LNP batch sizes (micro to milligram RNA) and cell culture vessels (0.1 to 45 million cells), demonstrating scalability of both LNPs and cell treatment. Editing efficiency and potency was affected by the type of culture media. LNP delivery to T cells for gene editing was optimized in two commercially available T cell culture media yielding similar EC50 values (0.3 to 0.5 µg/million cells). **Conclusions:** The results from this study further support the utility RNA-LNPs for the genetic engineering of primary T cells. The simple and gentle nature of LNP cell treatment allows for multiple genetic engineering steps for simultaneous expression and deletion of proteins for future cell therapies. These LNPs can be easily manufactured from small-scale screening of RNA libraries to rapid scale-up for clinical translation.

Thursday Posters: Targeted Gene Insertion

1191. Tridimensional Scaffolds Preserve Hematopoietic Stem and Progenitor Cell Polarity and Restrains DNA Damage for Efficient Gene Therapy Applications

Federico Midena[1], Laura Alessandrini[2], Roberta Vacca[1], Matteo Barcella[1], Emanuela Jacchetti[3], Edoardo Carsana[1], Claudio Conci[3], Teresa Tavella[1], Ivan Merelli[1], Manuela Raimondi[3], Raffaella Di Micco[1]

[1]SR-TIGET, Milan, Italy,[2]Università Vita-Salute San Raffaele, Milan, Italy,[3]Politecnico di Milano, Milan, Italy

Hematopoietic Stem and Progenitor Cells (HSPC) engineering, and particularly long-range gene correction, requires *ex vivo* activation to achieve a sizable fraction of edited cells. However, we recently discovered that *ex vivo* culture inadvertently leads to ROS-dependent proliferative stress and DNA damage, resulting in stem cell dysfunctions and potentially jeopardizing the efficacy of genetic engineering for HSPC-based clinical applications. We reasoned that these detrimental responses may arise from HSPC removal from the hypoxic bone marrow niche and be exacerbated by growth on bidimensional substrates. Thus, we employed a biocompatible tridimensional scaffold to counteract culture stress and reported mitigation of DNA damage accumulation, increased clonogenic potential, and enhanced multi-lineage repopulating capacity upon transplant into immunocompromised mice. Surprisingly, RNA-Seq analyses revealed massive transcriptional changes upon 3D culture, including upregulation of genes belonging to cytoskeleton organization and adhesion, and concomitant downregulation of oxidative phosphorylation and protein synthesis, which are associated with more primitive stem cells. Mechanistically, 3D scaffolds prevented HSPC deformation and squashing occurring in bidimensional culture, conserving nuclei morphology and tubulin polarity, potentially facilitating adaptation to *ex vivo* culture and preservation of stemness properties. Most importantly, pre-culture on 3D scaffolds ameliorated HSPC functionality upon Cas9/AAV6-mediated GE, endowing edited cells with increased multi-lineage output at single-cell resolution *in vitro* and superior engraftment into NSG mice. Similar results were obtained across different HSPC sources, such as cord blood and mobilized peripheral blood, as well as in multiple therapeutic settings, including lentiviral gene transfer and HSPC expansion, which is particularly relevant for certain diseases in which only a limited number of cells can be harvest from patients. Overall, our results highlight the importance of tridimensional interactions for the maintenance of HSPC biological properties, especially during *ex vivo* manipulation for gene therapy applications.

1192. Assessing the Best Strategy and Timeframe for *In Vivo* Genome Editing of Progressive Familiar Intrahepatic Cholestasis Type 2

Chiara Simoni[1,2], Elena Barbon[1], Francesco Starinieri[1], Martino Alfredo Cappelluti[1], Justine Nozi[3], Camilla Negri[1], Elisabetta Manta[1], Mauro Biffi[1], Matteo Mori[2], Rossana Norata[1], Martina Rocchi[4], Francesca Sanvito[4], Giuseppe Ronzitti[3], Angelo Lombardo[1,2], Alessio Cantore[1,2]

[1]SR-TIGET, Milan, Italy,[2]Vita Salute San Raffaele University, Milan, Italy,[3]Genethon, Evry-Courcouronnes, France,[4]IRCCS San Raffaele Scientific Institute, Milan, Italy

Progressive familiar intrahepatic cholestasis type 2 (PFIC-2) is a monogenic metabolic disorder caused by mutations in *ABCB11* gene encoding the Bile Salt Export Pump. Loss of function of this protein results in impaired secretion of bile from the liver leading to jaundice, pruritus, fibrosis and ultimately to liver failure. PFIC-2 treatment consists in alleviation of pruritus, external or internal biliary diversion or ileal transporter inhibitors. Since liver transplantation has shown to be beneficial but is associated to several possible complications, liver-directed *in vivo* genome editing may represent an alternative treatment, potentially allowing for stable and precise gene correction in paediatric patients. Here, we aim to compare different strategies based on the CRISPR-Cas9 platform, to select the most efficient to integrate *ABCB11* cDNA under the control of its endogenous promoter. We plan to deliver *in vivo* donor DNA with viral vectors and Cas9 mRNA and guide RNA (gRNA) with lipid nanoparticles (LNP). Firstly, we designed 12 gRNAs targeting the first intron of *Abcb11* to address most of the disease-causing mutations and maintain as much as possible physiological gene regulation. We screened the gRNAs based on their cutting efficiency in hepatocyte cell lines and selected the best performing one. Afterwards, we used mCherry as reporter gene, to compare different donor DNAs designed to exploit homology directed repair (HDR) or homology-independent targeted integration (HITI). *In vitro*, we observed the highest integration rate with the HDR-based donor DNA. *In vivo*, we systemically administered adeno-associated viral vectors (AAVs) delivering the different donor DNAs configurations to Cas9 transgenic mice. We observed slightly higher integration rate, mCherry expression and mCherry-positive liver area exploiting HDR-based donor DNA. We obtained the same results in 2-week- and 2-day-old Cas9 mice. Since patients affected by PFIC-2 develop liver fibrosis even before one year of age in the most severe forms, we set out to assess its impact on *in vivo* genome editing. Indeed, liver fibrosis may negatively affect the delivery efficiency of genome editing components, either by viral vectors or LNP, and therefore the efficacy of the treatment. Thus, we evaluate the impact of fibrosis on viral vector transduction, exploiting both chemically induced and genetic mouse models of liver fibrosis. We intravenously administered lentiviral vector (LV) or AAV encoding for a reporter gene to CCl_4 treated mice, which display intermediate to severe pericentral fibrosis. In this model we observed reduced LV transduction while AAV transduction was not affected. In mice treated with DDC diet, which display intermediate to severe periportal fibrosis, we observed reduced transduction efficiency by both LV and AAV. Moreover, we intravenously administered LV to 6-month-old PFIC-2 mice, displaying mild periportal fibrosis, and showed a slight

reduction of LV transduction. These findings highlight the need for early intervention in diseases like PFIC-2 which rapidly develop liver fibrosis. Overall, these data provide the basis to further develop *in vivo* genome editing approaches and to assess the best timeframe for the treatment of PFIC-2 patients.

1193. Dual AAV-Delivered, Meganuclease-Mediated Gene Insertion Approach for the Treatment of Phenylketonuria - Proof-of-Concept Study in Rhesus Macaques

Benedicte Rousseau[1], Celensia Tolson[1], Rucha Fadnavis[1], Taku Moriwaki[1], Katrina M. Ralph[2], Kathryn Shelton[2], Luke J. Segura[2], Rick A. Finch[2], R. Jason Lamontagne[1], Jenny A. Greig[1], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]MD Anderson, Bastrop, TX

Phenylketonuria (PKU) is an autosomal recessive metabolic disorder caused by missense mutations in the gene encoding phenylalanine hydroxylase (*PAH*), a hepatic enzyme that catalyzes the conversion of phenylalanine to tyrosine. Although PKU does not directly shorten life expectancy, high phenylalanine concentrations in tissues and blood can result in symptoms ranging from a musty odor from the skin and urine to intellectual disability, epilepsy, and hyperactivity/behavioral problems. Most PKU cases are detected at birth via newborn screening; overall incidence is 1/15,000 in the United States, but this rate varies around the world. Dietary and pharmacological treatment options exist, but their life-long, inconvenient nature can raise compliance and efficacy issues. Our aim was to establish a long-lasting, single-administration treatment for PKU. Here, we developed a dual adeno-associated virus (AAV) vector-delivered, meganuclease-mediated gene insertion approach to insert the human PAH transgene into a previously identified safe harbor site for sustained expression. Our approach comprises two AAVrh79 vectors. The first vector encodes an engineered ARCUS meganuclease, M2PCSK9, that targets the human *PCSK9* gene. The second, a "donor" vector, encodes a codon-optimized version of the human PAH transgene flanked by 500 bp homology arms on either side of the meganuclease target site in the human genome (and ~95% homologous to rhesus macaques) to aid insertion at the meganuclease-induced double-strand break. We evaluated our approach in a proof-of-concept study in 6-9-month-old rhesus macaques following intravenous administration. The dual AAVs were administered at a total dose of 4 x 10^{13} GC/kg (n=2; high dose) and 1.33 x 10^{13} GC/kg (n=2; low dose) with a 3:1 donor-to-meganuclease vector ratio. Another cohort received a 1:1 ratio for a total dose of 2 x 10^{13} GC/kg (n=2; intermediate dose). Liver biopsies were performed at three months post dosing, and animals were followed with bi-monthly blood tests to monitor liver function. Transient, mild elevations of liver enzymes (alanine transaminase and aspartate transaminase) that resolved without intervention were observed and blood chemistry analyses showed no signs of decreased liver function. As the M2PCSK9 meganuclease specifically disrupts PCSK9 expression, we used total serum PCSK9 protein levels as a surrogate readout of target site editing. We observed a consistent decrease in PCSK9 levels in all three dual treatment cohorts, with the intermediate- and low-dose cohorts achieving a > 50% reduction compared with the donor-only group. We conducted amplicon sequencing to directly assess target site editing at the three-month biopsy timepoint. At the meganuclease target site, we detected an insertion/deletion frequency of 5% in the low-dose, 21.1% in the intermediate-dose, and 11% in the high-dose cohorts compared with 0.08% in the donor-only group. *In situ* hybridization from liver biopsy tissue analyses of the dual treatment cohorts showed expression of human PAH RNA in 4.5% of cells in the low-dose, 8.5% in the intermediate-dose, and 3.1% in the high-dose animals, and 4.5% in the donor-only group; we confirmed this expression pattern via RNA transcript levels. These results indicate that our dual AAV vector-delivered, meganuclease-mediated gene insertion approach can safely introduce a PAH transgene into the liver of nonhuman primates. This finding raises the possibility that our combination therapy may represent a single-administration treatment approach for PKU that is PAH-mutation-agnostic and that may provide a less restrictive, improved quality of lives for those affected by PKU.

1194. Dual AAV-Delivered, Meganuclease-Mediated Gene Insertion Approach for the Treatment of Citrullinemia Type 1

Mark Urban[1], Rucha Fadnavis[1], Celensia Tolson[1], Hongwei Yu[1], Katrina M. Ralph[2], Kathryn Shelton[2], Luke J. Segura[2], Rick A. Finch[2], Lili Wang[1], R. Jason Lamontagne[1], Jenny A. Greig[1], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]MD Anderson, Bastrop, TX

Citrullinemia type 1 (CTLN1) is characterized by elevated blood citrulline levels caused by mutation of the argininosuccinate synthetase 1 (*ASS1*) gene. ASS1 converts citrulline and aspartate to argininosuccinate in the urea cycle. Loss of functional ASS1 results in a failure to clear ammonia through urea, which can lead to hyperammonemia, which, in turn, can cause lethargy, increased intracranial pressure, seizures, coma, and death. Liver transplantation currently represents the only effective curative treatment. Here, we developed a dual adeno-associated virus (AAV) vector-delivered, meganuclease-mediated gene insertion approach to insert the human ASS1 transgene into a previously identified safe harbor site for sustained expression. Our approach comprises two AAVrh79 vectors. The first vector encodes an engineered ARCUS meganuclease, M2PCSK9, that targets the human *PCSK9* gene. The second, a "donor" vector, encodes the native human ASS1 transgene flanked by 500 bp homology arms on either side of the meganuclease target site in the human genome (and ~95% homologous to rhesus macaques) to aid insertion at the meganuclease-induced double-strand break. We evaluated our approach in infant (2-3-month-old) rhesus macaques following intravenous administration at a total dose of 1.33 x 10^{13} genome copies (GC)/kg (n=2) or 4 x 10^{13} GC/kg (n=2). An additional animal was injected with 3 x 10^{13} GC/kg of the donor vector alone (n=1). Vector administration was well tolerated with normal serum biochemistry and liver function indices throughout the study, indicating that our approach posed a low risk to liver integrity and function. As the M2PCSK9 meganuclease specifically disrupts PCSK9 expression, we used total serum PCSK9 protein levels as a surrogate readout of target site editing. We found a dose-dependent reduction in PCSK9 serum levels compared with the donor-only control during the first year following treatment. Liver biopsies were taken at ~3 months and

one-year post-injection to determine ASS1 transgene expression and editing at the *PCSK9* locus. Amplicon sequencing of the meganuclease target site revealed an insertion/deletion frequency of 12.3% (low dose) and 30.7% (high dose) compared with 0.02% in the donor-only group, indicating efficient meganuclease-mediated target site editing. Levels of hepatic ASS1 RNA and protein expression were found to positively correlate with vector dose. *In situ* hybridization analyses at 3 months showed ASS1 RNA expression in 7.1% (low dose) and 20.8% (high dose) of cells in macaques dosed with the dual vector approach, but only in 1.6% of cells in the donor-only macaque. Human ASS1 protein expression, assessed by immunofluorescence, was present in 8.9% (low dose) and 14.5% (high dose) of cells at 3 months in macaques dosed with the dual vector approach, but only in 1.5% of cells in the donor-only animal. Follow-up analyses (currently ongoing) suggest that these expression levels were maintained for at least 12 months. Previous studies in multiple models of CTLN1 indicate that achieving 5%-10% of normal ASS1 activity confers a therapeutic effect, suggesting that our dual AAV vector approach can achieve clinically relevant, stable ASS1 expression in the liver of NHPs. These results add to a growing body of data that supports the use of our dual AAV vector-delivered, meganuclease-mediated gene insertion approach to treat various genetic metabolic conditions using disease-specific donor vectors in combination with our *PCSK9* safe harbor site-targeting meganuclease.

1195. Efficacy and Toxicology Studies of a Clinical Candidate Dual AAV-Delivered, Meganuclease-Mediated, Gene Insertion Approach for Ornithine Transcarbamylase Deficiency

Claude C. Warzecha[1], Lili Wang[1], Peter L. Bell[1], Yanqing Zhu[1], Cecilia D. Dyer[1], Elizabeth L. Buza[1], Rucha Fadnavis[1], Zhenning He[1], Hong Zhang[1], Celensia Tolson[1], Katrina M. Ralph[2], Carolyn L. Hodo[2], Kathryn Shelton[2], Luke J. Segura[2], Rick A. Finch[2], R. Jason Lamontagne[1], Jenny A. Greig[1], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]MD Anderson, Bastrop, TX

Ornithine transcarbamylase deficiency (OTCD) is an X-linked urea cycle disorder that causes neurotoxic ammonia buildup, which can result in potentially fatal episodic hyperammonemia crises. Current medical management of OTCD includes a protein-restricted diet and ammonia-scavenging agents. Liver transplantation is the only available curative treatment option. However, these options may be insufficient or unavailable to alleviate hyperammonemia crises in infants; thus, there is an unmet clinical need for a long-term curative treatment. Here, we report two investigational studies with a dual adeno-associated virus (AAV) vector-delivered, meganuclease-mediated gene insertion approach to treat OTCD. Our approach comprises two AAVrh79 vectors that insert the OTC transgene into a previously identified safe harbor site for sustained expression. The first vector encodes an engineered ARCUS meganuclease, M2PCSK9, that targets the human *PCSK9* gene. The second "donor" vector encodes a codon-optimized human OTC transgene flanked by 500 bp homology arms on either side of the meganuclease target site in the human genome (~95% homologous in rhesus macaques) to aid insertion at the meganuclease-induced double-strand break. We conducted a minimal effective dose study in *spf^{ash}* mice, an OTCD model, to assess efficacy. Neonatal *spf^{ash}* mice received intravenous (IV) injections of one of three total vector doses ($4.4x10^{12}$ genome copies [GC]/kg, $1.3x10^{13}$ GC/kg, or $4.0x10^{13}$ GC/kg) of a murine surrogate (homology arms include mouse genomic sequences) or $3.0x10^{13}$ GC/kg of the donor vector alone for human OTC expression; additional *spf^{ash}* mice and wild-type mice received vehicle as controls. *spf^{ash}* mice injected with the intermediate or high doses of the dual vector survived 9.6 days or 8.6 days, respectively, during a 10-day high-protein diet challenge, whereas vehicle-treated mice survived an average of 7.6 days. We achieved OTC enzyme activity levels in the livers of high-dose-treated *spf^{ash}* mice up to 29% of wild-type levels, compared with 10% or 13% of wild-type levels in *spf^{ash}* mice injected with vehicle or OTC donor vector only, respectively. These increases in OTC enzyme activity and survival demonstrated the advantage of our approach over traditional gene therapy (as modeled by donor vector alone). We also performed a toxicology study in infant rhesus macaques whereby 6-12-week-old macaques received IV injections of $4.0x10^{13}$ GC/kg of our dual vector (n=4) or vehicle (n=2). Three animals (one vehicle and two vector-injected macaques) were necropsied on Day 91; the remaining three animals had a liver biopsy on Day 91 and were necropsied one-year post-injection. *In situ* hybridization and immunostaining assays revealed stable human OTC mRNA and protein expression, respectively, from Day 91 through one-year post-dosing, in which 25%-30% of hepatocytes were positive for human OTC. Vector administration was well tolerated, with no impact on clinical pathology parameters except for transient, mild liver enzyme elevations that resolved without intervention. Comprehensive histopathological examination at Day 91 and one year revealed vector-related, non-adverse mild mononuclear cell infiltrates in the portal area of the liver and minimal neuronal degeneration/necrosis in the trigeminal and dorsal root ganglia. In conclusion, these data suggest that our dual AAV vector-delivered, meganuclease-mediated gene insertion approach could safely and efficiently insert a human OTC transgene into the genome in a mutation-agnostic manner to treat OTCD.

1196. Leveraging Homology Directed Repair with the Use of an AAV Donor and CRISPR/Cas9 Nuclease to Accelerate Efficacy in Wilson Disease Mice

Amy M. Bastille[1], Jay Wang[1], Shreya Amarwani[1], Kirsten Lapoint[1], Don Foster[1], Neil Huang[1], Megan Ericson[1], Ogooluwa Ojelabi[2], Shiliang Hu[1], Bei Cheng[3], Matthew Chiocco[1], Mariana Nacht[1], Mathias Hebben[1]

[1]Alexion Genomic Medicine, Astra Zeneca Rare Disease, Lexington, MA,[2]LogicBio Therapeutics, Lexington, MA,[3]Astra Zeneca, Waltham, MA

Wilson Disease (WD) is a progressive, rare monogenetic disease resulting in excessive copper accumulation in tissues such as liver, brain and eye. This disease is caused by a mutation in the ATP7B gene and transmembrane protein, resulting in copper toxicity. We previously used GeneRide, our AAV insertion technology, to demonstrate that correction of hepatocytes in a mouse model of WD resulted in lowering of urinary copper and the level of plasma liver injury marker, ALT. To accelerate metabolic correction and reduce disease pathogenesis, we investigated the combination of GeneRide with a CRISPR/Cas9-based

nuclease delivered by lipid nanoparticles (LNPs). Our historical data with GeneRide demonstrated therapeutic benefit in this model, relying on the phenomenon of selective advantage that can be observed in diseases with severe liver damage such as methylmalonic acidemia (MMA) and hereditary tyrosinemia type I (HT-1). Selective advantage and hepatocyte expansion occurs because GeneRide-edited hepatocytes are healthier than mutant hepatocytes, and the repopulation of the liver by edited hepatocytes drives efficacy over time. However, due to the low initial editing rate with GeneRide alone, it can take many months to see benefits of the therapy in some models of metabolic disease. With the addition of a nuclease to GeneRide, in wild-type mice we observed an up to 1000-fold increase in the amount of ALB2A, a circulating technology-specific biomarker that correlates with the number of edited hepatocytes. To see if editing more cells initially, in conjunction with selective advantage, would accelerate time to efficacy, we evaluated this strategy in the WD mouse model. We observed an increase of 750-fold in ALB2A expression, compared to GeneRide alone, at one-week post-dose which suggests a higher editing efficiency. This level of ALB2A expression corresponded to a level achieved after 7-8 months with GeneRide alone. Furthermore, a longitudinal analysis of the surrogate biomarker ALB2A and ATP7B expression directly, along with key WD-associated biomarkers shows an acceleration of metabolic correction and therapeutic benefit. This suggests that combining a nuclease with GeneRide technology could be used to treat a variety of metabolic diseases.

1197. Endogenous Human TRIM Protein Expression Influences Potency of a Knock-In Human-Rhesus Chimeric Protein Expression Platform for CXCR4-Tropic HIV-1 Cure

Amanda M. Dudek[1,2], Elena J. Sasu[1,2], Matthew H. Porteus[1,2]

[1]Pediatrics, Stanford University School of Medicine, Stanford, CA,[2]Institute for Stem Cell Biology & Regenerative Medicine, Stanford University School of Medicine, Stanford, CA

Allogeneic hematopoietic stem and progenitor cell transplant (Allo-HSCT) of naturally *CCR5*-null cells has cured HIV-1 infection in some individuals, however these instances have been extremely rare due to several limitations, including high prevalence of dual co-receptor and CXCR4-co-receptor using viral strains. We have recently reported a Cas9/AAV based multi-target genome editing strategy which can be used in an autologous HSCT setting (therefore preventing the risk of graft verses host disease), and which shows greater than 2,000-fold reduction in viral load upon CXCR4-tropic challenge of primary human peripheral blood derived CD4+ T cells. During challenge of individual components of this multi-editing strategy, we identified that single knock-in of the inhibitory protein hRhTRIM5α (a humanized chimera of the species specific restriction factor TRIM5α) showed high donor-to-donor variability in efficacy for restricting CXCR4-tropic replication. While highly effective at restricting HIV replication in some T cell donors (>200- fold reduction in free virus concentration), this individual factor showed very little effect in other donors (~3-fold reduction), suggesting donor-specific or cell-biology-specific differences in restriction factor function. As a multi-meric and catalytically active protein, we hypothesized that endogenous human TRIM5 and TRIM5-interacting proteins may be influencing the efficacy of the therapeutic hRhTRIM5α protein after knock-in. Using peripheral blood CD4+ T cells from previously characterized donors, we have identified that knock-out of the endogenous human TRIM5 locus decreases the efficacy of hRhTRIM5α restriction, causing a roughly 20-fold reduction in restriction factor potency in multiple T cell donors. Reduction in restriction factor efficacy was observed not only by reduction in fold-change of free-virus concentration, but additionally in reduced inhibition of intracellular HIV DNA copy accumulation. These data highlight an interesting mechanistic result that human TRIM5 may facilitate a cooperative effect that augments the effective concentration of restrictive TRIM proteins in primary CD4+ T cells. Additionally, these results have significant implications for improving the efficacy of various gene editing and cell therapy strategies in development using this family of proteins, and highlights the importance of personalized medicine in development of effective therapies for HIV-1.

1198. *In Vivo* Transgene Expression from Hematopoietic Cells in Primatized Mouse Models Following Non-Viral Knock-In

Jack M. P. Castelli[1,2], Rachel Cunningham[1,2], Molly E. Cassidy[2], Youngseo Jwa[2], Matthew D. Gray[3], Mark R. Enstrom[2], Jonathan D. Linton[2], Anthony Rongvaux[2,4], Justin J. Taylor[5], Jennifer E. Adair[2,6]

[1]Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA,[2]Translational Science and Therapeutics Division, Fred Hutchinson Cancer Center, Seattle, WA,[3]Vaccine and Infectious Diseases Division, Fred Hutchinson Cancer Center, Seattle, WA,[4]Department of Immunology, University of Washington, Seattle, WA,[5]Beirne B. Carter Center for Immunology Research, University of Virginia, Charlottesville, VA,[6]Department of Medical Oncology, University of Washington, Seattle, WA

Broadly neutralizing antibodies (bnAb) against HIV are capable of providing immune protection in non-human primate (NHP) models and clinical trials but require repeated injections. Endogenous production of bnAbs could provide life-long treatment for controlling HIV, ideally as a single intervention. We previously presented editing of the immunoglobulin heavy chain locus in NHP hematopoietic stem and progenitor cells (HSPC) to achieve knock-in and expression of long transgenic sequences, without the use of viral vectors. Here, we show that edited HSPCs are capable of engrafting in primatized mouse models and differentiating into cells of the lymphoid lineage. Comparing 10 different CRISPR/Cas guides using 2 different nucleases, we show that the Cas12a ribonucleoprotein can achieve perfect homology-directed repair (HDR) events at a frequency of 2.4% (Fig.a). Cas9 guides displayed lower editing on average (27.4% ± 15.3%) and a higher frequency of imperfect HDR (96.2% ± 1.7% of total HDR events) than Cas12a guides (39.8% ± 12.1% and 68.3% ± 13.1% respectively). Electroporation assays in $CD34^+$ cells isolated from healthy donor pigtail macaques (*Macaca nemestrina*) showed that single-stranded oligodeoxynucleotide templates achieved 4-fold more HDR events than double-stranded DNA of the same length. Edited NHP HSPC (n=2 donors) were injected into a sub-lethally irradiated neonatal immunodeficient mouse model. Cell doses of 1.5×10^5 per

mouse achieved engraftment of up to 3.1% ± 0.5% NHP-derived cells in the blood (n=19 mice), allowing for study of lymphocytes (Fig.b). As previously observed, B cell populations start at a higher fraction and steadily decline over time until T cells become the dominant lineage at 12-14 weeks (Fig.c). A fluorescent eGFP reporter under the control of a B cell specific antibody heavy chain gene promoter shows expression patterns that match this shift, with 0.41%-0.77% of NHP-CD45$^+$ cells expressing eGFP in some mice before week 14 (Fig.d). Ongoing studies are focused on NHP HSPCs edited with a transgene encoding the bnAb 10-1074, an investigational drug for treatment and prevention of HIV. In addition, we show that edited NHP HSPC (n=1 donor) can engraft in a non-irradiated adult immunodeficient mouse model and result in higher than 73% B cell populations (n=8 mice). We anticipate that higher B cell populations with similar editing levels will enable us to track expression patterns and bnAb production in this model. These studies will pave the way for future validation of one-time gene therapy treatments in NHP models of HIV infection.

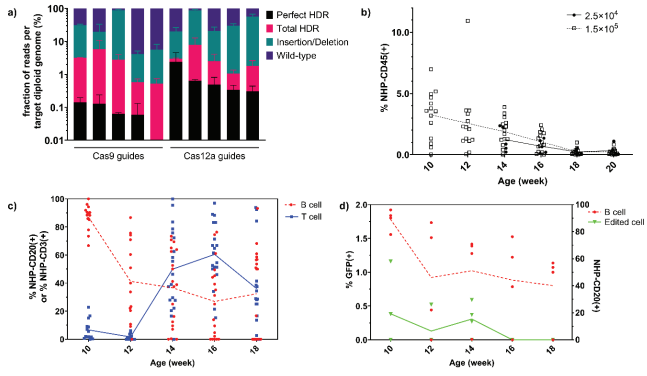


Figure. a) Cumulative frequency of editing outcomes at target locus in NHP lymphocyte cell line. b, c) Engraftment rate of mice at varying NHP-CD34$^+$ cell doses and lymphocyte populations as a fraction of NHP-derived cells in the blood. d) Transgene expression levels and corresponding B cell population in mice showing any level of eGFP out of 8 mice injected with edited HSPC.

1199. Innovative IDLV Design for Transgene Targeted Integration via Microhomology-Mediated End Joining Pathway

Giulia Scalisi[1,2], Aboud Sakkal[1,2], Lacombe Laurie[1,2], Alexandra Tachtsidi[1,2], Guillaume Corre[1,2], Giulia Pavani[3], Peter Lenting[4], Anne Galy[5], Mario Amendola[1,2,6]

[1]Genethon, Evry, France,[2]Université Paris-Saclay, Univ Evry, Inserm, Généthon, Integrare Research Unit UMR_S951, Evry, France,[3]Children's Hospital of Philadelphia, Philadelphia, PA,[4]UMR_S1176,Inserm, Univ. Paris-Sud, Université Paris-Saclay, Le Kremlin-Bicêtre, France,[5]ART-TG, Accelerator of Technological Research in Genomic Therapy - Inserm US35, EVRY, France,[6]Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy

Ex vivo gene correction of hematopoietic stem and progenitor cells (HSPCs) is a promising therapeutic approach for numerous genetic disorders. While the combination of the CRISPR-Cas9 system with various DNA donors has enable gene correction and genomic integration, several pitfalls are still present, including the limited size of the DNA payload and low editing efficiency in HSCs. Integrase-defective lentiviral vectors (IDLV) offer a notable advantage in delivering large transgenes compared to AAV6. However, their integration still relies on homology direct repair (HDR) mediated integration with low efficiency in HSCs. Here we introduce a new class of IDLVs as DNA donor vector that leverages the microhomology end joining (MMEJ) DNA repair pathway. We designed an IDLV where the transgene expression cassette is flanked by a gRNA target site (23 bp), and a short (35 bp) sequence homologous to the intended genomic integration site (microhomology arm, MH). The underlying hypothesis is that the Cas9/gRNA cutting of IDLV will expose the MH for recombination with the genomic double strand brakes (DBS), to achieve a directional and precise target integration (TI). Compared to HDR, which requires cell cycle, MMEJ is active in all cell cycle phases and should be proficient in quiescent HSCs and the use of a small MH-arm will be an asset in terms of space for insertion of longer therapeutic cassettes. For linearizing the episomal IDLV, we used the same gRNA required to introduce DSB at the selected genomic locus, thus simplifying gRNA delivery and reducing potential off-targets associated with the use of two different gRNA.As proof of concept, a reporter gene was targeted to a safe harbor site (AAVS1) and a clinically relevant genomic locus (HBA) in K562 and T-cell lines. We showed that combining the MH-arm and the linearization of the IDLV we obtained efficient on-target, directional and seamless GFP TI (as measured by ddPCR, FACS and by Sanger sequencing of genome-IDLV junction PCR). In addition, using targeted nanopore long-read sequencing (LRS), we demonstrated the correct integration of the whole DNA expression cassette at the expected locus. To assess the contribution of the gRNA target and MH sequence alone, we generated IDLV with each one of these sequences and we demonstrated that both are required to achieve efficient and precise integration. All these data were further confirmed by molecular analysis of single cell clones. We then moved to clinically relevant human HSPC. After optimizing transduction and editing condition, we achieved and GFP integration efficiency in both genomic loci that was similar to the classical HDR based IDLV integration. Again, molecular analysis, LRS and single colony PCR confirmed the efficiency and precision of the strategy. We then challenged our IDLV to deliver and integrate a FVIII cDNA (5kb) and demonstrated once more efficient and precise integration and proficient protein expression. We are currently testing this strategy using additional therapeutic transgenes. We also have preliminary data that NHEJ inhibitors can increase IDLV integration, and we are exploring the use of different IDLV envelope to increase HSC transduction. In conclusion, we propose an MH-IDLV system as an effective platform for cell editing, alternative to the HDR-based TI, which may have important therapeutic implications in the treatment of genetic and tumour pathologies.

1200. Hematopoietic Stem and Progenitor Cell Gene Editing for Congenital Dyserythropoietic Anemia Type II

Mercedes Dessy Rodríguez[1], Sara Fañanas-Baquero[1], Carmen Contreras[1], Rolf Turk[2], Garret Rettig[3], Giandomenico Turchiano[4], Nathan White[4], Yi-Ting Hu[4], Michael Tadros[5], Jose-Carlos Segovia[1], Oscar Quintana-Bustamante[1]

[1]CIEMAT, Madrid, Spain,[2]Integrated DNA Technologies, Coralville, IA,[3]Integrated DNA Technologies (IDT), Coralville, IA,[4]University College London, London, United Kingdom,[5]Danaus GT, Madrid, Spain

Congenital dyserythropoietic anemias (CDAs) are a group of inherited anemias that affect the development of the erythroid lineage. CDA type II (CDAII) accounts for around 60% of all CDA cases. CDAII patients suffer from ineffective erythropoiesis, morphologic abnormalities in erythroblast within the bone marrow, hemolysis, and iron overload. CDAII is caused by mutations in the *SEC23B* gene, with more than one hundred pathological variants described throughout the gene. Allogeneic hematopoietic stem cell transplant (HSCT) represents the only curative option for this disease, but it implies serious side effects. Autologous HSCT of genetically corrected cells would mean a definitive treatment for CDAII. To address the genetic correction of most CDAII patients, we have established a gene editing approach based on knock-in wild-type *SEC23B* cDNA at the endogenous locus in hematopoietic stem and progenitor cells (HSPCs). First, we designed and tested different sgRNAs targeting the initial sequence of the *SEC23B* locus in human HSPCs. We selected the most active sgRNAs and performed GUIDE-Seq analysis to assess their off-target potential. A sgRNA showed only 0.8% off-target events. Then, we performed CAST-Seq analysis for this sgRNA to reinforce its safety profile. A low chromosomal aberration frequency, with most of the translocation events in less than 0.01% of alleles, was detected with this sgRNA in human HSPCs. Based on the target sequence of this sgRNA we designed two rAAV-based knock-in donors to insert the wild-type SEC23B cDN or a turbo GFP (tGFP) cDNA, together with a splicing acceptor signal and the bovine growth hormone polyadenylation (bGH polyA), into the sgRNA genomic target sequence to express the exogenous cDNAs under the control of the *SEC23B* promoter. We nucleofected the sgRNA and transduced healthy HSPCs with the rAAV knock-in donors. We detected up to 70% edited cells with wild-type SEC23B cDNA and up to 80% HSPCs expressing tGFP. We confirmed the expression of wild-type SEC23B-bGH-polyA mRNA in HSPCs gene-edited with the wild-type SEC23B donor by RT-PCR assay. This result showed the correct expression of the exogenous *SEC23B* cDNA by the endogenous SEC23B promoter. Furthermore, to evaluate the erythroid potential of our gene editing strategy, we performed an *in vitro* erythroid differentiation in healthy donors HSPCs. We observed that the editing with SEC23B cDNA did not alter the erythroid differentiation phenotype of the healthy donor cells. However, the editing with the tGFP cDNA caused the reduction of SEC23B protein and hampered erythroid differentiation *in vitro*, indicating the disruption of the *SEC23B* gene. In summary, we have established a gene editing approach for SEC23B deficiency, which might be used as potential gene therapy for CDAII.

1201. Development of Evolutionarily Conserved Viral Integration Sites as Safe Harbors for Human Gene Therapy

Marco Antonio Quezada-Ramirez

Gene Therapy Center, UMASS Medical School, Worcester, MA

Gene transfer into CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) involving integrating viral vectors has unpredictable outcomes including adverse events like leukemogenesis. Therefore, identifying and characterizing genome safe harbor (GSH) sites where exogenous gene material can be safely integrated is critically important for therapeutic gene addition in hematological diseases. To date, few GSHs have been characterized and applied in preclinical HSPCs-gene addition studies (*AAVS1*, *hROSA26*, and *CCR5* loci). Thus, to extend the options for gene knock-ins while reducing the risks of insertional mutagenesis, we use a novel approach to identify GSHs based on a proven system of stable transgene insertion: the evolutionarily conserved integration of parvovirus DNA into the genome of host species. From a dataset of 199 unique endogenous parvovirus (EPV) integration events bioinformatically identified in vertebrate species, 102 loci were mapped to the human genome, and 17 were experimentally evaluated as GSHs in primary CD34$^+$ HSPCs from the bone marrow of adult healthy donors. Targeting each candidate GSH was performed through electroporation with Cas9:gRNA complex and ssDNA templates to insert an eGFP expressing cassette, or by combining Cas9:gRNA nucleofection with rAAV6 transduction delivering the same expression transgene. Following this approach at least nine promising GSHs for blood disorder therapies were identified. Similar to the reference locus *AAVS1* (an EPV-derived site), little effect on cell viability was observed after gene addition while maintaining the multipotency and persistent transgene expression. Of the nine GSHs, six targeted loci displayed widespread expression in both erythroid and immune phenotypes while three clearly exhibited immune branch specific-regulation after the differentiation of edited cells. Remarkably, transcriptional disturbances were minimal when targeting intergenic located GSHs and no evidence of malignant transformation was observed through RNAseq. According to these results, additional GSHs are likely to emerge from the remaining mapped loci. Furthermore, since it is unlikely that the lineage restricted transgene expression from some GSHs is exclusive to hematopoietic cells, other tissue-specific GSH sites are likely to emerge. Thus, our approach extends the available locations for gene knock-ins while reducing the risks of insertional mutagenesis, unpredictable transgene expression profiles, effects on differentiation, and increasing the therapeutic effects. This approach is also intended to allow the multiplex editing of primary cells in order to increase the traits carried by edited cells aside the therapeutic transgene, such as selective resistance of modified cells or tracking phenotypes for animal studies.

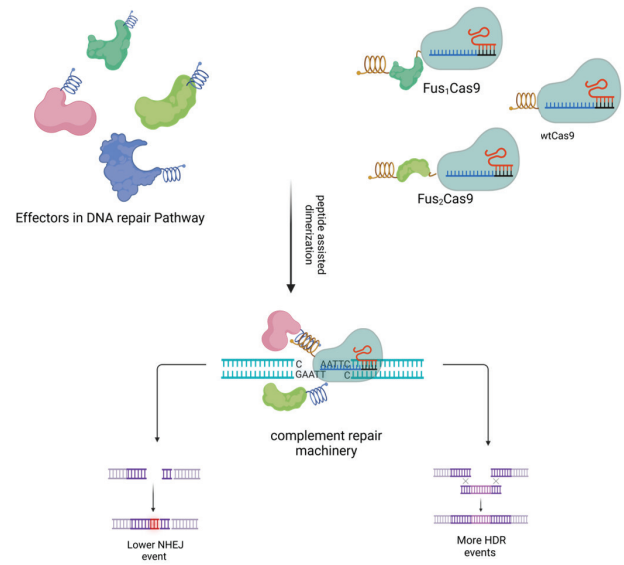
Thursday Posters: Gene Targeting and Gene Correction New Technologies

1202. Peptide-Assisted Tethering of DNA Repair Effectors to Cas9 for Precise Genome Editing

Sibtain Haider[1], Ali Faiq[1], Toni Cathomen[2], Claudio Mussolino[2]

[1]Institute for Gene Therapy, Freiburg, Germany,[2]Medical Center - University of Freiburg, Freiburg, Germany

CRISPR-Cas9 has emerged as a versatile gene editing tool in the field of cell and gene therapy. Its ability to induce dsDNA breaks can be exploited to revert a disease-causing mutation via the homology-directed repair (HDR) pathway. However, such strategies suffer from the intrinsically low frequency of HDR compared to the alternative non-homologous end-joining (NHEJ) repair pathway at target site. Efforts to enhance HDR efficiency include the use of drugs, that inhibit NHEJ, or the development of Cas9 fusion proteins, that alter the normal resolution of the Cas9-induced DNA break to eventually favour HDR. While promising, the efficacy of these approaches varies greatly across cell types, possibly related to different expression profiles of DNA repair factors. To address cell type specificity, we introduce an innovative CRISPR Cas9 strategy designed to exploit the dimerization capability of synthetic peptides. The proposed CRISPR Peptide Assisted Localization (PAL) system comprises two essential elements: 1) a Cas9 nuclease fused to a small synthetic peptide and 2) a library of DNA repair effectors fused to a complementary peptide. Here, we demonstrate how CRISPR-PAL can be used to identify the most effective combination of effectors tailored to a particular cell type. Distinct nuclease and effector combinations resulted in up to a 2.5-fold increase in HDR events compared to unmodified Cas9 across diverse cell types. Furthermore, the dimerization capability of CRISPR-PAL system enabled the use of novel combinations that were unamenable through mere overexpression of effectors. Our findings highlight the versatility of this system that can be optimized to maximize precision editing across wide range of cell types. Ongoing experiments explore the efficacy of the CRISPR-PAL system in correcting Fanconi Anemia (FA) specific mutations by enriching the missing effectors at target site in patient-derived cells. We anticipate that the CRISPR-PAL approach will prove instrumental in the development of more effective, tailored genome editing solutions with broad therapeutic applications.



1203. Decoding Rhodopsin Gene Regulation to Build Innovative Retinal Gene Therapies

Rosa Maritato, Emanuela D'Andretta, Alessia Medugno, Enrico Maria Surace

Department of Translational Medical Sciences (DiSMeT), Univesity of Naples "Federico II", Napoli, Italy

Rhodopsin-Autosomal Dominant Retinitis Pigmentosa (RHO-ADRP) is a progressive photoreceptor degenerative condition leading to irreversible vision loss. In our efforts to develop a treatment for RHO-ADRP, we successfully established a robust proof of principle for gene therapy using Adeno-associated virus (AAV) vectors, combining gene silencing and gene replacement strategies. This innovative approach involves the incorporation of two expression cassettes in a single AAV vector. The silencing step employs an unconventional transcriptional repression system, by using DNA-binding domain (DBD) (ZF6) which operates without repressor domains. Through the sole binding of ZF6 to short and accessible DNA Cis Regulatory Element (CRE), which is unbound from endogenous transcriptional modulators, we achieved effective RHO gene silencing at low doses with minimal off-target effects and limited engagement with cellular systems. Building on this success, we conducted a comprehensive exploration of this CRE consisting of CRX and NRL DNA binding sites with a 22bp (linker) sequence interposed, bound by ZF6. We named this complex Rho regulatory unit RRU (CRE-RRU). Manipulation of factors such as orientation, order, spacing, and sequence composition of this RRU (CRE-RRU syntax) demonstrated a broad spectrum of modulation in transcriptional outputs. In conclusion, by deciphering the gene regulatory code inherent in these CREs, we achieved a controlled expression of silencing and replacement transgenes. Importantly, this control mechanism remained effective across a wide range of vector doses, ensuring a safer and more efficacious gene therapy for RHO-ADRP.

1204. Targeting the Sense and Antisense Pathogenic C9ORF72 Transcripts as Therapy for C9ORF72 ALS

Vanessa Zancanella[1], Kristel van Rooijen[2], Ines Lopes Pereira[2], Cynthia Brouwers[2], Noortje Heidinga[2], Soraya Smit[2], Warner Hoornenborg[2], Rhode Erbrink[2], Irena Bockaj[2], Greg Dobrynin[2], Jovana Kovacevic[2], Morgane Wartel[2], Stijn Waaijer[2], Tycho Hoogland[2], Mercedes Valls Seron[2], Melvin M. Evers[2], Ying Poi Liu[2]

[1]UniQure biopharma B.V., Amsterdam, Netherlands,[2]uniQure biopharma B.V., Amsterdam, Netherlands

Amyotrophic lateral sclerosis (ALS) is a devastating and fatal neurodegenerative disorder affecting the upper and lower motor neurons in the brain and corticospinal tract leading to muscle atrophy, paralysis and eventually death. The most frequent genetic cause of familial ALS is a hexanucleotide GGGGCC repeat expansion in the C9orf72 gene. These expanded repeats are bidirectionally transcribed into repeat containing transcripts, which forms sense and antisense RNA foci. These repeat containing transcripts can also be translated in a non-ATG dependent manner into different dipeptide repeat proteins. Both the RNA foci and dipeptide repeats (DPRs) proteins contribute to disease by acquiring toxic properties. Using our novel linQURE™ technology, we developed an AAV gene silencing strategy based on concatenation of 2 miQURE®-based microRNAs to silence both the sense and antisense repeat containing transcripts (V1 and V3). This approach used our learnings from our previously reported sense C9ORF72-targeting miQURE® expression and target engagement in ALS mouse models where we demonstrated a sustained and dose-dependent effect. These second generation linQURE™ constructs are designed to specifically target the toxic transcripts to prevent the formation of RNA foci and DPRs, while preserving healthy C9ORF72 levels. After careful optimization of linQURE™ and characterization and selection of the most potent synthetic miQURE® combinations, we have shown expression of the novel linQURE™ miRNAs for targeting both sense and antisense repeat containing C9orf72 transcripts. Further investigation of AAV-linQURE™ is under way to show efficacy, tolerability, and safety in larger animals. miQURE is a registered trademark in the US and other jurisdictions. linQURE is a trademark pending in several jurisdictions.

1205. The Efficacy of AAV-Mediated Genome Editing, and Undesired Vector Integrations Are Influenced by R-Loops Formation

Francesco Puzzo[1], Madzia Crossley[2], Aranyak Goswami[1], Katja Pekrun[3], Feijie Zhang[1], Karlene Cimprich[2], Mark A. Kay[1]

[1]Pediatrics and Genetics, Stanford University School of Medicine, Stanford, CA,[2]Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA,[3]Pediatrics and Genetics, Stanford University, Stanford, CA

AAV vectors have been reported to stimulate homologous recombination (AAV-HR). Moreover, they can randomly integrate into numerous regions throughout the genome.Our lab previously demonstrated that the use of a promoterless AAV vector with host genomic homology arms, flanking a therapeutic coding sequence, could induce homologous recombination in mouse liver. While this approach achieved therapeutic efficacy in some animal models, the percentage of edited cells remains low (~ 1.0%). To enhance the efficiency of AAV-HR, we performed a genetic screen (deAlencastro et al., 2021) that led us to identify the Fanconi Anemia complementation group M (FANCM) as one of the major determinants influencing the AAV-HR. FANCM is among the helicases required to resolve DNA/RNA hybrids, also known as r-loops, that can occur when an RNA filament anneals to a single strand DNA. R-loops form primarily during DNA transcription, and if not tightly regulated may trigger the cellular DNA damage response and induce unscheduled DNA breakage. However, these hybrids are resolved by specific helicases or also enzymes belonging to the RNase H family which degrade the RNA strand of the DNA/RNA hybrid. Here, by inhibiting two enzymes, FANCM and SRSF1, involved in r-loop dissolution we demonstrated a significant improvement of AAV-HR in vitro. In addition, the use of a small molecule (topotecan) increased the formation of r-loop levels in a murine hepatoma cell line (HEPA1-6) and mouse liver. Interestingly, treatment with topotecan resulted in a significant improvement of AAV-HR, 8-fold and 2.5-fold in HEPA1-6 and liver respectively. We showed the mechanism underling the enhancement of AAV-HR efficiency was indeed due to r-loop formation as co-treatment with RNAseH1 resulted in a 1.5-fold decrease in AAV-HR. Furthermore, we mapped and quantified r-loops throughout the genome. To this end, we performed DNA/RNA immune precipitation sequencing (DRIP-seq) experiments in HEPA1-6 and mouse liver. To our knowledge, this is the first genome-wide r-loop map of an intact tissue. Particularly, we detected a remarkable r-loops levels in the region of the *Albumin* region (exons 12-14) where we designed homology targeting arms (Barzel et al. Nature 2015). Conversely, we did not observe any integration when targeting a different *Albumin* region with low r-loops levels. In addition to enhanced AAV-HR, our data suggest that genomic regions enriched for r-loops may represent hotspots for "random" AAV integration. Indeed, recent studies showed AAV vector integrated in genes with relatively high level of r-loops upon liver gene transfer. Bioinformatic analyses are ongoing in order to identify prospective genomic features that might further explain the interplay between vector integration and r-loops. These findings may shed light on processes that could improve the efficacy of genome editing and possibly enhance our ability to predict regions most susceptible to insertional mutagenesis with therapeutic viral vectors.

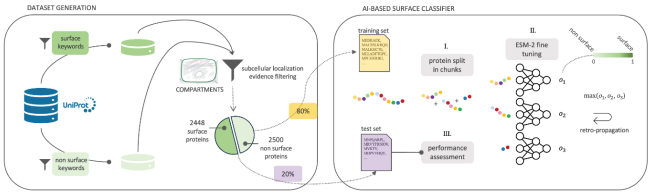
1206. An Adapted Protein Language Model for Cell Surface Protein Identification in Genomic Therapies

Chloé Canavate, Lélia Polit, Oscar de Felice, Dina Zielinski, Julien Cottineau, David Del Bourgo

WhiteLab Genomics, Paris, France

Genomic therapies hold great promise for providing a durable cure for untreatable diseases but require precise targeting of the affected tissue or cells. Advances in target specificity, for example by designing binders through rational or guided strategies [1] have led to improved efficacy. However, experimental validation is prohibitively expensive. Thus, identifying specific cell surface receptors is a crucial step to increase the transduction of target cells. It aims at enhancing efficacy, reducing immunogenicity, and avoiding the transduction of certain

tissues to reduce off-target effects. We aimed at accelerating research and development of vectors for gene therapies with a rationally guided approach by optimizing the identification of target-specific receptors to limit the number of potential candidates and reduce associated costs. To this end, we developed a deep learning method that predicts whether a protein is at the cell surface or not, based only on its amino acid sequence and can be applied to any protein, regardless of length. Numerous *surfaceomes*, databases of proteins located at the cell surface, have been documented in the literature, including experimental approaches [2], *in silico* methods [3] and combined. Current approaches are based on several pre-computed features [3] or predict other topological domains rather than surface proteins. However, a reliable gold standard is currently lacking, and each of these databases represents a finite set which is a strong limitation to scale these approaches. Here, we employ a comprehensive approach integrating biological context, a highly curated training dataset, and advanced deep learning methodologies without the need for feature selection, which can limit model generalizability.



To construct an appropriate dataset, we leveraged UniProt, by identifying keywords associated with either cell surface proteins or non-cell surface proteins which were then cross-checked in datasets with high confidence subcellular localization data from COMPARTMENTS. This resource integration enhances the reliability of our selection, limited to proteins with well-established surface or non-surface localization, which is the basis for developing a well-performing and generalizable model. The backbone of our deep learning approach consists of protein language models based on the amino acid sequence as input. These models have been adapted to accommodate proteins of variable length, enhancing their versatility in capturing intricate sequence patterns. Through fine-tuning, we adapted the models to our classification task of discerning between surface and non-surface proteins. This adaptation leverages the inherent features of protein sequences, enhancing the models' discriminatory capabilities for more specific applications in genomic therapies. The final ensemble model is composed of 100 ESM-2 learners, obtained via cross-validation and hyperparameter search. The model is able to discriminate between surface and non-surface proteins with an accuracy of 84% on the internal test set requiring only the protein sequence as input. This work contributes to the ongoing development of targeted therapies, by accelerating and de-risking their development, with potential implications in the discovery of candidate receptors for advanced therapeutics. [1] Pupo A et al, *Mol Ther*. 2022. [2] Bausch-Fluck D et al, *PLoS One*. 2015. [3] Bausch-Fluck D et al, 2018

1207. Isolation of AAV-Derived Inverted Repeats for Enhanced Gene Editing

Naveen Vridhachalam[1,2], Brian Golitz[3,4], Matthew L. Hirsch[1,2]

[1]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Department of Ophthalmology, University of North Carolina at Chapel Hill, Chapel Hill, NC,[3]Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC,[4]University of North Carolina School of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC

Previous studies have shown that a DNA double strand break (DSB) at or near a chromosomal target site enhances homologous recombination (HR) mediated genome editing. As such, designer endonucleases that recognize specific DNA sequences and generate a DSB at a site of interest have rose in popularity, however, concerns exist for in vivo therapeutic applications including the immune response to foreign epitopes and off-target mutagenic activity. A reported alternative to site-specific endonucleases are proximal inverted repeats (IRs), structured DNA elements processed to DSBs via endogenous DNA repair. To date, the influence of IRs engrafted on the DNA repair molecule have not been well characterized in a gene editing context. The hypothesis of this work is that DSBs and/or inverted repeats on the repair template will enhance gene editing towards the objective of adeno-associated virus (AAV) recombinant genomes enhanced for homology directed repair. In this work, the effect of a nuclease-induced DSB or adeno-associated virus (AAV) derived inverted repeats located on a repair plasmid was investigated for chromosomal gene editing in human embryonic kidney cells. The results demonstrate that, in conjunction with a DSB at the chromosomal target site, inducing a DSB on the repair molecule significantly increased the gene editing frequency. Consistent with reports that IRs are processed to DSBs, the presence of the AAV serotype 2 ITR sequence on a repair plasmid significantly increased chromosomal gene editing compared to a no structure control. Deletion analysis of the AAV IR identified the viral origin of replication as necessary for optimal gene editing and putative stem length was directly correlated to HR. Next, a truncated AAV IR enhanced for editing was randomly mutated in a manner that conserved sequences necessary for vector production thereby generating a mutant ITR library of $>10^6$ diversity. High throughput screening of individual mutant ITRs in a gene editing selection identified AAV IR sequences decreased and enhanced for gene editing compared to the library parent and the wt AAV IR sequence. Consistent with the AAV ITR2 deletion analysis, the recovered mutant IR most enhanced for editing contained mutations in the AAV origin of replication. Currently, bioinformatic analyses correlating sequence/structure to chromosomal repair efficiencies and AAV vector production are in progress. Together, these results demonstrate that a DSB or viral IR sequences on the repair molecule stimulate gene editing and offer mechanistic insights into the generation of AAV viral vectors that mediate high efficiency genetic engineering in the absence of a site-specific endonuclease.

1208. Aldevron's Nanoplasmid™: Big Benefits in a Small Package

Jeremy Luke[1], Tyler Kozisek[1], Jason Devlin[1], Patrick Alfredo Paez[1], Denis Arutyunov[1], Aaron Carnes[2], James Williams[1], Seshidhar Reddy Police[3], Venkata Indurthi[1]

[1]Aldevron, Fargo, ND,[2]Aldevron, Lincoln, NE,[3]Aldevron, Boston, MA

Aldevron's Nanoplasmid™ vector backbone (<500 bp) is a replacement for a previous generation of *Escherichia coli* plasmid backbones that improves manufacturing workflows, safety, and performance of gene and cell therapeutics. Nanoplasmid vectors do not include antibiotic resistance markers, which improves safety compared to plasmids or minicircles that rely on antibiotic resistance for bacterial selection during the manufacturing process. Nanoplasmid vectors contain a 70 bp RNA-OUT antisense RNA cassette that confers sucrose resistance to the host cells by suppressing the mRNA translation of sucrose toxicity gene *sacB*. This allows antibiotic-free plasmid selection in sucrose-containing media. Nanoplasmid vectors also contain a small (300 bp) specialized bacterial R6K replication origin in place of the traditional pUC replication origin and can only replicate within the specialized *E. coli* host strains. The latter feature makes the risk of horizontal gene transfer by the Nanoplasmid vectors significantly lower than that of traditional systems. An alternative R6K replication origin-based Nanoplasmid vector backbone also dramatically improves fermentation yields of the clinically important vectors, namely adeno-associated virus (AAV) gene therapy vectors and mRNA vaccine vectors, which are known to have several challenges from a manufacturing standpoint. Namely, AAV transfer plasmids contain highly structured palindromic Inverted Terminal Repeats (ITRs), flanking the 'payload' transcription unit. These ITR repeats, which contain a viral replication origin and viral packaging signals, are unstable and difficult to replicate in *E. coli*, creating challenges for AAV transfer plasmid manufacture. On the other hand, mRNA vaccines are manufactured from poly (A) tail-repeat containing template plasmid vectors that contain an antigen gene upstream of a poly (A) tail repeat. Similarly to ITR repeats, the long (80-120 bp) polyA repeats are prone to deletions, and mRNA vaccine vectors are often low-yielding in *E. coli*. These challenges appear to be due to the traditional backbone pUC replication origin having difficulty replicating closely positioned structured DNA sequences. Nanoplasmid vector backbones can be easily retrofitted into the existing clinically proven AAV transfer vectors or mRNA vaccine vectors to replace the pUC origin with the R6K origin and an antibiotic resistance marker with an RNA-OUT cassette while not changing the sequence of the vector payload. In addition, the Nanoplasmid vector host strains were engineered to produce R6K replication protein, which initiates replication from the R6K origin, which drives high-yield fermentation (>1 g/L pDNA) of Nanoplasmid vectors in the Aldevron HyperGRO™ fermentation process. The next generation REVIVER™ host strain supports the propagation of R6K origin-based Nanoplasmid vector backbones while increasing the yield and replication stability of structured DNA sequences even further. REVIVER strain shows higher fermentation productivity compared to the standard recombination deficient hosts such as SURE, Stbl3™, or, Stbl4™ where multiple mutations characterizing these strains lead to very poor biomass and vector yields in the large-scale fermentation required for the production of vectors for clinical and commercial applications.

1209. Novel CRISPR Effectors and Reverse Transcriptases Discovered from Metagenomics Enable Extensive Remodeling of the Human Genome

Morayma M. Temoche-Diaz[1], Leanna R. Monteleone[1], Rebecca C. Lamothe[1], Sourab Kulkarni[1], Sarah M. Laperriere[1], Anu Thomas[1], Kaitlyn Tsai[1], Whitney Krey[1], Shailaja Chadha[1], Lisa M. Alexander[1], Hannah T. Engel[1], Keirstinne Turcios[1], Ketaki D. Belsare[1], Margaret Hackney[1], Jennifer S. Oki-O'Connell[1], Steve J. Flammer[1], Jerry Hung-Hao Lo[1], Chloe O. McCollum[1], Dominic T. Castanzo[1], Owen Perot Janson[1], Catalina Alonso-Martinez[1], Steffen Durinck[1], Cristina N. Butterfield[1], Cindy J. Castelle[1], Daniela S. A. Goltsman[1], Megan van Overbeek[1], Alan R. Brooks[1], Brian C. Thomas[1], Christopher T. Brown[2]

[1]Metagenomi, Inc., Emeryville, CA,[2]Metagenomi, Emeryville, CA

CRISPR is revolutionizing biotechnology and human therapeutic development. Multiple clinical trials have been conducted for therapeutics created using CRISPR nucleases, and recently the first approval has been granted by the United States Food and Drug Administration. Despite significant advances, many diseases are not yet treatable owing to a need for additional specific and efficient gene editing systems, including those that are capable of introducing complex edits. In vivo gene editing has the potential to cure patients by leveraging existing, safe RNA delivery technology. Such diseases include transthyretin amyloidosis, hemophilia, Wilson's disease and alpha-1 antitrypsin deficiency. Here, we demonstrate the ability of a novel gene editing platform for therapeutically-relevant knock-out, correction, and large gene insertion using all-RNA delivery. This was accomplished by mining the natural environment for novel programmable CRISPR systems as well as reverse transcriptases (RTs) for converting editing templates delivered as RNA into DNA. We identified a novel CRISPR nuclease capable of efficient genome editing and validated therapeutically-relevant guides in vivo in a mouse study using lipid nanoparticles (LNP) for delivery. No off-targets could be identified for top-performing guides based on an unbiased cell-based assay, further establishing the utility of this system for therapeutic applications. Given the in vivo translation, efficiency, and specificity of this CRISPR nuclease, we used the system in conjunction with novel RTs to accomplish prime editing as well as large, targeted integrations. Millions of RTs were characterized to identify candidates capable of accomplishing either small replacements or large integrations. Novel RTs showed improvements in prime editing efficiency and specificity, compared with industry-standard controls across diverse and therapeutically relevant edits and cell types. Furthermore, we believe we are the first to demonstrate programmable large integration of up to 1 kb into the genome of human cells using a system delivered entirely as RNA. Taken together, this flexible platform, which we refer to as RIGS (RNA-mediated integration systems), is compatible with readily available LNP delivery systems and has the potential to cure numerous diseases through in vivo genome editing.

1210. Synthetic AAV DNA Mediates High-Efficiency Homology-Directed Genome Editing

Christopher Luis Chavez
Visgenx, Santa Cruz, CA

AAV-based genome editing offers a promising alternative to CRISPR methods due to its enhanced safety profile and precision. Unlike CRISPR, AAV vectors integrate genetic material into the host genome in a more controlled manner, reducing the risk of off-target effects and unintended mutations. However, AAV-based genome editing is very inefficient, often reported below 0.1%. To overcome this limitation in efficiency, a synthetic AAV DNA system has been developed. Synthetic AAV DNA is a single-component, completely synthetic alternative to CRISPR/Cas9 or AAV-based genome editing tools that takes advantage of many of the inherent properties and versatility of AAV-based genome editing while bypassing its limitations (low editing efficiencies and manufacturing costs). In addition, synthetic AAV DNA is not bound to the payload size limitations of a standard AAV system, allowing for targeting of previously untreatable genetic diseases. Synthetic AAV DNA is produced utilizing enzymes in a cell-free system and results in a linear closed-ended double-stranded DNA molecule with full AAV ITRs at each end (Fig1). Genome editing with the synthetic AAV DNA does not require exogenous proteins or guide RNAs for editing and does not induce double strand breaks in genomic DNA. This eliminates on-target cutting of unedited alleles and off-target cutting effects resulting in unintended mutations. Genome editing is directed to the target sequence by homology arms matching the target sequence within the synthetic AAV DNA sequence. *In vitro* experiments utilizing a P2A-GFP synthetic AAV targeting the highly expressed ALB locus in HepG2 cells have consistently demonstrated GFP gene addition efficiencies between 40-50%. By placing GFP in-frame with the ALB gene, GFP expression is controlled by native ALB gene regulatory elements (Fig2). Gene addition in this manner demonstrates the potential applications for treating hemophilia for Factor VIII or Factor IX deficiencies. The synthetic AAV DNA system can also be used to repair point mutations and deletions. A synthetic AAV was designed to target and swap a TA pair to an AT pair at the AAVS1 locus. This substitution removes an NheI site and allows for editing efficiency to be visualized by restriction analysis. Editing rates at the AAVS1 locus in HeLa cells are currently ~30% for this two base-pair swap. Base substitutions of this manner have potential to repair point mutations like those responsible for sickle cell disease. Junctional analysis for both the GFP gene addition and the two-base substitution, reveal seamless editing with no inclusion of AAV ITR sequences detected. The synthetic AAV DNA system can also be used as a gene therapy vector to express genes over 4.7 kb in size. An example synthetic AAV ABCA4 (6.8 kb) expression vector was assembled to show this feature of the system. An ABCA4 synthetic AAV could be used to treat Stargardt's disease. Other oversized synthetic AAV DNA vectors have potential to treat diseases such as Usher's (16.6 kb) and DMD (11.1 kb). A suitable delivery system will be needed to deliver the synthetic AAV system to the appropriate target tissues.

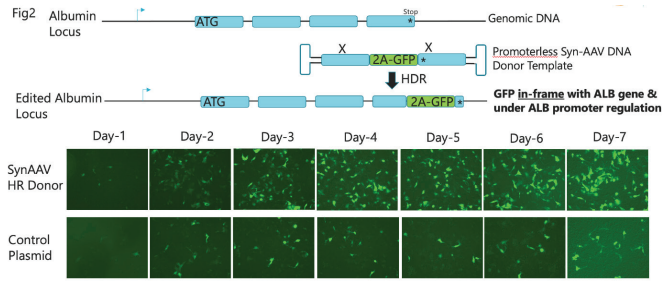
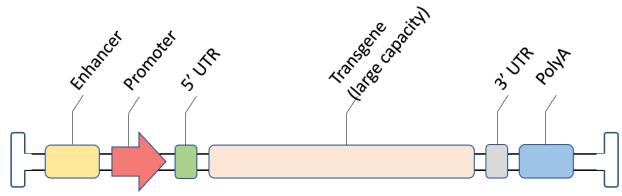


Figure 1



1211. Synthetic, Enzymatically Produced DNA and Non-Viral Delivery Systems for Gene Editing Applications

Amine Bouchareb, Samantha Mnkandla, Ana-Vanina Hangu, Anca-Paula Ristin, Tejaswini Mahadeva, Elena Dyankova, Amy Walker
4basebio, Cambridge, United Kingdom

The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production of mRNA, and DNA templates used in gene editing applications. In addition to worldwide lack of capacity and long lead times, complex sequences or long polyA tails are difficult to propagate in bacteria. 4basebio has developed a proprietary, scalable, fast and fully enzymatic synthesis process for the production of linear DNA constructs. The process is size and sequence independent. Unlike plasmid DNA, 4basebio DNA eliminates contamination from endotoxins or host proteins. Hermes™, 4basebio's proprietary non-viral delivery system, is a nanoparticle vector that can encapsulate a range of payloads, including synthetic DNA, mRNA, RNP complexes, or a combination thereof, and can be customised to target cells or tissues of interest. The particles additionally offer long term stability at 4°C, overcoming cold chain requirements. Here, we present the combination of synthetic DNA and Hermes™ nanoparticles to integrate a GFP template. Furthermore, synthetic opDNA™ was used for the production of Cas9 mRNA. High template integration efficiencies and high cell viability were observed across a range of cell types. Importantly, a reduced DNA template size, owing to a lack of bacterial backbone, allows for a copy number advantage over plasmid DNA, facilitating reduced DNA doses and improved toxicity profiles. 4basebio's double-stranded and single-stranded DNA offerings enable superior editing efficiencies in cells. In combination with the Hermes™ nanoparticle delivery platform, 4basebio offers an alternative to viral vectors and a promise to advance non-viral gene editing therapies both *ex-vivo* and *in-vivo*.

1212. Comprehensive Genomic Profiling of Human CD19 CAR T-cells Engineered with a Mammalian Transposon

Alisa O. Vershinina[1], Geneva Young[1], Francisco Navarro[1], Joshua LaMora[1], Rebecca Panitch[2], Hugues Bernard[1], Argus Athanas[2], Shanfu Xie[1], Joseph J. Higgins[1], Oleg Iartchouk[1]

[1]SalioGen Therapeutics, Lexington, MA,[2]Watershed Informatics, Cambridge, MA

Introduction: Existing gene therapy modalities cause unintended off-target events (e.g. chromothripsis, random insertions, deletions, chromosomal rearrangements) that increase the risk for oncogenesis and mutagenesis. To overcome these safety concerns and limitations in payload size, we engineered the only active mammalian transposable element (TE) derived from bioengineered *Myotis lucifugus* (*bMLT*) with the ability to target specific octanucleotide sites in the human genome and deliver large payloads by a mechanism referred to as Gene Coding technology. **Methods:** Primary T-cells from three human donors were electroporated with an engineered *bMLT* mRNA and a *CD19 CAR* plasmid DNA element (modified group), the *CD19 CAR* DNA element alone (control group) or were left untreated (unmodified group). *CD19 CAR* expression and copy number were measured by FACS and ddPCR in all T-cell groups. Efficacy studies were conducted in Nalm6 xenografted NSG™ immunodeficient mice. To assess the genomic safety profile, we applied chromosomal analyses, targeted NGS (Illumina), and hybrid capture long-read third generation sequencing (PacBio) to compare the profiles of modified CD19 CAR-T cell lines (n=3) to the unmodified group (n=6). To investigate structural alterations, we conducted genomic hybridization screening (KromaTiD), optical genome mapping (Bionano), and G-banding of metaphase chromosomes. We tested CD19 CAR-T cells by in-solution hybridization capture utilizing 7,816 oligonucleotide probes. The probes spanned 800 kb across 127 genes, which are associated with 12 tumor tissue types (IDT). **Results:** Modified CD19 CAR-T cells showed expression levels up to 62%, an average of 5.62 vector copies/cell, and persistent tumor suppression in xenografted mice treated for up to 70 days. About 80% of integration sites were within intergenic and intronic regions on one allele with the majority (>98%) at specific octanucleotide motifs. Long read sequencing showed no detectable structural variants in the human genome within 3 kb of the integration sites. Further analysis showed normal 46, XX female karyotypes in modified and unmodified T-cells without evidence of chromosome loss, chromothripsis, rearrangements, or activation of oncogenes. Across the three donor cell populations, we identified 68 low-to-high frequency somatic variants of which 15 were small non-pathogenic indels not reported in ClinVar. Out of 53 single nucleotide polymorphisms (SNPs), we discovered 16 novel and 37 previously reported variants. The SNPs overlapped with 63 genes, 432 transcripts, and 15 regulatory features. Most of the SNPs were in regulatory regions with 39% downstream or upstream of genes, and 18% in 3' UTRs. The remainder resulted in transcriptional modifications with 70% of variants leading to synonymous and 30% to missense changes. We identified copy number variation (CN) in 25 genes. These SNP and CN genomic variations were present in unmodified and modified samples with no increase in mutational burden attributable to modifying T-cells by Gene Coding. **Conclusions:** We performed comprehensive genomic profiling of primary human T-cells modified by a *bMLT* TE using Gene Coding technology to generate *CD19 CAR*. These human T-cells delivered therapeutic levels of *CD19 CAR* expression without exacerbating the risk of oncogenic mutations, cancer-associated somatic mutations or increasing the mutational burden. These findings suggest that Gene Coding technology may provide an avenue for therapeutic DNA insertion that overcomes many common safety concerns with current gene therapies.

1213. Space Health Innovations: Using Gene Therapy to Induce Metabolic Torpor in Human Organoids

Jonathan Diaz[1], Ritu Ramamurthy[1], Caleb Heathershaw[1], Gemma Nomdedeu-Sancho[1], Olivia Latham[1], Jacolby Roddey[1], Frido Welker[2], Jorsua Bethencourt[2], Antonis Bartsiokas[3], Vijay Gorantla[1], Tom Shupe[1], Alan Jacobson[1], Paul F. Wilson[4], Joshua T. Maxwell[1], Anthony Atala[1], Graça Almeida-Porada[1], Christopher D. Porada[1]

[1]Wake Forest University School of Medicine, Wake Forest Institute for Regenerative Medicine, Winston Salem, NC,[2]Globe Institute, University of Copenhagen, Denmark,[3]Democritus University of Thrace, Komotini, Greece,[4]Pacific Northwest National Laboratory, Richland, WA

To ensure astronaut health on long-duration space missions, it is necessary to identify means of protecting astronauts from the unique environmental (microgravity, space radiation) and psychological (isolation, distance) stressors, which can profoundly impair multiple tissues/organs. Torpor induction could significantly mitigate these stressors and reduce resource consumption and waste production during extended space missions. The ability to induce metabolic depression/ torpor also has the potential to transform medicine on Earth. Our research adopts an innovative approach, utilizing gene therapy to induce metabolic torpor in human organoids. At the center of our approach are two key tools: 'omics data from the extinct S.H. hominins, who appear to have hibernated, and the FOXO1A gene, which plays a vital role in regulating metabolism, stress resistance, and cell longevity. We hypothesized that these 'omics data (when compared to closely related non-hibernating hominins, and the FOXO1A gene network could serve as blueprints of pathways that can be leveraged to induce metabolic torpor in modern humans. We developed a lentiviral vector system that integrates a cumate switch to precisely control expression of FOXO1A (and ultimately genes identified as unique to the S.H. hominins) linked to a GFP reporter for real-time tracking of gene expression. This system (with FOXO1A) is currently being tested in human liver, cardiac, and trabecular bone organoids to explore gene therapy's potential for modulating metabolic rate and inducing a state of torpor. Experiments to-date in liver organoids using a battery of viability, functionality, and metabolic assays have revealed that 5'-AMP plays a critical function in safely maintaining hypometabolic states, particularly under stress conditions. This parallels its role in hibernating animals, where it helps reduce metabolic rates and conserve energy - a mechanism we aim to exploit to induce torpor in modern human tissues. Cardiac organoids exposed to decreasing glucose exhibited a clear threshold of reduced contractility and altered calcium transients, providing valuable baselines for metabolic management in

space and terrestrial medical scenarios, using FOXO1A and/or gene pathways identified in S.H. hominins. The implications of our research are broad and multifaceted. In space medicine, the ability to induce hypometabolism in human tissues could revolutionize how astronauts' health is managed. Looking beyond space exploration, the potential terrestrial applications of this technology are equally groundbreaking. This includes the potential to revolutionize treatment paradigms for metabolic disorders, enhance strategies in critical care, and improve outcomes in scenarios such as organ transplantation and trauma care. Our studies thus mark an advancement in the fields of gene therapy, space health, and regenerative medicine. While our findings are currently in preliminary stages, they establish a robust foundation for future investigations, they underscore the pivotal connection between space exploration and advancements in global healthcare, and they may ultimately pave the way for a future where the boundaries of human resilience and medical innovation are continually expanded.

1214. L-PGI: A DNA Ligase Mediated Gene Editing System for Versatile and Precise Genomic Integration

Jenny Xie[1], Angela Nan[1], Chris Bartolome[1], Sandeep Kumar[1], Shakked Halperin[2], Michael Chickering[2], Parbir Grewal[2], Leonard Chavez[2], Jonathan D. Finn[1]

[1]Tome Biosciences, Inc., Watertown, MA,[2]Replace Therapeutics, LLC, a direct and wholly owned subsidiary of Tome Biosciences, Inc., Watertown, MA

The efficiency of genome modifications using CRISPR nucleases and reverse transcriptase (RT) is limited by RT processivity, template complexity, and intracellular dNTP levels, which can become rate limiting in non-dividing cells and tissues of therapeutic interest. To overcome these challenges, we present Ligase-mediated programmable genomic integration (L-PGI), a CRISPR-guided DNA ligase system for highly precise replacement/integration of a targeted sequence of pre-synthesized DNA containing desired edit. L-PGI uses 5 components: (1) Cas9 nickase, (2) DNA ligase, (3) pre-synthesized DNA donor template that contains the desired edit, (4) splint, and (5) ligase mediated guide RNA (lmgRNA). Once delivered to the cell, Cas9 nickase forms an RNP with the hybridized lmgRNA-splint-donor complex, binds to the genomic target, and nicks the DNA, which releases the genomic flap. The splint acts as a bridge between the lmgRNA, genomic target site, and donor DNA, precisely aligning all of the components in the correct spatial configuration, allowing the DNA ligase to ligate the genomic flap and donor. The ligated donor displaces the endogenous strand and results in incorporation of the edits encoded in the donor into the genome. L-PGI does not induce significant double stranded breaks, is not subject to RT limitations, and is efficient in both dividing and non-dividing cells. The single-step direct integration of pre-synthesized DNA can incorporate a variety of edits from small nucleotide conversions to deletions and insertions up to greater than a hundred base pairs, all with higher efficiency and lower incidence of unintended edits than existing gene editing platforms. Due to its versatility, L-PGI has the potential for broad therapeutics application in the treatment of a variety of genetic diseases, ranging from single mutation correction to whole gene replacement to exon displacement.

1215. Genetic Medicines Have Arrived. How Do We Afford Them?

Manar Zaghlula

UC Berkeley/Innovative Genomics Institute, Berkeley, CA

Eighteen genetic therapies have been approved by the U.S. Food and Drug Administration to date, a number that now includes the first CRISPR genome editing therapy for sickle cell disease, CASGEVY. This extraordinary milestone is widely celebrated because of the promise for future genome editing treatments of previously intractable genetic disorders and cancers. At the same time, such genetic therapies are the most expensive drugs on the market, with list prices exceeding $3 million per patient. Although all approved cell and gene therapies trace their origins to academic institutions, reliance on for-profit pharmaceutical companies for subsequent development and commercialization results in prices that prioritize recouping investments, paying for candidate product failures, and meeting investor and shareholder expectations. To increase affordability and access, sustainable discovery-to-market alternatives are needed that address system-wide deficiencies. Here, we present recommendations of a multi-disciplinary task force assembled to chart such a path. We describe a pricing structure that, once implemented, could reduce per-patient cost at least tenfold and propose a business model that distributes responsibilities while leveraging diverse funding sources. We also outline how academic licensing provisions, manufacturing innovation and supportive regulations can reduce cost and enable broader patient treatment.

Thursday Posters: Oligonucleotide Therapeutics

1216. miX^{on}, a System for Tunable Control of miRNA Expression by Drug-Induced Splicing

Ryan C. Giovenco[1], Alejandro Mas Monteys[2], Ellie Carrell[3], Beverly L. Davidson[3]

[1]Children's Hospital of Philadelphia, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, PA,[3]Children's Hospital of Philadelphia, Philadelphia, PA

MicroRNAs (miRNAs) are small noncoding RNAs that modulate gene expression by blocking translation or inducing decay of target mRNAs through complementary base pairing. Mature miRNAs are processed from long primary miRNAs (pri-miRNAs) in a multi-step process in which the RNAseIII endonucleases, Drosha and Dicer, cleave a stable hairpin to generate functional 20-24 bp sequences. Ultimate strand-loading preference into the RNA-induced silencing complex (RISC) is determined by multiple factors, including the identity of the 5' base and low 5' thermodynamic stability within the RNA duplex. Endogenous pri-miRNA scaffolds have been successfully adapted to host artificial miRNAs that, when delivered using adeno-associated viruses (rAAVs), lead to sustained knockdown of disease-related transcripts and protein for therapeutic benefit. Although we previously demonstrated that artificial miRNAs mitigate the toxicity of short hairpin RNAs (shRNAs) (McBride et al. 2008), toxicity remains possible due to unintended silencing (Monteys et al. 2014). Thus, a system to fine-tune miRNA

expression following AAV delivery would be a beneficial addition to the RNA interference (RNAi) toolkit. Previously, we developed X^{on}, a drug-induced splicing switch that controls translation initiation in response to the small molecule LMI070 (Monteys et al. 2021). Here, we modified X^{on} to provide drug-inducible control over miRNA expression, which we present as miX^{on}. The miX^{on} cassette was designed to control the expression of miS1, our well-characterized RNAi sequence targeting human ATXN1. Following modifications to optimize strand biasing and sequence engineering to minimize background splicing, we developed a construct that showed 47% knockdown in a luciferase reporter system in response to LMI070, with no background knockdown in untreated, transfected cells. Moreover, this construct demonstrated reduced silencing efficiency in Drosha knockout cells, supporting miRNA processing by Drosha. Next, we engineered miX^{on} to target an endogenous mRNA, PCSK9. Transfection of a constitutively spliced miX^{on} cassette into HuH-7 cells led to a 22% knockdown of PCSK9. Interestingly, we observed a 12-fold increase in miRNA processing from a canonical miR-30 scaffold compared to our miX^{on} construct, with a 60% knockdown in PCSK9. This disparity between miRNA processing and knockdown could indicate an upper threshold for miRNA silencing by transfection and supports future efforts to optimize strand biasing and scaffold design for robust silencing. Overall, miX^{on} displays significant promise as a novel system for drug-inducible control over miRNA expression.

1217. Analytical Methods and Platforms for circRNA Vector Engineering to Improve Circularization Efficiency

Yali Sun, Anis Khimani, Yanhong Tong, Zhi-xiang Lu
Revvity, Waltham, MA

Background: Circular RNAs (circRNAs) have emerged as pivotal players in RNA medicine. Unlike linear counterparts, circRNAs possess a closed-loop structure, conferring them with enhanced stability and resistance to degradation. This resilience makes them promising candidates for diagnostic and therapeutic applications. The ribozyme-based strategy stands out as the predominant method for synthetic circRNA production. Optimizing the ribozyme backbones for enhanced RNA circularization is a critical aspect of upstream process development. This highlights the essential need for analytical solutions to precisely measure circularization efficiency. **Method:** T4 phage and Anabaena permuted intron-exon (PIE) were used as ribozyme backbones. Revvity RNA Pico reagent (Part#: CLS960012), and DNA 5K/RNA/CZE HT LabChip (Part#:760435) were used on LabChip® GX Touch™ (Part# CLS138160) for fragment analysis. Revvity Respiratory SARS-CoV-2 RT-PCR Panel 1 reagent kit (Part# SDX-56390) was used for reverse transcription and real-time PCR analysis (***Figure 1***). **Result and Conclusion:** We characterized peaks from circRNA processing samples in Labchip® electrophoresis (***Figure 2***). Our study illustrates the systematic analysis of circularization efficiency through the integration of the Labchip® electrophoresis fragment analysis and real-time PCR platforms (***Figure 1***). The analytical methods will capture the attention of industrial experts, particularly those focused on optimizing RNA circulation efficiency in upstream process development. Simultaneously, it will assist academic researchers accelerating studies and exploring key elements for ribozyme catalytic activity.

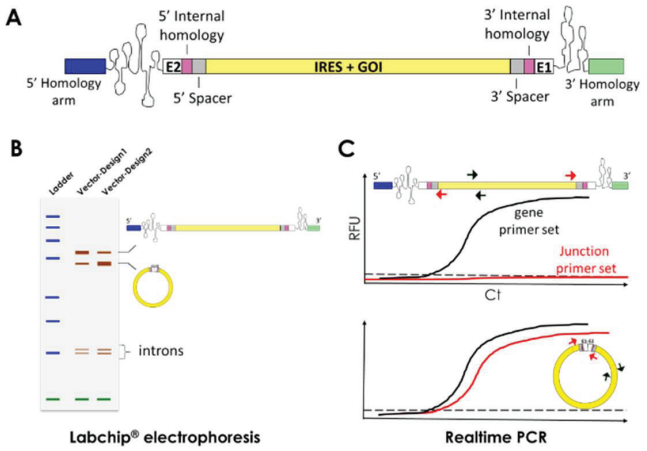


Figure 1: Analytical analysis workflow of synthetic RNA circularization. (A) A typical permuted intron-exon construct design for synthetic circRNA precursor. E1, E2: exon 1, exon2; IRES: internal Ribosome entry site; GOI: Gene-of-Interest; Catalytic intron as a preserve element critical for ribozyme folding is drawn as scribble lines and hairpin structures. **(B)** Schematic diagram showing fragment analysis of RNA species in RNA circularization on LabChip®. Construct variants by circRNA vector engineering are labeled as Vector-Design1 and Vector-Design2. **(C)** Real-time PCR primer design and amplification curve for measuring ribozyme circularization. Top diagram represents results from linear precursor or samples with no or low circulation yield; bottom diagram represents results from samples with success circularization. Red arrows represent junction primer sets; black arrows represent body primer set.

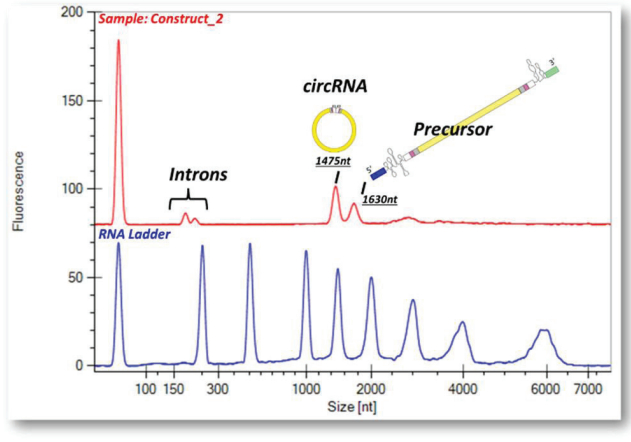
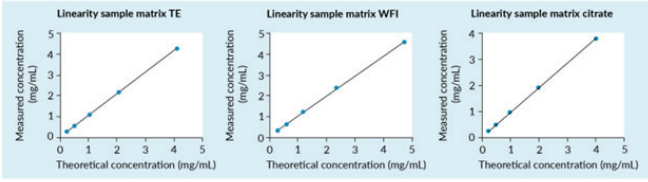


Figure 2: circRNA peak patterns using RNA pico reagents on LabChip®. *Top: one typical non purified RNA product by ribozyme-based circularization. Bottom: RNA Ladder from RNA Pico reagent. If size resolution of electrophoresis platform is optimal, assuming no 100% circularization efficiency, four bands are usually expected: small introns' peaks excised from 5' end and 3' end, one circRNA peak and one RNA precursor peak. The sizes labeled below RNA species are theoretical value based on sequence design.*

1218. mRNA Concentration Determination with the CTech™ SoloVPE® System

Paul Mania
Repligen, Jamison, PA

mRNA-based therapeutics differ from small molecules and other biologics and have significant analytical challenges. mRNA characterization for pre-clinical/clinical testing and lot-release are required to stay concurrent with regulatory standards, to be competitive in the marketplace. Traditional analytical methods used to quantify mRNA face challenges involving, dilution error, fixed-pathlength measurements and measuring among other UV-absorbing components in the matrix. These challenges can lead to variability making it difficult to transfer methods. There is an important demand in the industry to perform robust, repeatable mRNA concentration acquisitions in a single-test, utilizing low volumes and in a timely manner. The SoloVPE variable pathlength spectrophotometer is a preferable alternative to traditional UV spectrophotometry. Utilizing the versatility of the variable pathlength technology (VPT) allows measurements to be performed without dilution and without blanking with other matrices. The study shows how the SoloVPE can accurately and repeatably measure mRNA concentration among other matrices that would normally need to be blanked and diluted by traditional UV analytical methods. Validation results for linearity in different sample matrices are plotted in Figure 1, showing the SoloVPE can accurately measure concentration of different gradients in the presence of multiple different matrices. The SoloVPE technology is platform-able and allows for a single test method to be leveraged that can support multiple mRNA products without the need to redevelop and validate multiple methods. The versatility of the SoloVPE reduces the cycle time for product-specific work, helping to support streamlined products and allowing CDMOs to easily development and validate the platformed analytical method.



Data are reported as mean ± SEM).

1219. A Universal Assay Determination Method for Antisense Oligonucleotides - A New Approach Using Slope Spectroscopy

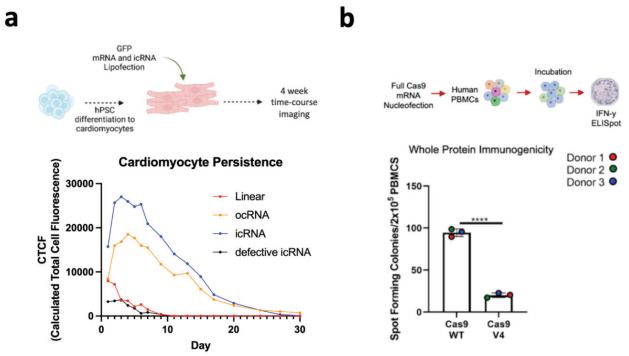
Kaitlin Alexander
Repligen, Oceanside, CA

Antisense oligonucleotides (ASO) are an important drug discovery platform for most major pharmaceutical companies. For drug safety and efficacy, an in-process control (IPC) assay of the active ingredient concentration is used to determine the quality of the ASO drug product. Standard HPLC-UV methods for IPC measurements are time consuming and have a relatively complex process. A novel method using the SoloVPE slope spectroscopy system was developed to have high repeatability, robustness, and significantly decreased the time to results. The following experiments were performed using multiple ASO samples with several chemistry modifications. One of the ASO samples was tested across several days and different analysts to assess intermediate precision of the method. This resulted in less than 1% RSD across test days and analysts. All samples were then tested within various buffer solutions and validated internally as well as at two different contract manufacturing organizations (CMO) to determine the method's repeatability, accuracy, and reproducibility. The method revealed to produce a 98%-102% recovery compared to the expected value and less than 1% RSD. The robustness of the method was also tested by adding several set-up adjustments, such as base, sugar, and linkage modifications. These experiments resulted in an overall difference of less than 2%, proving the SoloVPE method to be consistent and can be implemented universally. Using the SoloVPE method also saved approximately 4 hours of processing time and 67% cost/time reduction as compared to the former HPLC method. The SoloVPE method allows for the same accuracy and precision, compared to HPLC but with superior efficiency and a more universal IPC assay for ASO product measurement.

1220. Robust Protein Translation and Genome Targeting via HPLC-Free *In Situ* Circularizable and *In Vitro* Circularized RNA

Michael Tong[1], Hammza Khaliq[1], Aditya Kumar[1], Sangwoo Han[1], Nathan Palmer[1], Emma Finburgh[1], Madeleine K. Wing[1], Camilla Hong[1], Amir Dailamy[1], Joseph N. Rainaldi[2], Wei L. Chew[3], Ester J. Kwon[1], Prashant Mali[1]
[1]Bioengineering, University of California, San Diego, La Jolla, CA,[2]Biomedical Sciences, University of California, San Diego, La Jolla, CA,[3]Agency for Science, Technology and Research, Singapore, Singapore

RNAs are a powerful therapeutic class, but their inherent transience limits their activity. Circularization of RNA can improve their persistence, but simple and scalable approaches to achieve this are lacking. To facilitate the pursuit of circular RNAs, we developed two methods: "**o**utside developed" **c**ircular RNA (ocRNA) to enable robust in vitro circularization and in situ or "**i**nside developed" **c**ircular (icRNA) for in cell circularization. Importantly, we concurrently developed simple protocols to enable the successful purification of these circular RNAs without utilizing HPLC while retaining high yields (20-70%). These constructs enabled various applications (Figure A) demonstrating strong functional expression and persistence. We also further optimized protein translation from icRNAs and characterized significant expression differences from cap independent and cap dependent translation across cell lines, as well as relationships between immune response and protein expression. Leveraging this persistence, we enabled robust genome and epigenome targeting via both zinc finger proteins and CRISPR-Cas9 systems delivered as icRNAs. To enable compatibility between persistence of expression and immunogenicity of these delivered proteins, we next developed a novel **lo**ng **ra**nge multiple**x**ed (LORAX) protein engineering methodology to screen combinatorial variants, and demonstrated efficient genome targeting via deimmunized CRISPR-Cas9 (Figure B) delivered as icRNAs. We anticipate our highly simple, scalable, and versatile icRNA and ocRNA methodologies will have broad utility in basic science and therapeutic application.



1221. tRNA Therapeutics to Treat Stop Codon Disease

Stephen W. Eichhorn
Alltrna, Cambridge, MA

Nonsense mutations, which convert a sense codon within a protein-encoding mRNA into a stop codon and thus cause premature termination of protein synthesis, are the underlying cause of approximately 10% of all genetic diagnoses in humans. In recent years, transfer RNAs (tRNAs) engineered to read through premature termination codons (PTCs) and restore continued synthesis of the polypeptide chain have gained attention as an emerging novel therapeutic approach to potentially unify the treatment of thousands of rare and ultrarare stop codon diseases. To develop tRNAs with desired therapeutic features, such as high activity, specificity and durability, and low immunogenicity, Alltrna has built a unique platform based on *(i)* high-throughput screening of engineered tRNA oligonucleotides combining optimized sequence and chemical modification patterns and *(ii)* rational and machine learning-guided design principles. Preclinical data demonstrate productive PTC readthrough *in vitro* in a wide range of clinically relevant disease models spanning more than 15 different genes, and *in vivo* in PTC-containing mouse models across different stop codons. These preclinical proof-of-concept results are promising and provide an attractive programmable design framework for tRNAs as a novel class of medicines to restore disrupted protein production, regardless of target, for thousands of diseases with the same underlying genetic mutation. Recent preclinical data will be presented at the meeting.

1222. vMIX: Towards a "Plug-and-Play" Platform for Gene Silencing Therapy in Neurodegenerative Diseases

Catia Andreassi[1], Randa Elsayed[1], Agnieszka Ule[1], Alice Gennari[1], Deborah Ojutalayo[1], Lawrence Oliver[1], Joana Furtado[1], Zoe Walker[1], Yalem Bekele[2], Amrita Abhilash[2], Paniz Hosseini[2], Samantha Correia[2], Philip Coan[3], Olivier Brock[4], Carlos J. Miranda[3], Romain Joubert[1], John Isaac[1], Chris E. Shaw[1,4]
[1]Discovery, AviadoBio Ltd., London, United Kingdom,[2]Vector Sciences, AviadoBio Ltd., London, United Kingdom,[3]PreClinical, AviadoBio Ltd., London, United Kingdom,[4]UK DRI, King's College London, London, United Kingdom

Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) are devastating neurodegenerative diseases that may present with distinctive clinical manifestations while sharing common underlying genetics and pathology. To date there are no therapies able to make a major impact on survival. Presence of abnormal protein aggregates is an hallmark of many neurodegenerative diseases. In 95% of ALS and tau-negative FTD cases, widespread dysregulation in mRNA splicing due to cytoplasmic aggregates of the RNA binding protein TDP-43 is observed. Aggregation of TDP-43 is facilitated by another RNA binding protein, Ataxin-2 and reduction of *ATXN2* mRNA has been protective against TDP-43 toxicity in cellular and animal models. Aggregates of mutant superoxide dismutase 1 (SOD1) protein are causative in a substantial proportion of familiar cases of ALS and reduction in mutant *SOD1* mRNA has also proved beneficial *in vitro* and *in vivo* studies. Therefore, we have developed an adeno-virus associated (AAV) vectorised gene silencing platform, vMIX, that expresses proprietary microRNAs (miRNAs) capable of down-regulating target mRNA levels as a potential therapy for ALS and FTD. MicroRNAs are able to recognise specific mRNA sequences and engage the RISC complex leading to mRNA degradation or stalling in the translation, and decreased protein expression. The miRNA biogenesis is a complex, multi-step process that starts with the synthesis of a longer pri-miRNA precursor sequence and continues with its maturation to pre-miRNA by sequential cleavage reactions in the nucleus , and then in the cytoplasm to generate a mature miRNA duplex. Because of the rule of complementarity between target mRNA and miRNA, only one (called guide) of the 2 sequences in the mature miRNA duplex will be able to pair with the target mRNA. When loaded in the RISC effector complex, this sequence will be stabilized and by binding to the target mRNA, it allows the execution of the silencing activity. The complementary sequence (passenger) will be degraded. Therefore, an efficient RNAi based silencing approach relies on precisely processed miRNA precursors. However, the maturation of the miRNA precursors is a complicated process and not all the variables that control it are well understood. The sequences of the miRNA itself, of the flanking stems and of the loop that connects the miRNA duplex do have a major impact on the maturation process and affect the precision with which the miRNA is generated. In order to generate precise miRNAs able to accurately target the mRNAs of interest, we have developed a battery of miRNA candidates targeting *SOD1* or *ATXN2* mRNAs, and studied the impact of sequence variations on the maturation process. Using Next Generation Sequencing (NGS) technologies, we have assessed the effectiveness of alternative sequences on: - the cleavage precision,

guide sequence, and guide to passenger ratio of the candidate miRNAs; - the silencing efficiency of the candidate miRNAs; - the expression of endogenous miRNA repertoire. In addition, we investigated the species-specificity of the miRNA processing by conducting parallel experiments in cell lines originating from different species, and further compared the results of in *in vitro* and *in vivo* studies. By combining these analyses with a rational candidate design, we are endowing the vMIX platform with nucleotide-precise targeting abilities, developing a versatile, ready-to use miRNA-based gene therapy approach.

1223. Divalent siRNA Therapy for Huntington's Disease Demonstrates Robust Safety and Efficacy in Pre-Clinical Assessment in an Ovine Model

Hector Ribeiro Benatti[1], Rachael Miller[1], Toloo Taghian[1], Dimas E Moreno[1], Jillian Gallagher[1], Erin F. Hall[1], Abby McElroy[1], Nathan K. Yingling[1], Vania Anagnostakou[1], Ellen Sapp[2], Marian DiFiglia[2], Anastasia Khvorova[1], Heather L. Gray-Edwards[1], Neil Aronin[1]

[1]UMass Chan Medical School, Worcester, MA,[2]MassGeneral Institute for Neurodegenerative Disease, Charlestown, MA

Background: Huntington's Disease (HD) poses a significant challenge as an autosomal dominant neurodegenerative disorder, resulting from the expansion of CAG repeats on chromosome 4. This expansion triggers the translation of a mutant huntingtin (mHTT) protein. Its chronic expression leads to protein aggregation in the central nervous system (CNS), and subsequent cell death. The severity and onset of HD correlate with the size of the CAG repeat, manifesting in various symptoms like choreiform movements, behavioral disturbances and dementia. Despite the absence of therapies capable of altering the onset or progression of the disease, there remains an urgent demand for safe and effective interventions. A prevailing hypothesis suggests that diminishing mHTT expression within the CNS could alleviate the pathological features of the disease and improve the overall clinical condition. **Methods:** Our study addresses the therapeutic potential of a non-allele selective divalent small-interfering RNA (di-siRNA) scaffold designed to reduce normal huntingtin (HTT) and mHTT expression in the CNS. Utilizing this di-siRNA, notable efficacy was achieved in mice. However, acute seizures were noted, likely due to siRNA's negative charge. To address this issue we optimized the formulation to include calcium and magnesium cations and corrected the seizure phenotype. Advancing to a pre-clinical stage, safety and efficacy assessments were conducted in wild type sheep (n=28 divided in 7 cohorts) through intracranial ventricular (ICV) and intrathecal injections. Cohorts included a low ICV dose (25mg) and dose escalation groups (50, 75, and 100mg). Lumbar intrathecal injections offer an alternative less invasive route; therefore we tested a moderate 50mg dose. Safety and HTT knockdown (mRNA and protein) were analyzed 2-months and 6-months post-injection. Safety monitoring included real-time holter electroencephalography (EEG), histopathology and pre/post-MRI analysis. **Results:** EEG revealed a dose-response toxicity: 25 and 50mg doses demonstrated safety, while 100mg resulted in seizures. Tissue analysis revealed efficacy in the striatum of sheep, one of the regions of the brain most severely affected by HD, demonstrated by HTT mRNA and protein reduction. The 50mg dose demonstrated superior efficacy compared to the baseline dose, revealing a notable reduction of approximately 50% in mRNA and an approximate 75% decrease in HTT expression. Intriguingly, doses >50mg did not increase knockdown further. After 6 months silencing in the cortex and hippocampus was evident, but not in the striatum. **Conclusion:** Here we demonstrated the potential of a di-siRNA as a therapeutic approach for HD. The observed dose-dependent seizure phenotype emphasizes the necessity for meticulous safety EEG assessments. The long-term durability findings suggest a need for repetitive dosing <6 months to sustain the therapeutic efficacy in the striatum. Next, we will test the impact of repetitive dosing using the most effective and safe dose (50mg). Looking toward future clinical trials, we will also test magnesium-only formulation, addressing the unavailability of USP-grade calcium and magnesium buffers. These findings not only advance our understanding of HD treatment but also pave the way for future clinical trials.

Thursday Posters: Nonviral Therapeutic Gene Delivery and Synthetic/Molecular Conjugates

1224. Silicon Nanoneedle Microarray-Based Delivery of Cell Gene Therapies

Salma Sami

Mekonos, Inc., Alameda, CA

The field of cell and gene therapies is quickly revolutionizing modern medicine by redefining treatment modalities. Genetic disorders previously deemed incurable are now open to newly developed treatment options. At its core, cell gene therapy involves the delivery of genetic material into the cell for the purpose of correcting or improving an underlying genetic problem. Over the years, multiple vehicles of gene delivery have been developed, with varying degrees of success. Among these, viral vectors, electroporation and transfection are most common. Unfortunately, these methods are inefficient, cumbersome, stressful to the cells and, in case of viral vectors, may lead to downstream genomic instability and potential adverse effects like secondary cancer. To address these bottlenecks, Mekonos has capitalized on the nanoneedle cell delivery technology and developed a high throughput gene delivery system based on solid silicon nanoneedle microarray chips. Mekonos' novel cell engineering platform side-steps the problematic use of canonical modes of gene delivery by utilizing an array of solid silicon nanoneedles to enable delivery of biomolecular payloads of various compositions and sizes. Our method of silicon nanoneedle fabrication utilizes semiconductor technology to produce needles of consistent height and width. Cells of interest are first "trapped" (temporarily immobilized) on the system membrane using microfluidics, then the payload-coated chip is placed on the cell array, thereby ensuring one needle-one cell interaction and delivery. The workflow is scalable, efficient and causes minimal cell damage. Additionally, our method has been demonstrated to be independent of cell or cargo type and has been utilized to deliver a plethora of cargo (CRISPR-Cas reagents, nucleic acids) into a variety of cells (immortalized, primary, stem). The

advantage of Mekonos' silicon nanoneedle-based delivery platform is that it allows for scaled up workflows, efficient and controlled payload delivery to various cell types, as well as the ability to deliver multiple cargo modalities, singly or in multiplexed arrays. Moreover, unlike other gene delivery vehicles in use today, our platform enables the delivery of very large cargo, for example >100kb plasmids, in a dose-controlled manner to various cell types of interest, with minimal stress and loss in viability. A unique advantage of our platform is the ability to multiplex heterogenic cargo for one-time or sequential delivery into multiple cell types with no additional steps. This allows for the delivery of both plasmids and RNPs on the same needle, at the same time, in any iteration controlled by the end-user, allowing for multiple gene edits while minimizing risk of genomic instability. Perhaps the most unique aspect of our technology is its cross-platform functionality since it can be used to manipulate a wide variety of cell types, including mammalian, plant and yeast, among others. We envision that the combination of our semiconductor, microfluidic, and surface chemistry technologies will redefine cell therapies, facilitating the precise and scalable engineering of all cell types.

1225. DELiveri: A Novel High-Throughput Screening Platform for the Discovery of Molecules That Promote Extra-Hepatic Nucleic Acid Delivery

Omer Ziv[1], Adrej Alendar[1], Shoval Tirman[2], James Murphy[1], Liv Austenaa[1], Chiara Barberio[1], Omer Weissbrod[2], Yaniv Erlich[1], Paloma H. Giangrande[3]

[1]Eleven Therapeutics, Cambridge, United Kingdom,[2]Eleven Therapeutics, Tel-Aviv, Israel,[3]Eleven Therapeutics, Cambridge, MA

Cell-specific delivery is the holy grail of RNA therapeutics. Currently, small chemical conjugates such as GalNAc have been successful in achieving liver-specific delivery. However, delivery to other tissues/organs remains a major hurdle. DNA-encoded chemical libraries (DELs) have the potential to provide hypothesis-free screening methods to discover new RNA conjugates that can enable extra-hepatic delivery. Here, we present DELiveri, a groundbreaking DEL-based screening platform for identifying nucleic acid conjugates for productive cytoplasmic delivery in a massive parallel fashion. DELiveri harnesses a synthetic biology genetic circuit that converts cells into molecular radars. This circuit creates a strong and unique signal when the DEL barcodes are bioavailable in the cytoplasm. After extensively validating our circuit in small-scale experiments, we utilized our technology to simultaneously screen over 35,000 delivery molecules in human cells. Our results showed high consistency among biological replicates (R >0.9), strong separation between negative and positive controls, agreement with the expected chemoinformatic trends (i.e., molecular structure, hydrophobicity content), and strong chemical similarly between the hits. Importantly, we identified molecules with cell-specific signatures that are currently being evaluated as cell-specific delivery ligands for various RNA therapeutic modalities. In summary, our strategy quantitatively decodes the structure-activity relationship (SAR) of cell-specific delivery molecules and opens an avenue for the use of generative AI tools in designing new molecules that enable extra-hepatic nucleic acid delivery.

1226. Skeletal Muscle *liveGT* Enhances Therapeutic Expression Levels over Several Months

Michael Francis[1], Jacob Hensley[2], Alex Otten[2], Anna A. Bulysheva[2]

[1]University of South Florida, Tampa, FL,[2]Medical Engineering, University of South Florida, Tampa, FL

Gene electrotransfer is an established physical method for delivery to various tissue and organs *in vivo* including melanoma tumors, skin, cardiac muscle, and skeletal muscle. GET advantages include nonimmunogenic and nonintegrative gene delivery to targeted tissues. Disadvantages include low expression levels, short-lived/transient expression, and muscle stimulation. Skeletal muscle, localize in vivo electro gene therapy (*liveGT*) as a next generation GET, was utilized with a monopolar electrode and optimized pulse sequences to determine longevity of expression and feasibility of reaching therapeutic blood levels of expression. *LiveGT* was administered directly to the skeletal muscle of Sprague Dawley rats, with plasmid DNA encoding luciferase or human insulin and glucokinase co-delivery. Expression levels were monitored with bioluminescence imaging as well as directly from serum samples via ELISA over nine months post initial *liveGT*. Additionally, to evaluate therapeutic potential blood glucose measurements were also monitored. Two-way ANOVA and Tukey multiple comparison tests were used to determine statistically significant differences, with $p<0.05$ considered significant. Control groups received vehicle, or no delivery at all. Reporter expression was observed to be maintained for over six months with significantly higher (over 100-fold) than respective controls. Exogenous human insulin expression and blood glucose modulation were maintained over three months, with significant reduction in mean circulating glucose compared untreated animals. Therefore, our preliminary findings implicate *liveGT* skeletal muscle reprogramming as a viable target for secretory protein therapies as therapeutic levels of expression can be achieved and maintained for several months without risks of integration and immunogenicity.

1227. Gene Electrotransfer Increases Mammalian Cell Glucose Consumption in an Insulin-Dependent Manner

Jacob W. Hensley[1], Michael Francis[2,3], Alex Otten[1], Tina Gagliardo[1], Anna Bulysheva[1]

[1]Department of Medical Engineering, University of South Florida, Tampa, FL,[2]Department of Orthopaedics, University of South Florida Morsani College of Medicine, Tampa, FL,[3]Asante Bio, Tampa, FL

More than 9 million adults are diagnosed with Type 1 Diabetes worldwide. Traditional therapies utilize insulin protein injections and continuous blood glucose monitoring. Research advancements for alternative treatment include Beta cell encapsulation, artificial pancreas systems, FDA approved pancreatic islet cellular therapy, Lantindra, and delivery of insulin and glucokinase via AAV, however, immunomodulation is required. The goal of this study is to deliver exogenous insulin and glucokinase via gene electrotransfer (GET) for glucose modulation. Transient transfection of C2C12 myoblasts with Nanoplasmid ® DNA encoding human insulin and glucokinase utilizing gene electrotransfer as the delivery vehicle was examined to overcome the need for immunomodulation. Following transfection of

myoblasts, media glucose levels were measured daily for seven days. Two-way ANOVA comparison and Tukey multiple comparison tests were used for quantitative statistical analysis. Immunofluorescence analysis measured transfection efficiency and percent co-localization. Exogenous co-expression of both insulin and glucokinase significantly increased media glucose depletion compared to respective controls, indicating that GET delivery of insulin and glucokinase is a viable therapeutic pathway. Of the myoblasts expressing either exogenous protein in the group transfected with both insulin and glucokinase on the same plasmid, there was 70% co-expression of both insulin and glucokinase. These findings are consistent with our hypothesis that cells can be reprogrammed to modulate blood glucose levels in an insulin-dependent manner, resulting in a potential treatment for Type 1 Diabetes without the use of immunomodulatory drugs.

1228. Targeted Non-Viral Gene Delivery through Transcutaneous Ultrasound Enables Rapid, Robust, Redosable, Titratable, and Durable Gene Expression in the Liver in Murine and NHP Models

Surabhi Rao, Victor Foster, Bert Frederich, Barry Campbell, Adel Alonso, Glenda Arauz, Celia Ortiz, Oliver Chen, Charlotte He, Elizabeth Del Greco, David Satyadi, Anagha Rao, Dhruv Bole, David Kim, Katie Benthall, Margarita Krivega, Julia Poniatowski, Ivan Krivega, Zoya Gluzman-Poltorak, Steve Feinstein, Kenneth Greenberg

SonoThera,Inc, South San Francisco, CA

Introduction: Robust delivery of DNA payloads to the liver remains a significant challenge for the development of safe and effective nonviral genetic medicines. Current limitations include the ability to 1) achieve durable episomal gene expression in a safe, titratable, and redosable manner, 2) deliver large DNA payloads (>5kb), 3) bypass endosomal and cytoplasmic immune recognition and effectively transit the nuclear envelope, and 4) achieve targeted transgene expression in clinically relevant hepatic cell types. Transcutaneous ultrasound-mediated gene delivery (UMGD) is a noninvasive *in vivo* gene delivery approach that may overcome the key delivery challenges facing genetic medicines. SonoThera is developing a novel ultrasound-guided nonviral gene therapy platform based on UMGD that allows selective targeting of specific organs and tissues within the body in a safe, redosable, durable, and titratable manner. Methods: To evaluate the potential of UMGD for safe and efficient DNA delivery to the liver, we developed a technology platform that utilizes novel acoustic profiles and next-generation genetic payloads with FDA-approved ultrasound components. The delivery process involves intravenous co-administration of DNA payloads and ultrasound contrast agents (a.k.a microbubbles), coupled with targeted application of externally applied ultrasound energy to guide the DNA into specific tissues via UMGD. *In vivo* bioluminescence imaging of firefly luciferase reporter gene expression was evaluated using an IVIS imaging system at multiple timepoints in the mouse liver following UMGD. Mouse and monkey liver tissues were analyzed for reporter gene presence and expression. Multiple clinical and molecular safety assessments were evaluated using established methods. Results: Rapid and robust transgene expression was detected in the mouse liver within 3 hours following DNA sonoporation, which persisted for at least 6 months after a single treatment. Furthermore, repeated administrations resulted in significant increases in gene expression levels relative to single administrations. Gene expression levels were titrated effectively in a dose responsive manner. Reporter gene presence and expression were efficiently detected in the analyzed mouse and NHP liver tissues. Safety assessments including clinical observations, ALT, AST, and a proinflammatory cytokine panel indicate an excellent tolerability and safety profile following single and repeat treatments in both murine and NHP models. Conclusion: These results position a novel UMGD platform as a promising technology for *in vivo* noninvasive transgene delivery using next-generation non-viral DNA payloads, thereby laying the foundation for the development of future redosable and titratable genetic medicines targeting the liver.

1229. Development of a Next-Generation Non-Viral Human FVIII Therapeutic Administered through Targeted Transcutaneous Ultrasound Enables Normal Physiological Levels of FVIII Transgene Expression in a Redosable and Titratable Manner

Margarita Krivega, Julia Poniatowski, David Kim, Elizabeth Del Greco, Ivan Krivega, Surabhi Rao, Victor Foster, Bert Frederich, Barry Campbell, Adel Alonso, Glenda Arauz, Celia Ortiz, Oliver Chen, Dhruv Bole, Katie Benthall, Charlotte He, Anagha Rao, David Satyadi, Zoya Gluzman-Poltorak, Steven B. Feinstein, Kenneth P. Greenberg

SonoThera, South San Francisco, CA

Introduction: Hemophilia A (HemA) is caused by the absence or deficiency of Factor VIII (FVIII) protein resulting in uncontrolled bleeding. The current primary standard of care for HemA consists of lifelong infusions of recombinant FVIII protein on a frequent basis. The recent approval of viral gene therapy for HemA provides an alternative, potentially once-a-lifetime treatment option. However, several limitations including high product cost, preexisting immunity to viral vectors, lack of durable FVIII expression, and lack of redosing capabilities suggest the need to develop alternative non-viral HemA gene therapy treatments to overcome these major limitations. Transcutaneous ultrasound-mediated gene delivery (UMGD) is a noninvasive *in vivo* gene delivery approach that may overcome the key delivery challenges facing HemA genetic medicines. SonoThera is developing a novel ultrasound-guided nonviral gene therapy platform based on UMGD that allows selective targeting of specific organs and tissues within the body in a safe, redosable, durable, and titratable manner. **Methods**: Using genetic engineering and screening of next generation DNA payload formats, we developed an optimized episomal DNA payload expressing a clinically validated human FVIII transgene. Transgenic FVIII expression was measured *in vitro* in human and mouse cells and *in vivo in mouse plasma*. To evaluate the potential of UMGD as a nonviral HemA treatment option, we delivered an optimized HemA payload using novel acoustic profiles, developed by SonoThera, and FDA-approved ultrasound components. The delivery process involves intravenous co-administration of DNA payloads and ultrasound contrast agents (a.k.a microbubbles), coupled with targeted

application of externally applied ultrasound energy to guide DNA into specific tissues via sonoporation. **Results**: The next-generation optimized DNA payload was determined to yield high human FVIII expression levels and secretion in human and mouse cells *in vitro* in a dose responsive manner. Additionally, the protein was determined to be functionally active via an established coagulation assay. By optimizing the UMGD *in vivo* delivery acoustic profiles, we have been able to efficiently deliver the optimized FVIII payload to the liver and reach sustainable therapeutic levels of FVIII in mouse plasma without triggering liver toxicity. Repeated UMGD treatments led to a significant increase in transgenic FVIII levels in plasma suggesting a redosing treatment regimen for elevating or maintaining a normal physiological level of circulating transgenic FVIII. **Conclusion**: These favorable efficacy and safety results achieved using a novel UMGD platform suggest further translation of the optimized non-viral human FVIII payload and delivery method into clinical development for the treatment of Hemophilia A.

1230. Safe and Redosable Non-Viral Gene Therapy in Hemophilia A: Transcutaneous Ultrasound-Mediated FVIII Delivery as a Promising Therapeutic Approach

Ting-Yen Chao, Meng-Ni Fan, Yuanyi Xu, Xiaohe Cai, Carol H. Miao

Seattle Children's Research Institute, Seattle, WA

Hemophilia A (HemA) is an X-linked clotting disorder characterized by a deficiency of functional factor VIII (FVIII) protein. Gene therapy is promising for treating hemophilia but the safety concerns and challenges brough by the pre-existing antibodies against viral vectors when using such vectors. Ultrasound-mediated gene delivery (UMGD) has emerged as a promising non-viral method that utilizes therapeutic ultrasound (US) and microbubbles (MBs) to facilitate gene transfer. This study aims to develop a noninvasive intravenous non-viral gene delivery protocol in combination with transcutaneous UMGD (tUMGD) in Hemophilia A mouse models to achieve efficient transfection of FVIII plasmids in the liver. To achieve sustained FVIII expression, FVIII plasmid was mixed with RC2K cationic MBs for 5 minutes, and subsequently injected intravenously into groups of mice via retro-orbital injection with tUMGD to induce cavitation in the liver for 60 seconds. The US parameters used in our experiments include: a center frequency of 1.1 MHz, a pulse repetition frequency of 14 Hz, and peak negative pressure (PNP) between 1.2 and 1.5 MPa, with a pulse duration of 150 μs in a 1-second ON and 2-seconds OFF pulse train for a total treatment time of 60 seconds. Blood samples were collected weekly for detecting human FVIII activity and alanine aminotransferase levels. Initially, HemA mouse models were subjected to treatment with various human FVIII plasmid candidates to enhance FVIII activity following hydrodynamic injection. The HemA mice treated the selected high-expressing FVIII plasmid achieved an impressive FVIII activity ($1.3x10^4$ %), representing a 10-fold increase compared to the mice treated with the other FVIII plasmid (1200%). Subsequently, we assessed US conditions using the high-expressing FVIII plasmid in HemA mice. Administering a total of 50 μg DNA with repeated US treatments using a PNP of 1.2 MPa resulted in significantly higher FVIII activity (19.5%), mirroring the outcomes observed in mice treated with a total of 75 μg DNA under the same US conditions. It was noted that a plateau in expression level was achieved with 50 μg plasmid DNA. Variant US conditions, ranging PNP between 0.5 and 1.5 MPa, were explored, and the optimal condition was identified as PNP 1.5 MPa. To evaluate the feasibility of redosable tUMGD using the FVIII plasmid, we utilized immunodeficient NSG HemA mice to perform tUMGD experiments with optimized US conditions and DNA doses for plasmid transfection in the absence of antibody formation. Mice treated with 50μg high-expressing FVIII plasmid under repeat tUMGD, initially achieved approximately 40% activity, sustaining around 10% for a month. A subsequent treatment aimed to enhance FVIII activity in these mice, resulting in an initial 35% activity, which gradually stabilized at 18% for two months. A third treatment was administered for redosing purposes, a similar trend in FVIII activity was observed. Slightly elevated ALT levels post-treatment normalized within a week, affirming the safety of tUMGD. In conclusion, these results demonstrated that tUMGD is a highly efficient, stable, and redosable nonviral delivery method. This minimally invasive, non-viral, and effective method holds promise for translating into future treatments for hemophilia A patients.

1231. A Scalable Insect Cell-Dual Baculovirus System for Non-Viral Gene Therapy (NVGT) Production

Adhvait Shah, Swetha Kumar, Hyun-Woo Kim, Darrick Yu, Jeong Lee

Cell Culture Process Development, GMU CMC, Sanofi, Waltham, MA

Sf9 cells have been employed successfully as a viable alternative to produce AAV. Recombinant AAV can elicit immune responses, thereby preventing drug redosing, and have limited payload capacity, thereby limiting the number of disorders addressed. NVGT allows re-dosing with larger payloads. In addition, NVGT methods such as using lipid nanoparticles, polymers, etc. are alternative approaches that pose low cytotoxic and immunogenic risks. Moreover, the Sf9-baculovirus platform has a favorable safety profile due to lack of pathogenicity of baculovirus in humans, thus offering an excellent platform to manufacture NVGT products. We developed and optimized an Sf9-baculovirus upstream process for manufacturing NVGT products such as closed-ended DNA or ceDNA. Although the Sf9-baculovirus system has previously been used for AAV manufacturing, the restricted coding capacity of AAV prevents its use as a vector for a number of diseases. We showed better process performance controlling for several parameters, which are typically not controlled in Sf9 culture. The optimized process resulted in 5-fold improvement in ceDNA titers, significantly reducing the number of batches needed to meet the clinical demand while maintaining product quality and thereby reducing the cost of goods. This allows shorter timelines for the therapy to reach patients. In this work, we have compared two approaches to infection of Sf9: one-baculovirus and two-baculovirus systems. The two-baculovirus system have rep and transgene on two different baculoviruses while the one-baculovirus system has the transgene stably integrated in the Sf9 genome, with the rep gene encoded by a baculovirus. The two-Baculovirus system was found to be more robust and superior in performance compared to the one-baculovirus system. Various parameters including, but not limited to, viable cell density at infection, relative MOI, MOI ratio, medium composition, pH, and temperature were optimized for the two-bac system. This optimization

resulted in 5-fold improvement in ceDNA titers. This optimized process was scaled-up for evaluation and validation including Ambr ® 250 systems, bench top bioreactors and pilot scale reactors up to 500 L. We demonstrated robust performance of this process both regarding the growth of Sf9 and ceDNA manufacture while maintaining product quality and productivity. We developed a harvest process using a continuous centrifugation strategy to process pilot-scale volumes of cell culture, up to 500 L. We identified a combination of centrifugation parameters using a single-use continuous centrifugation strategy that enabled quick and efficient generation of concentrated cell suspension, with minimal loss to cell viability and product quality. In conclusion, two-Baculovirus Sf9- baculovirus system for ceDNA production offers greater flexibility with an ability to deliver large transgenes. Since the two-baculovirus system allows for easily swapping to new transgenes by substituting a different transgene-baculovirus construct, as opposed to transfection/isolation of a new Sf9 clone, time-to-clinic for new indications is drastically reduced. Our novel seven-day two-baculovirus process includes growth of insect cells, NVGT product manufacture, and concluding with single-use continuous centrifugation to harvest a concentrated cell suspension for faster purification. The improved process requires fewer batches to meet the clinical demand, thereby making NVGT more accessible to patients.

1232. Rapid Enzymatic Synthesis and Scaling of DNA Gene Therapy Molecules

Daniel Blackstock, John Durant, Joris Cipi, David Turkington, Adam Villa, Russell Monds, Matthew Stanton

Generation Bio, Cambridge, MA

Non-viral gene therapy technologies offer many manufacturing advantages over viral-based therapies. Since virus production is not required, the costly, complex, and hard to control cell-based viral vector productions can be eliminated. Additionally, because intracellular encapsidation is abolished, the synthesis of the payload nucleic acids can be separated from the production of the delivery vehicle allowing for enhanced quality control of the drug produced. Without the necessity for the viral vector, the potential to introduce a completely cell-free method for simplified and accelerated nucleic acid manufacturing becomes a possibility. As demonstrated with mRNA vaccines, enzymatic *in-vitro* transcription (IVT) technologies have resulted in platform synthesis methods which can enable extremely flexible, rapid, and scalable production of RNA pharmaceuticals. While DNA production has traditionally relied on plasmid cloning and *E. coli* fermentation for scaling, we have developed a platform analogous to IVT, facilitating synthetic DNA production. Our rapid enzymatic synthesis (RES) method enables the production of kilobase length DNA transgenes at cutting edge speed, with flexible-end compositions for desired functionality, and gram / liter scalability. Our RES process consists of (1) DNA template amplification, (2) selective digestion and ligation driven assembly of ITR oligonucleotides onto the ends of the transgene cassette, (3) residual DNA clean up using a nuclease. The RES process eliminates any extraneous DNA typically present with plasmid backbone and reduces sequence specific biases that are often observed with *E. coli* scaling. RES generates synthetic transgenes up to at least 7kbp in size with tunable end structures and composition that allows for incorporation of additional functionality (*e.g.* modified nucleotides, aptamers). The yields achieved are >1000x template input, enabling gram / liter outputs for reduced scale requirements and enhanced ease of production. RES produces DNA transgenes with >99% purity when processed using chromatographic methods and products have been expression validated *in vivo* with performance comparable to plasmid-based transgenes.

1233. *In Vivo* High-Throughput Pooled Screening of Transcriptional Regulatory Elements for Gene Therapy

Jessica Keenan, Jicong Cao, Anais Sidonia, Russell Monds

Molecular Biology, Generation Bio, Cambridge, MA

Identification of genetic elements that drive therapeutic levels of gene expression often requires testing candidates in a series of model systems of increasing relevance (e.g., cells lines, mice, non-human primates). Arrayed construct screening (individual testing of each candidate) limits the number of designs that can be tested in a single experiment, particularly in the most relevant models. As a result, the design space than can be interrogated is limited and evaluation of performance is heavily weighted on experiments in less relevant models. In this work, we utilize a pooled screening approach to simultaneously characterize thousands of transcriptional regulatory elements in a single mouse and directly compare our results to an *in vitro* model. We designed a library of 6,000 transcriptional regulatory element candidates ("enhancers") intended to increase expression when inserted upstream of the transthyretin (TTR) liver promoter. Each enhancer candidate was composed of a combination of 8 binding sites for 12 transcription factors that regulate liver gene expression. Candidates were each assigned a unique 10 nt barcode to facilitate enhancer-specific quantification of mRNA expression. The enhancer/barcode pairs were synthesized as an oligo pool and used to generate a plasmid pool with the transgene cassette structure: enhancer candidate|TTR promoter|luciferase gene|barcode|3' UTR|polyA signal. The plasmid library was delivered to CD-1 mice (n = 3) via hydrodynamic tail vein injection and liver mRNA expression was measured by amplicon sequencing of the barcode regions. Replicates were highly correlated ($r > 0.96$) and >10 barcode counts were recovered for 99.8% of candidates. Enhancers spanned a broad range of activity levels, with the top 1% of candidates showing >5-fold increase in expression compared to the median expression of 50 random size-matched sequences and >2.5-fold increase compared to a benchmark enhancer. To compare enhancer performance between *in vitro* and *in vivo* models, the same plasmid library was transfected into HepG2 human hepatocellular carcinoma cells and mRNA expression was measured by amplicon sequencing. Replicate HepG2 experiments were highly correlated ($r = 0.97$) and >10 barcode counts were recovered for 99.9% of candidates. Comparing between *in vivo* and *in vitro* pooled experiments, expression values were moderately correlated ($r = 0.54$), suggesting that translation between models in pooled experiments is enhancer dependent. Notably, many enhancer variants with high expression *in vivo* would not have been chosen for additional characterization based on *in vitro* data, highlighting the value of expanding the design space screened in the most relevant models. Increasing screening throughput also enables learning design rules. Our library included a subset of variants to assess the impact of number of transcription factor binding site repeats

and spacing between repeats, which we discuss here. We also briefly discuss a separate *in vitro* pooled screening experiment in which we assess all single nucleotide variants of a genomic liver enhancer and identify a conserved single nucleotide change that increases expression, highlighting the value of pooled screening for construct design and optimization. To better understand the potential and limitations of pooled construct screening, future studies will focus on assessing the translation between pooled and arrayed experiments, both *in vivo* and *in vitro*. Successful validation of pooled screening *in vitro* and in murine models would suggest translation of the strategy to non-human primates as a future direction, a potentially transformative approach for developing the most effective constructs for new therapeutics.

1234. Ultrasound Mediated Gene Delivery of Cas9 Plasmids in Hemophilia A Mice Display Therapeutic Levels of Endogenous FVIII

Savannah M. Lawton, Meng-Ni Fan, Ting-Yen Chao, Chun-Yu Chen, Yuanyi Xu, Xiaohe Cai, Carol H. Miao
Seattle Children's Research Institute, Seattle, WA

Hemophilia A (HA) is a bleeding disorder in which an individual cannot produce functional clotting factor VIII (FVIII). Correction of the disease-causing mutation through gene editing is an attractive option for HA because there are currently no robust, long-term, non-repeat treatments available. Ultrasound mediated gene delivery (UMGD) has been shown to be a safe and effective non-viral method of gene delivery, that has the capability to predominately transfect a specific cell type, depending on the plasmid and ultrasound condition used. In this study, we investigated the capability of UMGD to successfully target liver sinusoidal endothelial cells (LSECs)- the native production site of FVIII, using CRISPR/Cas9 plasmids to restore endogenous FVIII production. Previous UMGD experiments in mice have shown a pulsed, therapeutic, ultrasound (US) treatment at 1.1 MHz frequency, 14 Hz PRF, with 50W power, and 150 μs PD, results in primarily LSEC transection in the liver. We utilized these ultrasound conditions, in combination with RN18 microbubbles (MBs) to enhance gene transfer of a Cas9 plasmid via cavitation. A mixture of MBs and Cas9 plasmid are injected into the portal vein of mice for 30s, while simultaneously the US transducer is placed on the surface of the liver and treated for 60s. For our gene editing animal model, we used immunodeficient mice with a 5 base pair deletion in exon 1 in the FVIII gene (NSG HA mice). The sgRNA is designed to target this deletion location in exon 1 (mF8sgRNA) and included in the Cas9 plasmid. Blood was collected from the mice at various timepoints over 90 days and the FVIII activity was determined via the aPTT assay. The livers were perfused with a digestion buffer prior to collection, and the hepatocyte and LSEC cell populations were separated. DNA was isolated from each cell population and sent for sequencing to evaluate editing efficiency. Sanger sequencing of treated mice liver DNA (n=4) showed LSEC's had an average in-frame editing efficiency of 1.6% and hepatocytes had an average in-frame editing efficiency of 0.68%. Additionally, mice had an average FVIII expression of 5% over the 90 days followed. While this editing efficiency remains relatively low, it is noteworthy that LSECs exhibit a higher efficiency compared to hepatocytes. This observation aligns with the indication that the US condition employed predominantly facilitates the transfection of LSECs. These results indicate that UMGD successfully delivered a CRISPR/Cas9 plasmid into LSEC's of HA mice, facilitating effective gene editing and leading to therapeutic levels of FVIII

1235. Circularization of Non-Viral Single-Strand DNA Template for Gene Correction and Gene Insertion Improves Editing Outcomes in HSPCs

Alex Boyne[1], Arianna Moiani[2], Gil Letort[2], Diane Le Clerre[2], Cosimo Ducani[3], Roger Salvatori[3], Alexandre Juillerat[1], Aymeric Duclert[2], Philippe Duchateau[2], Julien Valton[2]
[1]Cellectis, Inc., New York, NY,[2]Cellectis, Paris, France,[3]Moligo Technologies, Solna, Sweden

Leveraging the TALEN® technology, we developed a gene editing process leading to highly efficient gene correction and gene insertion via homology directed repair, in hematopoietic stem and progenitor cells (HSPCs). We first assessed the potential of non-viral linear single-stranded DNA (LssDNA) donor template delivery strategies versus the more commonly used viral (AAV) delivery. Both strategies led to high and comparable efficiencies of gene correction in HSPCs *in vitro*. We then compared the use of LssDNA versus circularized single-stranded DNA (CssDNA). We found that circularization markedly increased the knock-in (KI) efficiency with respect to its linear counterparts. Interestingly, this increase of KI was correlated to higher viability and a lower knock-out (KO) in circular versus linear ssDNA edited cells, respectively. In-depth characterization of edited HSPCs by scRNAseq unraveled different transcriptomic profiles associated to different donor template formats when using LssDNA or CssDNA, compared to AAV. Overall, we showed that non-viral ssDNA delivery associated to TALEN® gene editing reduces the toxicity usually observed with viral DNA delivery and allows high levels of gene correction in long-term repopulating hematopoietic stem cells. Circularization of the ssDNA has the potential to further increase the rates of KI without impacting cellular viability and fitness, facilitating the development of next generation cell therapies.

1236. Nano-Enhanced Optical Delivery of Optogenes in the Central Nervous System for Pain Modulation

Darryl Narcisse[1], Bob Benkowski[1,2], Matthew Dwyer[3], Samarendra K. Mohanty[4,5]
[1]Opsin Biotherapeutics, Fort Worth, TX,[2]DesignPlex Biomedical, Fort Worth, TX,[3]Texas Christian University, Fort Worth, TX,[4]Nanoscope Therapeutics Inc, Bedford, TX,[5]Opsin Biotherapeutics, Bedford, TX

Purpose: Pain is a major health issue that causes serious deficits in the psychological and physiological state of patients worldwide. Earlier, we have demonstrated pain inhibition by optogenetic modulation of specific neurons in the Anterior Cingulate cortex (ACC) and spinal cord using AAV-based gene delivery. Localized expression of genes in targeted cells is valuable for achieving efficacy without the potential for off-target expression and deleterious effects. Here, we describe the use of far-red laser-based spatially targeted nano-enhanced delivery (NOD) of genes encoding for multi-characteristic opsin (MCO).

Method: MCO is a highly sensitive optogenetic actuator that can be activated by red light, which can penetrate deeper tissue with less absorption and scattering loss. NOD is a non-viral delivery technique that uses nano-enhancers that amplify the light intensity that creates hot spots in the vicinity of targeted cells to perforate the membrane leading to the uptake of the genes. We utilized Gold Nanorods (GNRs) as nano-enhancers along with a 650 nm laser to deliver GAD-MCO genes into dorsal regions of the ACC and lumbar spine. The mixture of GNRs and GAD-MCO plasmids was injected into the target tissue (intracranial for ACC and intrathecal for the spinal cord at the L5-L6 junction) before laser irradiation. The laser beam was delivered via an optical fiber to result in ~ 35 mW power at the surface. Optogenetic stimulation of MCO-sensitized ACC and spinal cord was achieved via a low-power (<1 mW) red LED. Both acute inflammatory (via formalin injection) and chronic neuropathic hyperalgesia (by sciatic nerve constriction) pain models were used to evaluate the therapeutic efficacy of laser-delivered and optically-stimulated MCO in ACC and spinal cord. Results: The qPCR-based evaluation of MCO-transgene biodistribution showed localized presence in the targeted tissues. Immunohistochemistry of targeted tissue shows robust expression of mCherry (reporter for MCO) in the inhibitory GABAergic neurons of the ACC and spinal cord regions targeted by laser irradiation. Red light stimulation (via LED) of MCO-expressing GABAergic neurons of localized regions in ACC and spinal cord led to inhibition of inflammatory pain (measured by formalin test of licking/lifting) as well as reduction of chronic pain (assessed via paw withdrawal threshold using Von Frey assay). Conclusion: Non-viral laser delivery of MCO to neurons in the ascending /descending pain pathway has the potential to improve the efficacy of optogenetic modulation by localizing the gene expression at the site(s) of interest. The NOD method will also enable control on duration of gene expression and redosing while avoiding immune rejection and adverse events typical of virally delivered gene therapies.

Thursday Posters: Other Nonviral Delivery

1237. Lipid Nanoparticles Encapsulating FVIII Transgene as Re-Dosable and Durable Non-Viral Gene Therapy in Hemophilia A

Meng-Ni Fan, Xiaohe Cai, Ting-Yen Chao, Yuanyi Xu, Carol H. Miao

Seattle Children's Research Institute, Seattle, WA

Background: Current viral vector-based gene therapy has demonstrated success in achieving therapeutic coagulation factor levels in both hemophilia A and B. However, the application is restrained by potential risks and challenges arising from pre-existing neutralizing antibodies against these vectors. Additionally, post-therapy development of neutralizing antibodies poses hurdles for vector re-administration, crucial for maintaining adequate coagulation factor levels over time. Non-viral gene therapy utilizing lipid nanoparticles (LNPs) emerges as a promising strategy for treating hemophilia without the concerns of vector-neutralizing antibody formation hindering the therapeutic effects. **Goal:** Our goal is to establish non-viral gene therapy by using lipid nanoparticles to deliver FVIII plasmid with re-dosable capability to achieve stable and therapeutic levels of FVIII expression. **Method:** LNPs were composed of either a biodegradable cationic lipid (LipidA) or the FDA-approved lipid, MC3 as control for formula evaluation. These LNPs encapsulated plasmids comprising a CAG promoter and either secreted luciferase (LNP-scLuc) or FVIII-X10 variant (LNP-FVIII). Expression of scLuc and FVIII were evaluated in LNP injected C57BL/6 mice and HA immunodeficient mice (HA-NSG), respectively. Alanine transaminase (ALT) level in plasma was measured to evaluate hepatotoxicity. **Result:** Significantly higher scLuc expression was observed in mice receiving LipidA-LNP-scLuc compared to MC3-LNP-scLuc at days 1 and 7, showing transfection in 84.4% of hepatocytes in the liver. HA-NSG mice injected with LipidA-LNP-FVIII at doses of 0.5 μg/g (low dose) and 1 μg/g (high dose) exhibited approximately 11.8% and 23.0% FVIII activity at week 1, respectively. Re-administration of LipidA-LNP-FVIII resulted in peak FVIII expression around 39.1% two weeks after the second injection in the high-dose group. Sustained and detectable levels of FVIII expression were observed for more than 3 months in both high and low dose groups. Importantly, no significant elevation in plasma ALT levels was detected in LipidA-LNP-FVIII treated HA-NSG mice during the initial 2 weeks, suggesting the absence of apparent hepatotoxicity. **Conclusion:** Our results highlight the potential of LNPs as a non-viral approach for hemophilia A gene therapy, mitigating the risks linked with viral gene therapy. Moreover, the LNP platform allows re-administration strategies to sustain the therapeutic benefits.

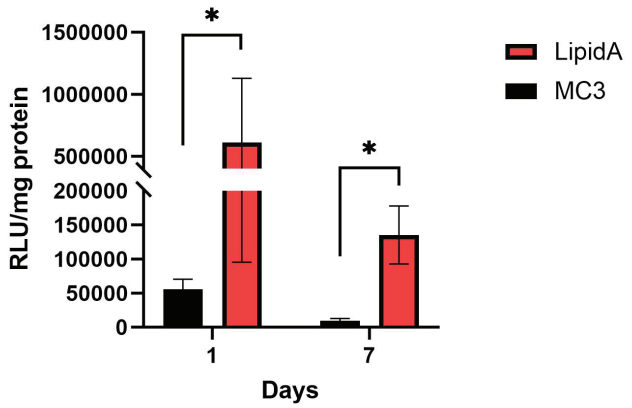


Figure 1. LNP formula comparison

C57BL/6 mice injected with LipidA-LNP-scLuc (shown in red) exhibited significantly elevated levels of scLuc expression in comparison to MC3-LNP-scLuc (shown in black) at both day 1 and day 7. Statistical difference was analyzed using Mann-Whitney test (*$p < 0.05$).

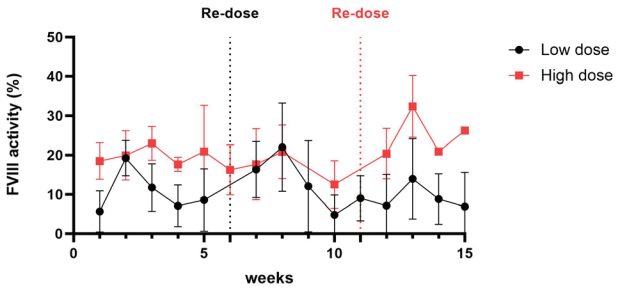


Figure 2. Plasma FVIII activity in HA-NSG mice administered with varied dosages of LipidA-LNP-FVIII

Plasma FVIII activity in HA-NSG mice receiving LipidA-LNP-FVIII at low dosage (0.5 μg/g, shown in black) or high dosage (1 μg/g, shown in red) was monitored over time. LipidA-LNP-FVIII was administered at week 0 and 6 for the low dosage group and at week 0 and 11 for the high dosage group.

1238. ARMMs as Non-Viral Vehicles for the Delivery of Therapeutic Payloads to Liver Sinusoidal Endothelial Cells

Ryan von Kleeck, Shu-Lin Liu, Carson Semple, Lucy Sun, Pearl Moharil, Wendy Zhao, Jennifer Badji, Emmanuel Appiah, Kevin Le, Steven Greenway, Qin Yu, Joseph Nabhan

Vesigen Therapeutics, Cambridge, MA

Viral and non-viral approaches including AAVs and LNPs have been used to deliver therapeutics to the liver. However, selective delivery of payloads to specific cell types, such as Liver Sinusoidal Endothelial Cells (LSEC), remains a challenge. We engineered a class of extracellular vesicles called ARMMs (ARRDC1 Mediated Microvesicles) to enable non-viral delivery of a wide range of payloads including RNA, proteins and gene editing protein/RNA complexes (Cas9/gRNA). To evaluate functional delivery of ARMMs to various liver cell populations *in vivo*, we intravenously administered ARMMs loaded with Cre recombinase protein to Ai14 reporter mice and evaluated downstream tdTomato reporter gene activation. We observed a high level of biodistribution of ARMMs to Kupffer Cells and LSECs with no Hepatocyte uptake. Furthermore, *in vivo* administration of ARMMs loaded with Cas9/gRNA showed notable gene editing efficiency in the LSEC population. These results suggest that delivering therapeutically-relevant payloads using ARMMs may be an effective approach to treat liver diseases involving the LSEC population, such as Hemophilia A.

1239. Optimizing Lipid Nanoparticle Formulations for Enhanced Non-Viral Gene Therapy: Overcoming DNA Delivery Challenges and Achieving High-Efficiency Transgene Integration

Hua Wang, Nicholas Cam, Alicia Davis, Khalid Hajj, Gopi Vemuri, Minghao Xu, Lijun Huang, Brian Truong, Sang Jun Lee, Johnnatan Tamayo, Eliana Estrada, Fernando Lopez Espinoza, Cindy Negron, Matt Nitzahn, Arturo Barcenas, James Clancy, Johannes Schwerk, Mu Li, Michael Bennett, Joshua Rychak

Poseida Therapeutics, San Diego, CA

Non-viral gene therapy technologies face significant challenges in achieving efficient gene correction or gene delivery. Despite recent advances with lipid nanoparticle technologies, safe and effective DNA delivery has proven very difficult. DNA is particularly challenging due to its unique physicochemical properties, its potent immunogenicity, its susceptibility to nuclease-mediated degradation in the cytosol, and the requirement of nuclear translocation. These challenges have been tackled through our focused optimization of DNA delivery to hepatocytes, in vivo, and genomic integration using the piggyBac® DNA insertion system and the super piggyBac transposase (SPB). We developed purpose-built lipid nanoparticle (LNP) formulations for efficient DNA encapsulation and delivery, using a design-of-experiment (DoE) approach. High-performing LNPs showed consistent physicochemical properties, but plasmid DNA (pDNA) required unique lipid-to-payload ratios, indicating a need for custom formulation. Exploring the role of ionizable lipids further, we created a library of 28 lipidoids combining common amine cores and novel terpene-based hydrophobes. While many lipids efficiently delivered mRNA, fewer were effective for pDNA. Further lipid design identified additional unique ionizable lipids with enhanced pDNA encapsulation and delivery capabilities. For hepatocyte targeting and immune cell avoidance, LNPs were modified with a GalNAc moiety, yielding dramatic decreases of IFN-gamma, liver transaminases (AST and ALT), and markers of biliary atresia (GGT). To address challenges unique to pDNA delivery and cellular trafficking, we evaluated the performance of LNPs in conjunction with a novel class of amphipathic compounds, with the hypothesis that such compounds would improve endosomal escape. We identified multiple compounds that provided a pronounced effect on LNPs encapsulating DNA plasmid reporters in HepG2 cells. An evaluation of one such compound yielded a nearly 2-log enhanced expression of a luciferase reporter, as measured by *in vivo* bioluminescence imaging. These LNP platform improvements were combined and applied to liver-directed gene therapies using the SPB transposase for transgene cargo integration. In a mouse model of Hemophilia A, characterized by a deficiency of FVIII, our optimized LNP-DNA formulations achieved FVIII levels much higher than conventional LNPs, reaching nearly 800% of normal. In another disease model, Arginase-1 deficiency, a urea cycle disorder that affects ammonia processing, the LNP platform yielded Arg1 expression in 20% of hepatocytes in juvenile mice, nearing the efficiency level of an AAV vector. In conclusion, DNA delivery requires unique LNP formulation parameters and optimized excipients for maximizing

cellular trafficking. Such optimization realizes the full potential of our non-viral DNA platform technology and piggyBac DNA insertion system for treating serious genetic disorders.

1240. Optimization of Ionizable Lipid Structure to Modulate ApoE Binding and Enable Stealth Lipid Nanoparticles

Daniele Vinciguerra, Douglas Rose, Sandy Serizier, Andrew Milstead, Nolan Gallagher, Viktoriya Syrovatkina, Ryan Delaney, Prudence Li, Di Bush, Matthew Stanton, Randall Toy

Generation Bio, Cambridge, MA

Lipid nanoparticles (LNPs) are the primary means of non-viral delivery of nucleic acid gene therapies. With the current generation of clinically validated LNP delivery systems, their efficacy is dependent on the activity of the serum apolipoprotein E (ApoE) (1). As an important fat transporter, ApoE binding may have a substantial impact on LNP circulation time and hepatic uptake. Therefore, modulating ApoE binding could be an important method by which hepatic LNP uptake can be circumvented, which is an important characteristic toward the development of stealthy, long-circulating LNPs. We hypothesized that ApoE binding is dependent on the LNP lipid composition and more specifically, on specific structural elements of the ionizable lipid. Starting from a parental ionizable lipid, we modified different chemical regions of the lipid and assessed the impact of these structural changes. To determine biologically-relevant ApoE binding, we leveraged the ApoE-HSPG-LDLR pathway, which enables liver-specific LNPs accumulation in hepatocytes (2) and developed a heparin affinity chromatographic method that allows for rank ordering of LNPs based on their ability to productively bind ApoE. Following serum incubation, LNPs with higher ApoE binding interact more effectively with the column, allowing for selection of ionizable lipids that do not effectively recruit ApoE. Specifically, we demonstrate that correct positioning of a degradable ester along the lipid tail can significantly change binding as compared to the parent ionizable lipid, whereas lengthening the ionizable lipid tails leads to a decrease in binding. Interestingly, rank ordering of lipid binding to the column changed when sera from different species was used, indicating that certain ionizable lipid structures may preferentially harvest species-specific ApoE isoforms. To directly assess the impact of modulating ApoE binding on uptake and expression in hepatocytes, we leveraged hepatocyte cell lines and measured uptake and expression using DiD-positive, GFP mRNA-containing LNPs. We found that following serum incubation, decreased ApoE binding is correlated to decreased hepatocyte uptake and expression. The properties of reduced ApoE binding and hepatocyte uptake, while maintaining endosomal escape are ideal for applications outside of the liver. Our stealth compositions that have been retargeted to other cell types such as T cells take advantage of ionizable lipid designs aimed at minimization of lipoprotein binding. (1) Akinc A. et al. Targeted Delivery of RNAi Therapeutics With Endogenous and Exogenous Ligand-Based Mechanisms. Mol. Ther. 2010, 18 (7), 1357 - 1364. (2) Sato Y. et al. Different kinetics for the hepatic uptake of lipid nanoparticles between the apolipoprotein E/low density lipoprotein receptor and the N-acetyl-Dgalactosamine/asialoglycoprotein receptor pathway. Journal of Controlled Release 2020, 10 (322), 217 - 226.

1241. Development of Cell-Targeted Lipid Nanoparticles Using Antibody Derived Ligands for Targeted Delivery to Hepatocytes and Immune Cells

Douglas Rose, Lalita Oonthonpan, Ryan Delaney, Nolan Gallagher, Andrew Milstead, Anthony Perniciaro, Luke Hamm, Matt Manganiello, Brandon Johnston, Di Bush, Anthony Brouillard, Pei-Ni Tsai, Nathaniel Silver, Connie Martin, Tiffany Tate, Raphael Gagne, Dan Garafola, Yao Xin, Thamara Desilva, Christian Slubowski, Matthew Stanton, Phillip Samayoa

Generation Bio, Cambridge, MA

The field of gene therapy has long sought a non-viral redosable delivery strategy that can reach both the liver and extra-hepatic cell populations. To that end, Generation Bio has developed a cell-targeted lipid nanoparticle (ctLNP) capable of delivering nucleic cargo to targeted cell populations. Our ctLNP comprises three primary components: a stealth LNP carrying a nucleic acid cargo, an optimized bioconjugation linker, and a modular targeting ligand that directs receptor-selective biodistribution of the ctLNP to the intended cell population. The stealth LNP is a key component of this technology and exhibits minimal protein corona formation and extended residence time in the blood, avoiding off-target clearance by liver and spleen and allowing for efficient, highly selective redirection to cells of interest. This profile may enable ctLNPs to target tissues and cell types that have been previously out of reach for genetic medicines. Our demonstration of cell-type-specific targeting began in the liver with the asialoglycoprotein receptor (ASGPR), which has been clinically validated as a target for delivery of small interfering RNA and anti-sense oligonucleotides to the liver. Most commonly, ASGPR is targeted using multivalent GalNAc ligands which have a high affinity for the multimeric ASGPR complex. Initial work with GalNAc targeting ligands on a ctLNP demonstrated delivery to hepatocytes. However, increasing levels of GalNAc led to increased delivery to Kupfer cells, a cell type that expresses CLEC10A (CD301), which contains a conserved carbohydrate recognition domain shared with ASGPR. To reduce off target delivery, we took a protein-based ligand approach and identified an antibody fragment that shows higher binding affinity towards ASGPR over CLEC10A, which were conjugated onto stealth LNPs. To maximize ctLNP performance, we optimized the ligand identity, bioconjugation linker chemistry, linker length, and ligand density and assessed their impact on the physicochemical properties and functional activity of the ctLNPs. We also evaluated conjugation process steps that influence reaction completion, avoid formation of degradation products, preserve LNP characteristics after conjugation, and remove unreacted materials. Interestingly, while high ligand densities typically lead to lower ctLNP stability, ligand density can be tuned to find the optimal amount of stability and functional activity. Additionally, the spatial orientation and local accessibility of the targeting ligands play vital roles in the functional activity of the ctLNP. We compared the performance of ctLNPs with multivalent GalNAc ligands against those conjugated to antibody-derived targeting ligands and observed enhanced delivery to hepatocytes both *in vivo* and *in vitro* when using our antibody-derived ligands. From this initial demonstration, we have built upon our ctLNP technology and demonstrated successful extrahepatic targeting of immune cells. Starting from the previously optimized conjugation conditions, we

further refined ligand conjugation for T-cell targeting and attached the most highly specific and high affinity T-cell receptor-targeted ligands to our stealth LNPs. The resulting ctLNPs were evaluated based on their ability to deliver a GFP-expressing mRNA cargo into T-cells. We demonstrated that ctLNPs targeting CD3 led to significant amounts of T-cell depletion while targeted delivery to other T-cell receptors of interest led to a dose-responsive level of GFP expression with minimal off target delivery to myeloid & B-cell populations.

1242. Development of AAV No-End DNA-Containing Lipid Nanoparticles for Improved Delivery and Transgene Expression

Porkizhi Arjunan[1,2], Jakob Shoti[1], Yamika Ramesh[1], Keyun Qing[1], Srujan Kumar Marepally[2], Arun Srivastava[1]

[1]University of Florida, Gainesville, FL,[2]Centre for Stem Cell Research, Vellore, India

We have described the development of an AAV DNA-based synthetic vector, termed no-end DNA (NE-DNA), which is devoid of AAV capsid proteins, and mediates sustained expression following transfection into human hepatic cells (*Frontiers in Microbiology*, 13: 1033615, 2022). However, since NE-DNA is unlikely to be efficacious *in vivo*, in the current studies, we synthesized a number of lipid nanoparticles (LNPs) - Lipo 1-Lipo 12, based on our previous observation that galactosylated lipids mediate delivery of nucleic acids to liver with higher precision (*Biomaterials*, 2009 30: 2369-2384, 2009). These LNPs were used to encapsulate linear and NE-DNAs containing firefly luciferase (FLuc) and enhanced green fluorescence protein (EGFP) reporter gene expression cassettes. The encapsulation productivity was characterized by gel-retardation and heparin-displacement assays, and the stability of encapsulated DNA was corroborated by DNase 1-sensitivity assay. Each of the LNPs was used to transduce a human endothelial cell line, SK-Hep-1 and a human hepatic cell line, Huh7. These results for FLuc expression are shown in the two cell lines in **Figure 1A** and **Figure 1B**, respectively. As can be seen, in both cell lines, the extent of transgene exprpression was significantly higher with most of the LNPs containing the NE-DNA than those containing the linear DNA, and Lipo 1 was found to be the most efficient. Cyotoxicity assays revealed >90% viability in both cell lines transduced with Lipo-1, and was used in all subsequent experiments. Similar results were obtained with Lipo 1-EGFP LNPs in both cell lines, which were similar to that with the commercially available cationic liposomes, Lipofectamine (LF) (**Figure 1C** and **Figure 1D**). The efficacy of Lipo 1-FLuc LNPs, containing linear and NE-DNAs is curently being evaluated in a mouse model *in vivo*. Experiments are also currently underway to encapsulate linear and NE-DNA containing human clotting factors (hFVIII and hFIX) under the control of a liver-specific promoter. The development of Lipo 1-NE-DNA presents a promising strategy for repeat administration of hFVIII and hFIX genes, for the potential gene therapy for hemophilia A and hemophilia B, respectively, in humans.

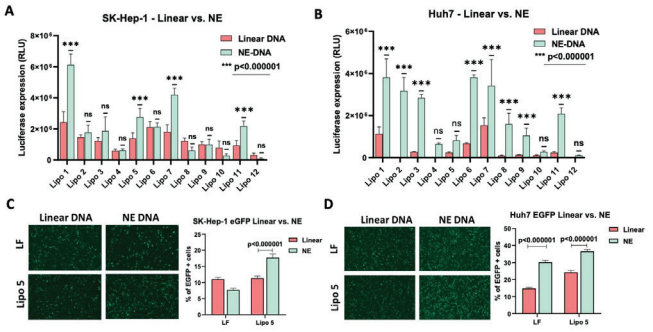


Figure 1: Firefly luciferase expression in human endothelial **(A)** and hepatic **(B)** cell lines mediated by LNPs containing linear- and NE-DNA. EGFP expression in human endothelial **(C)** and hepatic **(D)** cell lines mediated by Lipofectamine (LF) and Lipo 1 LNPs containing linear and NE-DNA. Transgene expression was determined 96 hrs post-transduction.

1243. Spatial Resolution of mRNA Lipid Nanoparticle-Based *In Vivo* Base Editing in the Liver of Mice and Monkeys

Thomas Chamberlain[1], Sharan Janjuha[2], Tatjana Haenggi[2], Kim Marquart[2], Merete Eisenhardt[1], Sean C. Semple[1], Gerald Schwank[2], Ying Tam[1]

[1]Acuitas Therapeutics Inc, Vancouver, BC, Canada,[2]Institute of Pharmacology and Toxicology, University of Zurich, Zurich, Switzerland

mRNA lipid nanoparticles (mRNA-LNP) enable effective delivery of gene editing systems following intravenous administration, particularly in the liver. This strategy offers several potential advantages compared to viral delivery approaches, including an improved safety profile (no likelihood of insertional mutagenesis, limited duration of expression), larger payload capacity, and the opportunity to repeat dose. In recent years, the mRNA-LNP platform has been utilized in preclinical and clinical studies to enable various gene editing tools, including CRISPR/Cas9 nucleases, zinc-finger nucleases, TALENs and base and epigenetic editors. However, to fully realize the potential of mRNA LNP delivered gene editors for treatment of diverse genetic diseases, it is important to rigorously assess, validate and optimize the in vivo activity of mRNA-LNP. For example, since there are differences in the properties and functions between hepatocytes in the liver (ie. pericentral and periportal), it is important to understand and quantify the relative editing that can be achieved in the different subpopulations of hepatocytes. To address this question, we adopted imaging-based in situ sequencing and quantified in vivo base editing rates in native liver tissues. Mice and non-human primates were treated with two doses of LNP encapsulating mRNA encoding base editors that installed A to G mutations at two different loci. The sequence of dosing (ie. first locus 1 and then locus 2 or vice versa) did not affect editing rates, and we frequently observed hepatocytes that were edited on both loci. Moreover, we found that base editing was similarly effective in both periportal and pericentral hepatocytes. These findings suggest that mRNA-LNP delivery of base editors following intravenous administration is a feasible approach for in vivo targeting of a wide spectrum of genetic liver diseases.

1244. Delivery of mRNA in Porcine Kidneys by Iontophoresis: A Promising Alternative Delivery Approach for Extra-Hepatic Genomic Medicine Delivery

Maria P. Torres-Gonzalez[1], Raquel Maeso-Diaz[1], Leigh Dement[1], Brendan Berkel[1], Justin Stegeman[1], Ahmed Hassan[2], Andrew S. Barbas[2], William Daunch[1]

[1]Research and Development, Focal Medical, Inc., Cary, NC,[2]Duke Department of Surgery, Duke University, Durham, NC

Among novel genomic medicines with promising potential, mRNA therapeutics are at the forefront for targeted treatments. However, delivering these molecules effectively to target cells without eliciting undesirable immune responses remains a significant challenge. The delivery of mRNA molecules by iontophoresis (IOP) represents a promising alternative to achieve targeted delivery to a specific organ, potentially minimizing off-target effects and extending therapeutic kinetics in comparison to standard delivery methods. The following study investigated the feasibility of IOP to deliver mRNA in a benchtop agarose model, *in-vitro* on porcine kidney cell lines, and *in-vivo* to porcine kidney tissue. The successful delivery of mRNA by IOP was first evaluated using a benchtop agarose gel model, in which numerous parameters were optimized including electrode type/material, electrode design/configuration, current, and treatment duration. After multiple iterations of these parameters, the optimum delivery of mRNA mimicking a kidney cortex delivery depth was obtained at 5mA treatment for 90min (Figure 1A). The transfection efficiency of IOP delivery of liposome-encapsulated EGFP-mRNA was optimized on porcine kidney cell lines embedded in 3D bioprinted GelMA Alginate hydrogel models. Representative microscopy data clearly shows that IOP-treated cells expressed fluorescence (i.e. EGFP) as opposed to cells treated by diffusion alone after 24hr of treatment (Figure 1B), which was further confirmed by One-step-RT-PCR (data not shown). Collectively, results obtained from these tissue surrogate models' experiments were used to systematically design *in-vivo* studies to deliver encapsulated mRNA by IOP directly to kidney tissue. Briefly, a prototype iontophoretic device was placed directly on the kidney of anesthetized Yorkshire female pigs and IOP treatment was administered at 5mA for 90min. After 24hr recovery, and following IACUC guidelines, necropsy was executed, and kidney tissue biopsies were collected and preserved for subsequent analysis. Overall animal health was excellent and no adverse kidney tissue/systemic toxicity reactions were observed. In agreement with results from tissue surrogate models, EGFP-mRNA expression was significantly upregulated in porcine kidney cortex biopsies when compared to passive diffusion delivery (Figure 1C), which was further confirmed by fluorescence microscopy (data not shown). Altogether these results validated the usefulness of surrogate tissue models used in the study by demonstrating that the device configuration selected, and the treatment conditions identified in these, were indeed mimicking *in-vivo* kidney delivery of mRNA, including tissue depth, coverage and transfection efficiency. Overall, the efficient delivery of functional mRNA by IOP was demonstrated across different models evaluated on this study. Potential benefits from this novel delivery platform include precise delivery, lower dose administered, greater efficacy, and minimized toxicity. Altogether, taking into consideration that extra-hepatic delivery of mRNA is desired to treat a vast array of common and rare, monogenic, and polygenic diseases, IOP technology represents a promising alternative to viral and non-viral vector delivery approaches to a wide range of solid organs other than the liver.

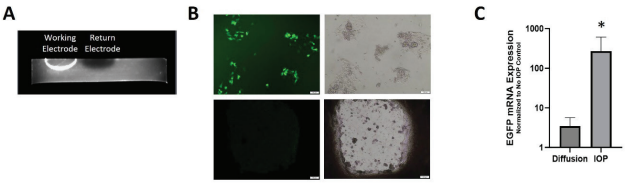


Figure 1. Delivery of EGFP-mRNA by IOP (A) mRNA delivery by IOP on benchtop agarose gel model with Ethidium bromide; (B) Fluorescence/phase contrast microscopy images of SKRT cells after 24hr of IOP treatment (top) and diffusion control (bottom); (C) EGFP-mRNA expression of porcine kidney cortex treated by IOP versus Diffusion control (* *p-value* < 0.05)

1245. Efficient Gene Editing in CD34+ Hematopoietic Stem and Progenitor Cells Using Non-Viral Lipid Nanoparticles

Aruna Balgi, Stella Park, Rita Zhao, Meenakshi Swaminathan, Nikita Jain, Anitha Thomas, Samuel Clarke, Angela Zhang, Reka Geczy

Cytiva, Vancouver, BC, Canada

Background and Aims: CRISPR-Cas9 gene knockouts in CD34+ hematopoietic stem and progenitor (HSPC) cells is emerging as a transformative tool for the correction of mutations that cause genetic blood diseases. This study expands on previous work, where we used a novel lipid nanoparticle (LNP) reagent for the multiplex gene editing of CAR T cells, yielding high viability and efficient editing. In this work, we explore the use of LNPs for the efficient CRISPR-Cas9 editing of CD34+ HSPCs, highlighting the vast potential of LNPs for pioneering gene therapeutics. **Methods:** LNPs encapsulating S.p. Cas9 mRNA and sgRNAs targeting CD33 or CD45 were formulated using the NxGen mixing platform allowing for scalable LNP production. The LNP composition was optimized for RNA delivery to HSC/HSPCs. Frozen, isolated human CD34+ cells were stimulated and RNA-LNPs directly added to the cells. Cells from various sources, such as mobilized peripheral, bone marrow and cord blood were evaluated. Further, various cell culture conditions, such as treatment kinetics, media and supplements were evaluated and optimized for efficient LNP delivery. Gene editing efficiency and cell viability was assessed via flow cytometry, proliferation using an automated counter, and differentiation potential through CFU assays. **Results:** Direct LNP addition to CD34+ HSPCs achieved an average of 84 ± 6% of CD33 and 81 ± 2% of CD45 knockout efficiencies in six separate experiments. Post-treatment, the cells retained an average of 95 ± 3% viability. The LNP-treated HSPCs showed strong cell proliferation, maintaining >90% proliferation relative to untreated controls. The CFU assays indicated unchanged lineage formation in both RNA-LNP and empty LNP controls. Additionally, the study successfully scaled up LNP production from research to pre-clinical scales using the microfluidic technology. **Conclusions:** This study demonstrates the potential of LNP-mediated CRISPR-Cas9 mRNA delivery as a promising approach for gene editing in HSPCs. The simple and gentle nature of LNP cell treatment allows for multiple genetic alterations for simultaneous expression and deletion of proteins paving the way for innovative gene therapies. The scalability of LNPs, enabled by novel mixing technology, permits efficient RNA library screening at small scales, and facilitates swift transition to large-scale clinical applications.

1246. Fractionation of Distinct Extracellular Vesicle Populations by Charge and Density to Evaluate the Heterogeneity and Purity of ARMMs

Kristin Luther, Adam Francouer, Leah Gens, Steven Greenway, Alysia Bryant, Ishaan Tiwari, Zachary Shelnitz, Gary Hao, Joseph Nabhan
Vesigen Therapeutics, Cambridge, MA

The class of extracellular vesicles (EVs) known as ARMMs (ARRDC1 mediated microvesicles) has recently been established as a platform for delivery of therapeutic proteins such as Cas9/gRNA complexes to specific tissues in vivo. This is accomplished by expressing the therapeutic protein fused with ARRDC1, leading to its incorporation in ARMMs. However, little is known about the physicochemical characteristics of ARMMs and how they differ from other types of extracellular vesicles, which can be concomitantly produced during upstream culture. Understanding this may enable us to achieve better separation of ARMMs from other vesicles, as well as provide insights into the unique properties of individual subclasses of EV. We used anion exchange and density gradient ultracentrifugation to fractionate crude EV preparations. We found that vesicles eluted in two distinct peaks during anion exchange (AEX), while during density gradient separation, they distributed over a range of densities from 1.02-1.11 g/cm3. Next we subjected the particles from both AEX peaks to density gradient separation. Both AEX peaks contained ARMMs, but the first peak had lower payloading, a less negative zeta potential, and lower gene editing activity than the second peak. With density gradient separation, the vesicles gradually became smaller and more negatively charged as the density increased until reaching 1.11 g/cm3, and the number of ARRDC1 molecules per particle increased. This increase was associated with enhanced gene editing potency in vitro and in vivo. Another population of particles was detected at a density of 1.17 g/cm3 that were of similar size to the vesicles, but had neutral zeta potential, and low expression of vesicle markers or ARRDC1. Taken together, the data suggest that ARMMs are smaller, denser, and more negatively charged than other types of EVs secreted by the current producer cells, and that combining purification methods can lead to higher enrichment of ARMMs in our product.

1247. ARMMs as a Versatile and Modular Non-Viral Platform for the Functional Delivery of Genome Editors

Qiyu Wang, Wendy Zhao, Mike Thomas, Shu-Lin Liu, Lucy Sun, Ryan von Kleeck, Zahra Baghbantaraghdari, Chou-Wei Chang, Jennifer A. Badji, Komal Vyas, Qin Yu, Joseph F. Nabhan
Vesigen Therapeutics, Cambridge, MA

An expanding array of genome editing tools and their derivatives are broadening the scope of potential therapeutic applications. However, a substantial majority of these applications face challenges in efficiently delivering gene editing technologies to tissues and cell types affected by diseases without inducing any toxicity. The primary obstacle lies in identifying non-viral carriers capable of ensuring safe, transient, and highly efficient functional delivery. In response to this challenge, we have developed ARMMs (ARrestin-domain 1 Mediated Microvesicles) as a targeted and re-dosable therapeutic platform for delivery of genome editors. This study details the successful loading of ARMMs with Cas9, ABE (Adenine base editor), and CBE (Cytidine base editors) complexed with guide RNA (gRNA) as fully functional ribonucleoproteins (RNPs) within ARMMs. We assess the functional delivery of these loaded payloads in vitro across various cell types, including post-mitotic cells. Moreover, our investigation extends to in vivo genome editing, revealing remarkably high levels, exceeding 60% in certain instances. Our data showcase robust editing of druggable or undruggable immune modulation targets, such as NLRP3 or IRF5, in both alveolar macrophages and Kupffer cells in mice and non-human primates (NHP). Importantly, in macrophages we demonstrate a significant reduction in downstream pro-inflammatory signals when we disrupt expression of NLRP3 or IRF5 using ARMMs. Collectively, our findings suggest that ARMMs serve as a versatile and modular non-viral platform for the functional delivery of genome editors, presenting a promising avenue for disease modification.

1248. Engineering ARMMs with Modular T Cell-Specific Engagers for Non-Viral Delivery of Therapeutic Payloads

Wendy Zhao, Lucy Sun, Qiyu Wang, Pearl Moharil, Mike Thomas, Silvia Piccinotti, Zahra Baghbantaraghdari, Chou-Wei Chang, Qin Yu, Joseph F. Nabhan
Vesigen, Cambridge, MA

Intracellular delivery of therapeutic payloads, such as RNA, protein, or genome editors, holds the key to treating a wide range of diseases. A major hurdle for payload delivery is the limited biodistribution of existing delivery vehicles to target cell-types. Overcoming this major hurdle, which involves identifying a versatile vehicle conducive to safe and functional delivery of these payloads to specific cell types through various administration routes, has been challenging. In this study, we engineered membranes of ARRDC1 mediated microvesicles (ARMMs) to display anti-CD8 or CD4 antibodies that interact with specific T cell populations. This approach demonstrated the ability to specifically retarget these vehicles to CD8+ or CD4+ T cells in in vitro cell lines and ex vivo primary cells. We also demonstrated targeted functional delivery of gene editing complexes to specific T cells in ex vivo primary cells, as well as robust detargeting of cells not expressing the target receptors. This demonstrated the adaptability of ARMMs to deliver therapeutic payloads to specific immune cell types and suggests that it may be possible to envision in vivo T-cell based therapeutic strategies.

1249. Engineering Directed Tropism in the Non-Viral ARMMs Delivery Platform

Silvia Piccinotti, Mike Thomas, Wendy Zhao, Nelly Valkov, Patrick Schupp, Qiyu Wang, Lucy Sun, Shu-Lin Liu, Zahra Baghbantaraghdari, Chou-Wei Chang, Hongru Zhou, Qin Yu, Joseph Nabhan
Vesigen Therapeutics, Cambridge, MA

Therapeutic macromolecules such as RNA, proteins and genome editors are poised to bring novel treatments for diseases with high unmet need. A persistent hurdle for the development of intracellular

large molecule therapeutics is their dependence on delivery vehicles with often limited biodistribution to introduce them into target cell-types. The biodistribution of adeno-associated viral (AAV) vectors and lipid nanoparticles remains largely limited to the liver and a few other tissue types. No major advances have been made to identify or engineer novel AAVs or to modify the tropism of LNPs. Therefore, many medically important tissues remain inaccessible. Pre-existing and emerging immunity to AAV and LNP components from natural or widespread vaccine exposure further limit the efficaciousness of these carriers. New delivery platforms amenable to tissue tropism engineering are crucial to sustain the development of macromolecular therapies. We report ARRDC1-mediated microvesicles (ARMMs) as a non-viral delivery platform with robust surface customization potential. We demonstrate that ARMMs can incorporate high densities of plasma membrane and endosomal fusogens as well as chimeric surface proteins engineered to bind specific cellular receptors. By altering surface binders, we have engineered ARMMs that selectively target cell types of interest in mixed in vitro and ex vivo cell cultures. These results suggest that ARMMs can be engineered to deliver therapeutic payloads to a variety of target tissues and cell types.

1250. An *In Vitro* Screening of Lipidnanoparticle (LNP) Formulations to Assess Intrinsic Cellular Targeting for Non-Viral Delivery

Anna Potenski[1], Gabriel Gamber[2], Lakshmi Raj[2], Hon-Ren Huang[3]

[1]Biologics Research Center, Novartis, Cambridge, MA,[2]Novartis, Cambridge, MA,[3]Novartis Pharma AG, Lexington, MA

Lipid nanoparticles (LNPs) have emerged as a promising non-viral delivery modality for cell and gene therapies due to their adaptability and chemical customizability. While there have been commercially successful uses of LNPs for vaccines and liver-targeted siRNA drugs, targeted delivery to extrahepatic tissues remains a challenge to expanding the development of this modality, and there is significant interest in understanding the inherent targeting capabilities of different LNP formulations. To understand the intrinsic cellular targeting preferences of different LNP formulations, we executed an *in vitro* screen of LNP formulations in multiple different cell lines, including primary cells. By utilizing GFP mRNA as the delivery cargo, we were able to use Incucyte imaging to track which LNPs are able to successfully transfect each cell type, as well as follow the kinetics of delivery and protein expression for each formulation. A screen like this has not been done before in this capacity and the data obtained provides a fingerprint of the cell type specificity of each formulation, which could identify lead LNPs for further development in extrahepatic indications.

1251. Streamlining Antibody Lead Generation: An Integrated Prioritization and Production Solution

Crystal Richardson[1], Ilaria DeVito[1], Anthony Moore[1], Andrea O'Hara[1], Zain Alvi[1], Pranay Vishwanath[1], Brian Sereni[1], Suhani Nagpal[2], Amy Migliori[2], M. Frank Erasmus[3], Laura Spector[3], Katheryn Perea-Schmittle[3], Michael Karney[1], Laure Turner[1], Sumit Kumar[1]

[1]Azenta Life Sciences, Burlington, MA,[2]OpenEye, Cadence Molecular Sciences, Santa Fe, NM,[3]Specifica, Santa Fe, NM

Harnessing the specificity and versatility of antibodies for cell and gene therapies as a delivery vehicle offers a transformative advance for precision medicines. From their ability for targeted delivery of therapeutic payloads to modulation of cellular interactions, antibodies hold immense potential in refining the precision, efficacy, and safety of future therapies. However, finding the right antibody candidate can be laborious and inefficient. Herein, we introduce an advanced and comprehensive solution to quickly identify and produce antibody leads for cell and gene therapy. This method involves the use of next-generation sequencing of outputs derived from both *in vivo* (i.e., B-cells and PBMCs) and *in vitro* (i.e., phage display) discovery campaigns. We pair this with an advanced bioinformatics platform that leverages machine learning to optimize the selection of potential antibody candidates. With this approach, it uncovered 5 - 50x more of the population diversity compared to traditional methods (e.g., random colony screening with Sanger). While next-generation sequencing expands the coverage of the underlying sequence diversity, top candidates are prioritizes using machine learning, selecting diverse leads from distinct clusters and prioritizing leads within clusters that have a reduced number of sequence-based liabilities. Once candidates are selected, they can be efficiently produced because we optimized expression vectors for high production rates. Thus, resulting in a quick and effective solution to generate candidates for vehicles in cell and gene therapy. In summary, we present our advanced end-to-end solution that uses next-generation sequencing, machine learning-based lead prioritization, and optimized antibody expression. Taken together, this platform consistently elevates the number of diverse leads and helps to uncover rare clones with favorable biophysical properties.

1252. Hermes™ Lipopolyplex Nanoparticles for Linear hpDNA™ Delivery

Emily Young, Amine Bouchareb, Gözde Tezcan, Matthew Walker, Hassan Idris, Amy Walker

4basebio, Cambridge, United Kingdom

Non-viral mRNA vaccines have been hugely successful, but instability of mRNA payloads and cold chain storage requirements remain a challenge. DNA is known to be more stable than mRNA. 4basebio's hpDNA™ is synthesised with a size and sequence independent fully enzymatic process, enabling GMP gram scale manufacture. Here, Hermes™ nanoparticles were optimised for hpDNA delivery. These formulations employ both lipid components & cationic ligands to drive payload encapsulation and stability in the liquid state. First, a one-step microfluidics method for combining lipids, ligand & hpDNA was developed. Notably, lipids and ratios used in conventional mRNA lipid nanoparticles (LNPs) precipitated following dialysis in PBS.

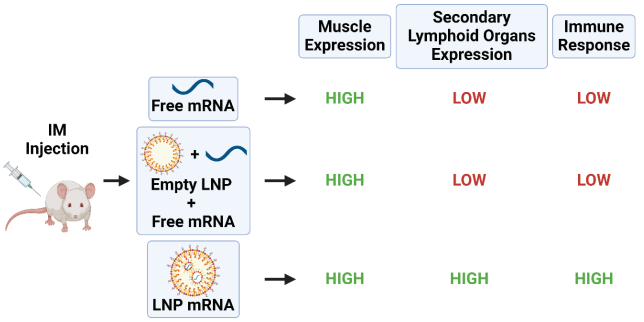
Using DOE, lipid & ligand molar ratios were optimised for hpDNA encapsulation. Including a fifth lipid prevented precipitation and had favourable biophysical characteristics and *in vitro* transfection efficiency. Bioluminescent imaging in mice noted luciferase expression >1-log higher with the optimised formulation than a commercial LNP control, and hpDNA™ formulations outperformed those encapsulating plasmid DNA. Finally, storage stability at 4°C of the optimised formulation was assessed. Hermes™ formulations including ligand maintained their size, PDI and encapsulation over the 28-day duration, whereas conventional LNPs displayed a ~40% drop in encapsulation efficiency. This enhanced stability at 4°C promises to overcome the cold chain issues of conventional LNPs. Combined with hpDNA™ stability and improved *in vivo* delivery, Hermes™ is ideally suited to both infectious disease and personalised medicine applications.

1253. The Main Driver of Immune Response to Lipid Nanoparticle-mRNA Vaccines after Intramuscular Injection is the Delivery and Expression of mRNA in the Secondary Lymphoid Organs

Asuka Takanashi

Drug Delivery Disposition and Dynamics (D4), Monash University, Parkville, Australia

Lipid nanoparticles, known as LNPs, have been the primary delivery system used in mRNA vaccines. Past studies have proposed the innate reactogenicity of LNPs to be responsible for producing the vaccine's adaptive immune response. However, it has not been clear whether the main drive for adaptive immune response is due to the adjuvant properties of LNPs in the muscle or the delivery of the formulation to the secondary lymphatic organs. To address this problem, we have formulated mRNA encoding either reporter gene (NLuc), OVA, or SARS-CoV-2 spike protein into LNP or coadministered the mRNA with empty LNP. We injected the formulations via intramuscular administration to correlate the delivery of the formulation with the adaptive immune responses. Compared to the unformulated mRNA or co-administered empty LNPs, high levels of nanoluciferase proteins were detected in the secondary lymphoid organs from LNP-formulated mRNA. Likewise, the LNP-mRNA encoding OVA showed the highest cellular immune response, while the unformulated mRNA or co-administered empty LNPs showed little to no response. Furthermore, only mice vaccinated with LNP-formulated mRNA encoding SARS-CoV-2 spike protein elicited robust cellular and humoral immune responses. Our results suggest that the delivery and the transfection of mRNA in the secondary lymphoid organs are the primary drivers for producing adaptive immune responses in mice. This study will help us design an mRNA delivery system that targets the secondary lymphoid organs for a better vaccine response.



1254. Comprehensive Characterization and Quantitation of Lipid Nanoparticle-Based RNA Formulations

William Penny, Michelle Chen

Waters | Wyatt Technology, Goleta, CA

The therapeutic potential of lipid nanoparticles (LNPs) as delivery vehicles has been demonstrated in recent years cumulating in the current emergency use of the mRNA based SARS-CoV-2 vaccines. In order to ensure the safety and efficacy of the LNP-RNA vaccine or therapeutics, various quality attributes of LNP-RNA products need to be measured throughput the product development cycle. In this poster, we will demonstrate the use of a dynamics light scattering (DLS) Plate Reader for fast screening and quality control of the LNP preparations, and multi-angle light scattering (MALS) combined with ultraviolet (UV) and refractive index (dRI) detectors following size exclusion chromatography (SEC) or field flow fractionation (FFF) separation for in-depth characterization. SEC or FFF provides sized based separation, and MALS, UV, and dRI detectors, on the other hand, enables biophysical characterization. We will show the data from the online detectors for the quantitation of particle size distribution, particle concentration, molecular weights of RNA and lipids, and sized-based RNA payload distribution of the LNP-RNA samples.

1255. An Innovative Cationic Lipid Library for Efficient and Tunable mRNA-LNPs

Quentin Huaulme[1], Margaux Briand[1], Kassandra Renaud[1], Eva Thiry[1], Malik Hellal[2], Claire Gueguen[2], Thibaut Ben Chimol[1], Melodie Seiler[1], Morgan Ziesel[1], Patrick Erbacher[1]

[1]Polyplus-Transfection, Illkirch Graffenstaden, France,[2]Polyplus-Transfection, Illkirch, France

Lipid nanoparticles (LNP) have demonstrated high efficiency in delivering RNA therapeutics *in vivo*. However, the properties of such nanoparticles obtained with conventional ionizable lipids are often difficult to modulate, and especially their biodistribution profile. These ionizable lipid-based nanoparticles often predominantly end up targeting the liver. One of the current challenges in the field consists of adjusting the chemical composition of the LNPs to the intended application. Here, we characterized a library of 10 innovative imidazolium-based cationic lipids as key component of cationic LNP (cLNP). We disclose their chemical structures and demonstrate

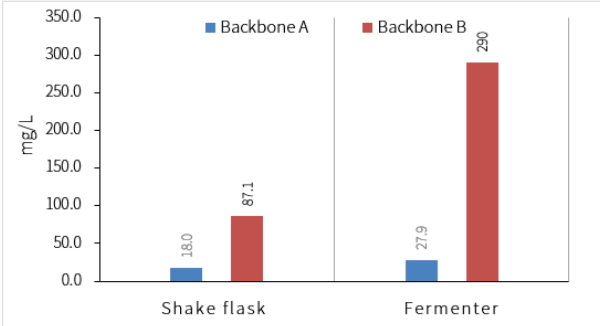
their efficacy in generating LNPs through characterization of their hydrodynamic diameters, Zeta potentials, and mRNA encapsulation efficiencies. The resulting particles display high transfection efficiencies and have little to no impact on cell viability *in vitro*, on HEK293 and CaCo-2 cell lines. *In vivo*, the biodistribution of the cLNP highly depends on the cationic lipid chemical structures, targeting mainly lungs and spleen. Among this library, we have also identified one cationic lipid as a potent additive in Moderna's Spikevax formulation.

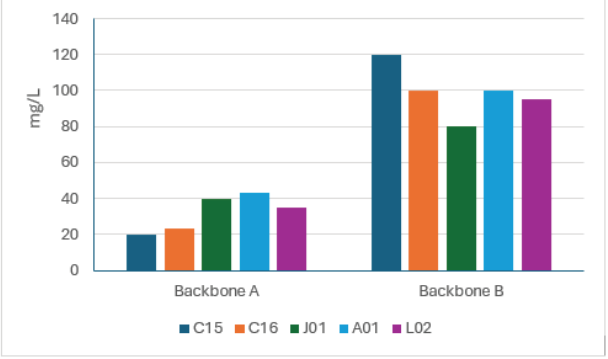
1256. Optimization of Vector Backbone for Increased Yield of Plasmid DNA for mRNA Generation

Guoze Chen

OBiO Technology (Shanghai) Corp., Ltd., Shanghai, China

The sequence elements of mRNA plasmids generally include resistance genes, replication initiation sites, and the GOI (Gene of Interest). The antibiotic resistance in plasmids is typically provided by kanamycin resistance gene to comply with regulatory requirements. Plasmid vectors used for mRNA template or virus packaging are usually high-copy-number plasmids including pUC, pBluescript, and pTZ vectors, typically with pMB1 or CoIE1 as the replication initiation sites. Using a commercial plasmid backbone to construct recombinant plasmids, followed by transformation into E. coli competent cells, and fermentation, we observed that significant differences in fermentation yield occurred if the same GOI gene was cloned into plasmid with different backbone. In a case study, the GOI of code C03 was constructed on backbone A and backbone B, respectively. After transformation into stbl3 competent cells, the cultivation of single clone in shake flasks at 37°C for 12 hours generated a different yield of 18 mg/L for plasmid with background A and 87 mg/L for plasmid with backbone. Moreover, fermentation of the same stain in a fermenter under similar conditions (37°C, 24h), the yields were 27.9 mg/L for plasmid with backbone A and 290 mg/L for plasmid with backbone B. This result indicates that changing the plasmid backbone can significantly increase the fermentation yield by approximately 5 fold in shake flasks and more than 10 fold in fermenters (see Figure 1). Figure1. Shake flask and fermenter yields of Plasmid C03 in backbone A and B. To further demonstrate whether the yield differences caused by plasmid backbones are universal, more Genes of Interest (GOI) were separately constructed into Backbone A and Backbone B, and subjected to shake flask cultivation under the same conditions. The yield of shake flask cultivation is shown in Figure 2. These results further confirm the impact of plasmid backbone on the fermentation yield of those plasmids used as mRNA template. Therefore, when constructing recombinant plasmids, it is essential to compare and optimize the plasmid backbone sequence in order to obtain a plasmid with excellent fermentation yield and superior quality attributes for construction the RCB of bacteria strain. Figure2. The plasmid yields from shake flask cultivation after cloning various GOI into backbone A and backbone B





1257. Novel Ionizable Lipids Derived from Biorenewable Sources Efficiently Deliver Candidate Vaccine mRNA *In Vivo*

Dylan Kairuz[1], Robin Klintworth[2], Aneesa Gani[2], Connor Stockly[2], Abdullah Ely[3], Kristie Bloom[4], Charles de Koning[5], Patrick B. Arbuthnot[4]

[1]University of the Witwatersrand, Johannesburg, South Africa,[2]School of Chemistry, University of the Witwatersrand, Infectious Diseases and Oncology Research Institute, Johannesburg, South Africa,[3]University of the Witwatersrand, Infectious Diseases and Oncology Research Institute, Johannesburg, South Africa,[4]Antiviral Gene Therapy Research Unit, University of the Witwatersrand, Infectious Diseases and Oncology Research Institute, Johannesburg, South Africa,[5]School of Chemistry, University of the Witwatersrand, Johannesburg, South Africa

Lipid nanoparticles (LNPs) are an important class of synthetic nucleic acid vectors. They demonstrated excellent properties as carriers of SARS-CoV-2 spike protein-encoding mRNA, and LNP-mRNA vaccines were vital to counter the COVID-19 pandemic. Nevertheless, clinical translation of LNP-mRNA therapies and vaccines is largely dependent on conveniently scalable and affordable production of the vectors. Traditionally four-component LNPs comprise an ionizable lipid, cholesterol, phospholipid and PEG-modified lipid. The ionizable lipid is an essential constituent that is required for stable particle assembly, cellular uptake and endosomal mRNA release. Currently available ionizable lipids are costly, difficult to prepare and derived from petroleum industry products. To address these limitations, we developed efficient methods for generating ionizable lipids from bio-renewable sources. Components of cashew nut shell liquid (CNSL)

were used as the primary supply of ionizable lipid building blocks. Useful features of CSNL are: 1) unlike other natural oils, CNSL contains only three related lipids, (cardol, cardanol and anacardic acid) which simplifies precursor purification, 2) CNSL is an abundant inexpensive agricultural waste product, and 3) the source is bio-renewable. Hydrogenated cardanol, which comprises a 14-carbon unsaturated lipid chain linked to a benzene ring, was purified from CNSL. A panel of ionizable derivatives of this compound was prepared with side groups on 1,2,4 or 1,3,5 positions of the benzene ring. To vary lipid shapes, different branched and unbranched side chains were added. Ionizable N atoms were included as part of the side chains or within coupled piperazine groups. This enabled pKas to be adjusted and provided further structural variation. Ester bonds were added to enhance metabolic degradation. Synthesis steps were high-yielding, and chromatographic purification was only necessary for the final product, which is useful for scalable synthesis. Four-component novel mRNA-containing LNP formulations were assembled using microfluidics. Encapsulation efficiencies of more than 90% were routinely achieved. Excellent particle diameters, typically approximately 80 nm with polydispersity indices less than 0.3, were consistently obtained. Injection of formulations containing lead ionizable lipids showed good reporter gene expression in vivo after intramuscular or intradermal injection (Figure 1), without evidence of toxicity. Immunogenic mRNA formulations encoding SARS-CoV-2 and *Mycobacterium tuberculosis* antigens are currently being formulated to evaluate immunogenicity and suitability for vaccines. Data from this work, which are being prepared for publication, lay the foundation for advancing a new class of LNPs with diverse applications. Low-cost and bio-renewable properties are unique features that address some of the limitations of current LNP technology.

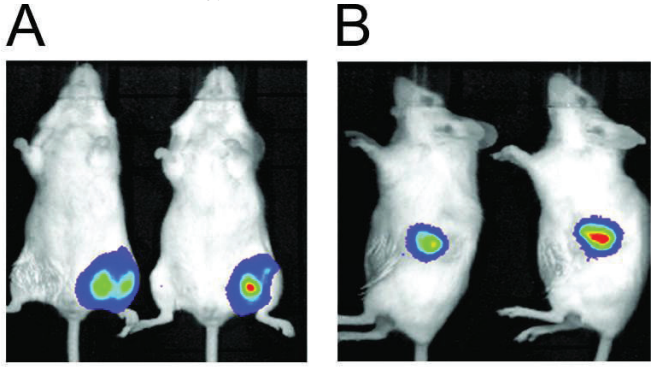


Figure 1. Representative example of Firefly luciferase (FLuc) expression in vivo following (A) IM and (B) intradermal injection of a four-component Fluc mRNA-LNP containing a CNSL-derived ionizable lipid. Imaging of mice was carried out 6 hours after injection.

1258. Strategies for Producing Clinical and Commercial RNA-LNP Drug Products

Samuel Clarke, Ian Johnston, Ashley Braun, Robert Young, Maria Kerin, Deepkanwal Singh, Braeden McDougall, Brian Ma, Anastasia Lazic, Leanna Yee, Curtis Robin, Felix Yuen, Pierrot Harvie, Suraj Abraham

Cytiva, Vancouver, BC, Canada

BACKGROUND The RNA-lipid nanoparticle (LNP) vaccines for the SARS-CoV-2 pandemic highlight the impact of genomic medicines deployed at scale. RNA-LNP applications also include gene expression and gene editing, which have utility for in vivo therapeutics for rare disease, and ex vivo engineering of cell therapies for cancer. Despite growing momentum, developing RNA-LNPs still faces significant manufacturing challenges. The mixing process to encapsulate RNA within LNPs is among the most difficult unit operations to scale-up to commercial throughput rates and batch sizes, while maintaining critical quality attributes (CQAs) such as size and biological potency. **METHODS** We have developed the new NanoAssemblr® commercial formulation system (CFS) for commercial-scale production of RNA-LNPs. This system utilizes the same NxGen™ mixing technology across the complete NanoAssembr suite of instrumentation to enable rapid scale-up and production. To demonstrate the scale-up capability of the suite, we produced and characterized a SARS-CoV-2 saRNA-LNP vaccine candidate under increasing flow rates and batch volumes to mimic the drug development process from discovery through to commercial production. The formulation flow rate was stepwise increased from 12 mL/min using the NanoAssemblr® Ignite+™ through to 800 mL/min using the new NanoAssemblr CFS. The LNP quality was evaluated by measuring size, polydispersity index (PDI), nucleic acid encapsulation efficiency, saRNA integrity, in vitro expression, and in vivo expression. **RESULTS** The saRNA-LNPs were of similar quality with sizes ranging from 61 - 83 nm, PDI < 0.18 and RNA encapsulation efficiency > 94%. ANOVA testing showed no significant differences in size, PDI, or encapsulation efficiency when comparing instruments or mixers. In vitro potency assays showed similar dose response curves between samples and consistent EC50 values between 1.3 to 3.6 ng/mL. Finally, in vivo immunization studies showed a robust SARS-CoV-2 specific IgG response in all instrument-mixer combinations with titers varying between 1.94x105 to 2.83x106 ng/mL. **CONCLUSIONS** This work demonstrates that production of nucleic acid-LNPs with consistent CQAs can be achieved on a wide range of scales using NanoAssemblr instruments and NxGen mixers. The NanoAssemblr CFS can prepare RNA-LNPs at the scale and throughput needed to meet commercial manufacturing goals for range of applications including vaccines, in vivo therapeutics, and ex vivo cell therapies.

1259. Elucidating the Mechanism in Microfluidics-Enabled Lipid Nanoparticle Dialysis Process via Single-Particle Cylindrical Illumination Confocal Spectroscopy

Fangchi Shao

Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD

Introduction Lipid Nanoparticles (LNPs) have emerged as highly efficient carriers for delivering mRNA-based vaccines and therapeutics. After the formulation of aqueous mRNA solution with an alcohol solution of lipids, dialysis is employed as a purification method to remove ethanol from the medium and exchange it with a physiological PBS buffer, rendering LNPs suitable for cell culture and *in vivo* administration [1]. Current dialysis approaches typically rely on dialysis membranes to enable the diffusion of buffer exchange, which can entail a lengthy process and add the risk of composition drift. Microfluidics as an emerging platform presents a promising alternative for LNP dialysis for precise fluidic exchange [2]. Here we develop a microfluidics-enabled LNP dialysis process and present a mechanistic understanding of the effect of medium component (salt, pH, and polarity) on LNP quality and transfection activity. **Materials and Methods** In this study, LNPs were formulated using T-junction. Dialysis was achieved in microfluidics with a flow rate ratio of 1:24 for LNP solution to PBS. Multi-color cylindrical illumination confocal spectroscopy (CICS) analyzed single-particle fluorescence burst quantification, enabling particle classification and mRNA payload characterization [3,4]. **Results and Discussion** Cy-5 mRNA and TopFluor-PC aided LNP categorization into three groups: empty, loaded, and free mRNA, corresponding to TopFluor-PC signal only, Cy-5 signal only, and coincident signals (Fig. 1A&B). Using a microfluidics system for on-chip dialysis (Fig. 1C), we compared its performance with a standard 18-h dialysis. Despite comparable sizes, slightly reduced empty particle fraction and increased the average payload per particle, microfluidic-based purification exhibited a lower polydispersity index (PDI) and a >10-fold increase in transfection efficiency (Fig. 1D). Additionally, increasing pH from 4.0 to 7.4, while holding other solvent conditions constant led to larger sizes but lower PDIs (Fig. 1E). Our data supports a mechanistic framework suggesting that ionizable lipids remain positively charged in acidic conditions, reducing merging events. At pH 7.4, neutral ionizable lipids have made merging events to happen that lead to increased size and payload, while decreasing empty particles and encapsulation efficiency. Similar approaches were applied to study effects of ionic strength and solvent polarity on LNP quality and bioactivity. This platform offers an unique opportunity to understand the structural feature and stability of LNPs and optimize their purification process. **References** [1] R. Vargas, *et al.*, *Colloids Interface Sci. Comm.* 54: 100709 (2023). [2] S. Haeberle and R. Zengerle, *Lab Chip*, 7(9): 1094 (2007). [3] K.J. Liu and T.H. Wang, *Biophys. J.*, 95(6): 2964 (2008). [4] S. Li, et al., *Nat. Comm.*, 13(1): 1 (2022).

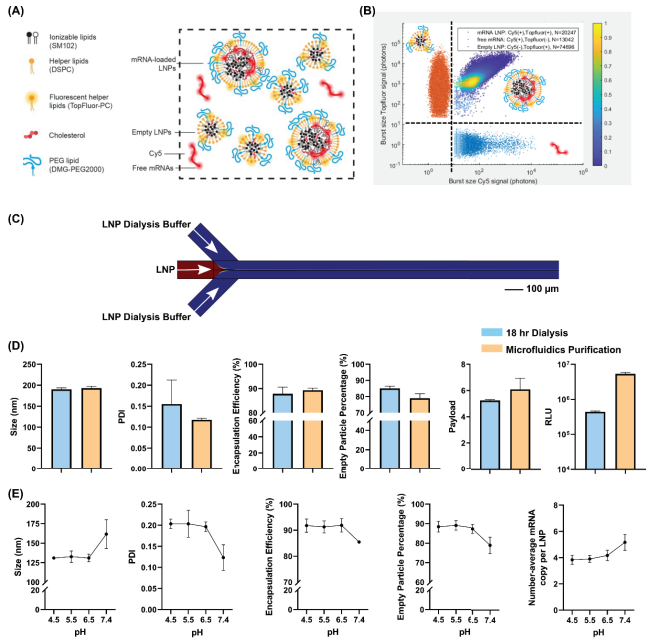


Figure 1. (A) Species of interest in LNP formulations including mRNA-loaded LNPs, empty LNPs, and free mRNA. (B) Classification of the LNP species by plotting TopFluor-PC signal against Cy5 signal intensity. (C) Microfluidic-enabled dialysis design. Two side channels are LNP dialysis buffer and the middle channel is LNP in acetate buffer with 25% ethanol. (D) Comparison between bulk dialysis method and microfluidic-enabled purification. In this study, we compared size, PDI, encapsulation efficiency, empty particle percentage, payload, and transfection. (E) Mechanism study regarding the pH of the dialysis buffer. By tuning the pH of the PBS buffer into 4.5, 5.5, 6.5, and 7.5, we compared the key performance metrics size, PDI, encap

1260. Detargeting Lipid Nanoparticle mRNA Therapeutics from the Liver Using miR-122 Targets

Brian J. Parrett, Nick M. G. Hoopingarner, Michael A. Barry

Mayo Clinic, Rochester, MN

Non-viral mRNA therapeutics have great potential to prevent and treat a variety of diseases making them an important tool in the molecular therapy toolbox. The most clinically advanced non-viral delivery systems are four component lipid nanoparticles (LNPs). A significant challenge in the development of LNP-mRNA therapeutics is strong liver tropism, limiting their use for non-hepatic molecular therapy. LNPs utilize the ubiquitous low density lipoprotein receptor (LDLR) and can transfect a variety of cell types, making them a potentially effective therapeutic for non-hepatic tissues if off-target effects in the liver can be avoided. Local injections, such as intramuscular injections (IM) of mRNA vaccines can reduce LNP deposition in the liver. However, it has been documented in preclinical studies that IM injection of the LNPs used in the COVID-19 LNP-mRNA vaccines can result in up to 21.5% of the injected dose escaping the muscle and transfecting liver. Given the problems of off-target mRNA expression in the liver, we use an endogenous liver specific microRNA-122 (miR-122) target sequence to ablate LNP-mRNA expression in the liver. We compared the effect of different quantities and locations of miR-122 target sequences on LNP-mRNA expression in the liver. miR-122 target sequences were inserted into the 5' or 3' untranslated region (UTR) of mRNAs encoding firefly luciferase. *In vitro* experiments were performed in hepatic (Huh7) and non-hepatic (293T, A549) cells with modified and unmodified LNP-mRNA to determine the effect of different quantities of miR-122 targets as well as their location in the 5' or 3' UTR on the translation

of luciferase mRNA. Modification of LNP-mRNA with miR-122 target sequences did not affect the normal translation of luciferase in non-hepatic cells but led to an ~80% reduction in hepatic cells. *In vivo*, mice injected intravenously (IV) with miR-122 modified LNP-mRNA showed up to a 99% decrease in luciferase activity in the liver. Injection of luciferase mRNA into the muscle and into other organs and sites resulted in significant off-target expression in the liver. In contrast, LNP-mRNA modified with miR-122 target sequences reduced liver luciferase activity as much as 95%. Due to the tolerogenic nature of the liver, leakage of LNP-mRNA could reduce the efficiency of immune responses to vaccine antigens. To test this, mice were vaccinated IM with LNP-mRNA encoding Ebola virus VP40 or VP40 modified with miR-122 target sequences to determine if off-target expression in the liver reduces the efficacy of vaccination. This data shows the utility of miR-122 target sequences to prevent off-target translation of LNP-mRNA therapies in the liver and has a broad range of applications in the development of vaccines and treatment of non-hepatic diseases.

1261. Lipid Nanoparticle Delivery of Plasmid DNA Elicits Robust Adaptive Immunity

Nicholas J. Tursi[1,2], Sachchidanand Tiwari[3], Nicole Bedanova[1], Elizabeth Parzych[1], Kevin Liaw[1], András Sárközy[3], Ebony Gary[1], Serena Omo-Lamai[4], Hiromi Muramatsu[3], Kerry Blatney[1], Ami Patel[1], Jacob S. Brenner[4], Norbert Pardi[3], David B. Weiner[1]

[1]Vaccine and Immunotherapy Center, The Wistar Institute, Philadelphia, PA,[2]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[3]Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[4]Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

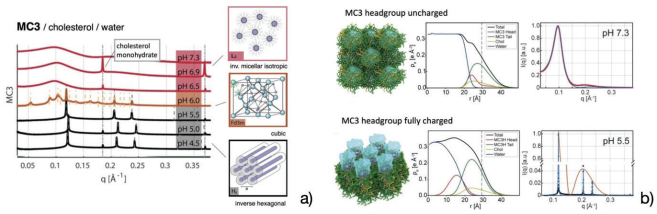
Gene-vectored vaccines induce immunity while circumventing manufacturing constraints of traditional platforms, exemplified by the approvals of lipid nanoparticle (LNP)-formulated mRNA, viral-vectored vaccines, and a DNA vaccine for SARS-CoV-2. DNA vaccines exhibit reduced manufacturing complexity but attenuated humoral immunity compared to mRNA-LNPs or adjuvanted protein. Here, we describe a four-component ionizable LNP-plasmid DNA formulation (DNA-LNPs) which induces similar adaptive immunity to mRNA-LNPs at the same dose. SARS-CoV-2 spike-encoding DNA-LNPs induced germinal center, serological, and T cell responses comparable to mRNA-LNPs. Extending these findings, Influenza hemagglutinin-encoding DNA-LNPs induced robust germinal center responses with functional serum antibodies equivalent to both mRNA-LNPs and adjuvanted protein. Moreover, DNA-LNPs induced superior hemagglutinin-specific $CD8^+$ T cell responses to these compared platforms. DNA-LNPs exhibited a favorable thermostability profile at 4°C or room temperature. This study identifies formulations for DNA-LNPs which induce robust immunity at doses comparable to mRNA-LNPs , exhibiting a unique profile important for additional development.

1262. How Mesoscopic Bulk Structure of mRNA Lipid Nanoparticles Relates to Gene Expression Kinetics at the Single Cell Level

Joachim Rädler

Ludwig-Maximilians-Universität München, München, Germany

Introduction Lipid nanoparticles (LNPs) developed into the most powerful delivery platform for mRNA based vaccination and therapies. Yet the physico-chemical mechanisms of pH-dependent endosomal fusion, mRNA release and subsequent expression are not fully understood. In particular the role of cationic ionizable lipids (CILs) is of interest as electrostatic interactions play a key role in the architecture of the LNPs core-shell structure. Using X-ray scattering we study the structures of the CIL/cholesterol/nucleic acid phases of three prototypical CILs, namely DLin-MC3-DMA (MC3), DLin-KC2-DMA (KC2), and DLinDMA (DD) and relate structures to all-atom molecular dynamics (MD) simulations and single cell gene expression timing and efficiency. **Results** Small angle X-ray scattering (SAXS) exhibits a rich pH-dependence of CIL-mesostructures (Fig.1a). In particular all three lipids exhibit a distinct order of liquid crystalline phases with decreasing pH: isotropic inverse micellar, cubic F_{d3m} inverse micellar, inverse hexagonal H_{II} and bicontinuous cubic P_{n3m}. In the presence of polyadenylic acid, in addition to the CIL/chol/water phases a condensed nucleic acid lipid H^c_{II} phase appears. The next-neighbor distance in the excess phase shows a discontinuity at the F_{d3m} inverse micellar to inverse hexagonal H_{II} transition occurring at pH 6 with distinctly larger spacing and hydration for DD vs. MC3 and KC2. MD simulations reproduce the observed inverse micellar to inverse hexagonal transition as a function of lipid head group charging (Fig.1b). We followed GFP-mRNA reporter gene expression mediated by CIL-LNPs taking time courses of single cell fluorescence in high-throughput by employing automated time-lapse microscopy in combination with micro-patterned surfaces. Late gene expression is found to correlates with lower expression levels. The data reveal a systematically delayed and hence less efficient gene expression of DLin-LNPs compared to MC3- and KC2-LNPs. **Conclusion** Our data suggest that the pH-driven F_{d3m}-H_{II} transition in bulk phases is a hallmark of CIL-specific differences in mRNA LNP efficacy. DLin lipid mesophases differ from MC3 and KC2 mesophases in distinctly larger next neighbor distances. Since DLin-LNPs also exhibits distinctly delayed and less efficient gene expression, it is hypothesized that the pH dependent structural transition is causal for endosomal fusion. Correlating physico-chemical properties with time resolved activity of LNPs in living cells provides a basis to further improve LNPs for development of improved non-viral mRNA delivery. **Reference** J. Philipp, A. Dabkowska, A. Reiser, K. Frank, R. Krzysztoń, C. Brummer, B. Nickel, C.E. Blanchet, A. Sudarsan, M. Ibrahim, S. Johansson, P. Skantze, U. Skantze, S. Östman, M. Johansson, N. Henderson, K. Elvevold, B. Smedsrød, N. Schwierz, L. Lindfors and J.O. Rädler. *PNAS* 2023 Vol. 120 No. 50 e2310491120. *Fig.1 a) SAXS data of MC3/cholesterol/water bulk phases as a function of pH b) MD simulations reproducing the inverse micellar to inverse hexagonal transition as a function of headgroup charge. Calculated electron densities agree with SAXS peak intensities.*



1263. Novel Humanized Particles for Efficient Delivery of CRISPR and Other Gene Editors

Peter K. Cabeceiras[1,2,3,4,5], Noah Adams[1], Tian Duo Wang[2,3,5,6], Christopher Woodilla[2,3,5], Caitlin Crandall[1], Bevan Bsharah[1], Xiaozhi Ren[1], Yanjing Li[1], Andrew Kane[1], Jeffrey Lee[1], Molly Riley[1], Annie Elliott[1], Vy Phan[1], Carrie Ng[7,8], Cosmin Mihai[1], Jason Auer[1], Casey A. Maguire[7,8], Melissa Bonner[1], J. Keith Joung[2,3,4,5,6]

[1]Nvelop Therapeutics, Cambridge, MA,[2]Molecular Pathology Unit, Massachusetts General Hospital, Charlestown, MA,[3]Center for Cancer Research, Massachusetts General Hospital, Charlestown, MA,[4]Program in Biological and Biomedical Sciences, Harvard Medical School, Boston, MA,[5]Center for Computational and Integrative Biology, Massachusetts General Hospital, Charlestown, MA,[6]Department of Pathology, Harvard Medical School, Boston, MA,[7]Department of Neurology, Massachusetts General Hospital, Boston, MA,[8]Harvard Medical School, Boston, MA

An urgent need exists for novel delivery modalities to fully realize the therapeutic potential of gene editing technologies including CRISPR. Recently described virus-like particles (VLPs) provide an alternative to more commonly used viral vectors such as AAV, offering advantages including delivery of a wide range of larger cargos (including ribonucleoproteins [RNPs]), transient cargo expression, and lack of potentially integrative DNA. However, VLPs still harbor multiple viral proteins, such as GAG, POL, and ENV, which may elicit immunogenicity and present potential challenges for manufacturing. Here we describe the development and optimization of Enveloped Delivery for Generalized Entry (EDGE) and humanized-EDGE (h-EDGE) particles, novel delivery platforms offering the same advantages of VLPs but harboring fewer or no viral proteins, respectively. EDGE particles do not contain viral GAG and POL proteins found in VLPs but instead utilize a compact human protein-derived architecture that we optimized to efficiently and non-covalently anchor cargos of interest to inner leaflet plasma membrane surfaces in EDGE producer cells. EDGE particles also harbor a viral ENV protein that enables high transduction efficiency and that distinguishes them from native extracellular vesicles. We show that EDGE particles can be used to deliver a wide range of gene editing cargos (CRISPR and non-CRISPR nucleases, base editors, and prime editors) and induce high frequency targeted edits into a wide variety of different cell types in culture (including primary human hepatocytes and primary human T cells). Importantly, we also show that EDGE particles can induce robust and efficient adenine base editing *in vivo* in mouse liver on a therapeutically relevant gene target (*PCSK9*). We also describe the initial development and engineering of h-EDGE particles, which use the same non-covalent membrane anchoring technology in EDGE particles but utilize one of several human-derived ENV-alternative (ENV-alt) proteins instead of viral ENV proteins to achieve high transduction efficiencies. We show that h-EDGE particles pseudotyped with different ENV-alt proteins exhibit varied cell-type tropisms. These h-EDGE particles can deliver CRISPR gene editing nucleases and base editors into a broad range of therapeutically relevant primary human cells including HSCs, neurons, hepatocytes, and skeletal muscle, inducing highly efficient editing of as high as 50% or more. Because h-EDGE particles lack any viral proteins, they offer the potential for reduced immunogenicity and redosing in addition to a range of novel cell-type tropisms. The EDGE and h-EDGE platforms provide new capabilities for delivering a wide range of diverse cargos to a variety of different cell types with potentially reduced immunogenicity and simplified profiles for manufacturing relative to VLPs. We anticipate that these new particles will be broadly useful for many research and therapeutics applications currently limited by the capabilities and characteristics of existing delivery modalities.

Thursday Posters: Cancer - Immunotherapy and Cancer Vaccines

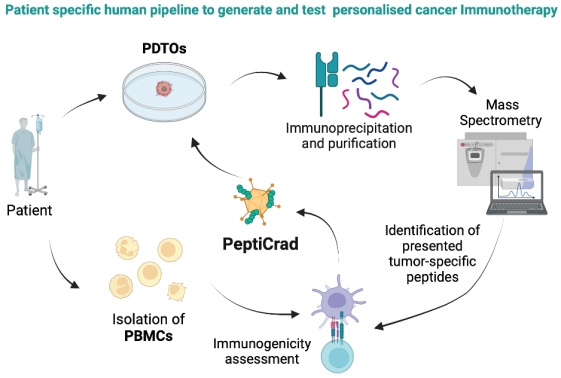
1264. Patient-Derived Tumor Organoids (PDTOs) as Preclinical Testing Platform for Personalized Cancer Immunotherapy

Gabriella Antignani

University of Helsinki, Helsinki, Finland

Currently available models to validate preclinical efficiency of Immunotherapies lack in mirroring or poorly recapitulate the original patient's tumor. Active immunotherapy, which is dependent on HLA-TCR interaction, cannot be tested in any other system than fully human based in vitro systems or humanized mice (complex, expensive, and time-consuming). Plus, only a fraction of patients will gain benefit from immunotherapy treatments and finding responsive patients in advance would be crucial. Therefore, we need to develop more sophisticated systems to address immunotherapies correct mode of actions in humans. Organoids are 3D multicellular structures that retain morphology and function of human tissues and organs. Patient-Derived Tumor Organoids (PDTOs) can be established from primary patient material, cultured long term, and cryopreserved. Successful in recalling the tumor heterogeneity and complexity in vitro, PDTOs can provide a robust ex vivo system for modelling human cancer, holding great potential as pre-clinical human platform for testing personalized therapies. Our work is focused on combining patient-derived immune cells and PDTOs to develop complex immuno-organoids that retain both patient's tumor unique features and immune system-mediated response. This allows to test tumor cell killing by the patient's own immune system after a personalized immunotherapy treatment. Moreover, after in-depth patient's tumor characterization, we design a personalized immunotherapy approach to specifically target the patient's tumor. Oncolytic vaccines have a dual mechanism: direct oncolysis of cancer cells and release of tumor antigens, causing cytotoxic T cell activation against cancer cells. To develop patient-specific oncolytic vaccine-based immunotherapies, our strategy is coating oncolytic vaccines with patient-specific tumor peptides (PeptiCRAd technology). We established PDTOs from

Renal Cell Carcinoma and Bladder Cancer, which we characterized by DNA sequencing, immunofluorescence, drug screens. HLA ligandome analysis of the PDTOs allowed the identification of 52 patient-specific tumor peptides. We validated the immunogenicity of these peptides by stimulating healthy donor Peripheral Blood Mononuclear Cells (PBMCs) and analyzing IFN-gamma secretion or CD107a degranulation. These peptides were not only capable of activating CD8+ T cells but also promoting the killing of tumor cells from the patients, showing their tumor specific activity. We also used patient-derived PBMCs to unravel the best candidate peptides able to recall a T cell antitumor response. Finally, we will investigate how an oncolytic vaccine decorated with patient-specific tumor peptides (personalized PeptiCRAd) is able to trigger tumor-specific adaptive immune response, measuring specific T cell killing of PDTOs with impedance-based Xcelligence system, LDH release and live cell microscopy. Establishing such a pre-clinical human model will pave the way for testing patient-specific responses, developing individualized treatments and accelerate their track to the clinic.



1265. Dendritic Cell Therapy in Pancreatic Adenocarcinoma Treatment: A Case Report

Taruna Ikrar

Medicine, Army Cellcure Center, RSPAD, Jakarta Pusat, Indonesia

Pancreatic cancer (PC) remains a devastating and fatal disease with high mortality rates. Immunotherapy offers a promising avenue for enhancing the treatment outcomes of this cancer. ***Methods and Results:*** This case report describes a 46-year-old male patient with pancreatic adenocarcinoma who received dendritic cell (DC) therapy as an immunotherapeutic approach to stimulate the patient's immune system. The number and proportion of viable cells were critical factors in determining the suitability of the therapy for the patient. The patient demonstrated improvement after treatment, such as an increase in hemoglobin levels to 10.4 g/dL and a decrease in CA 19-9 levels from 21.6 to 9.5 U/mL. ***Conclusion:*** Specific immunotherapies, such as DC therapy, can potentially prolong survival and enhance immune responses in the treated patients. Early diagnosis and timely treatment are crucial for improving the prognosis of PC.

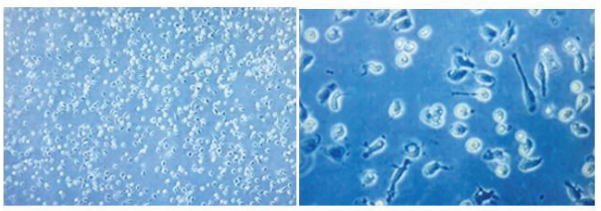


Figure 1 (A) 24 Nov. 2021 Figure 1 (B) 24 Nov. 2021

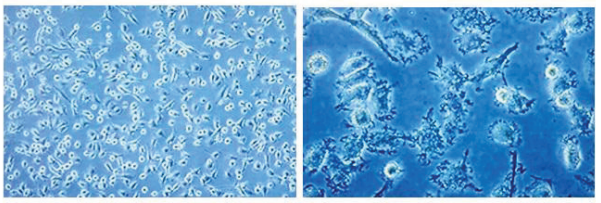


Figure 1 (C) 30 Nov. 2021 Figure 1 (D) 30 Nov. 2021

Figure 1.

Viable cell count and proportion of viable cells in the patient.

The patient had a viable cell count of 1246×10^7 cells, which is above the normal range of $1 \times 10^6 - 60 \times 10^6$. The high viable cell count indicates that the patient had enough cells to undergo certain therapies or treatments.

1266. Development of Immunotherapies for Osteosarcoma

Theresa Higgins[1], Daniel Patton[1], Timothy L. Eller[1], Isabella Shimko-Lofano[1], Payal Agarwal[2]

[1]Scott Ritchey Research Center, Auburn University, Auburn, AL,[2]Scott Ritchey Research Center, Auburn University, Auburn University, AL

Osteosarcoma (OS) is a highly aggressive and metastatic bone malignancy with a low survival rate and poor patient prognosis. In recent years, immunotherapies, such as chimeric antigen (CAR) T cells and conditionally replicative Adenoviruses (CRAds), have shown promise in treating various cancers. We have developed and characterized a next-generation conditionally replicative canine adenovirus CAV2-AU-M2 armed with anti-PD1 sdAb. The infectious and cytolytic effects of CAV2-AU-M2 were tested in four different canine OS cell lines in 2-dimensional (2D) and three-dimensional (3D) cell cultures. CAV2-AU-M2 showed selective replication in OS cells and induced efficient tumor cell lysis. Moreover, CAV2-AU-M2 produced anti-PD-1 sdAb that demonstrated effective binding to PD-1 receptors. This combination approach of two distinct immunotherapies is intended to stimulate and enhance the anti-tumor immune response in the tumor microenvironment. We also aim to develop CAR T cells targeting the cancer-specific B7H3 receptor. The cytotoxic effects of anti-B7H3 CAR T cells will be tested in 2-dimensional (2D) and three-dimensional (3D) cell cultures. We expect cell lysis only in B7H3-positive cell lines. We will also assay the immunogenic effects of CAR T cells by analyzing IFNγ, TNFα, and IL-2 cytokine levels in OS cell lines post-CAR T therapy.

1267. Inhibiting the PD1 Immune Checkpoint and the VEGFA Growth Factor Enhances the Cytotoxicity of Exhausted T Cells in Glioblastoma *In Vitro*

Shriya Madan, Anna Andrzejewska, David Gulisashvili, Graeme Woodworth, Debra Kukuruga, Yajie Liang, Piotr Walczak, Miroslaw Janowski
University of Maryland, Baltimore, MD

Introduction: Glioblastoma, a highly aggressive brain cancer, remains elusive to all therapeutic strategies, including the Nobel Prize-winning immune checkpoint inhibitors (ICIs). We hypothesize that the pro-tumoral growth factor VEGF-A may further inhibit the anti-tumor function of exhausted T cells. Therefore, we studied combinations of ICIs with bevacizumab (anti-VEGF-A antibody) in co-cultures of glioblastoma cells with T cells at various states (activated, primed, and bead- or cell-exhausted) in addition to assessing the levels of VEGF-A produced by GBM cells *in-vitro,* with or without the treatment of bevacizumab. **Methods:** HLA-matched T cells were activated using anti-CD3/CD28 beads, then primed through exposure to GBM1-luc cells and exhausted using consistent exposure to anti-CD3/CD28 beads or by culturing them with GBM1 cells. Co-cultures of these T cells at each of the four states with GBM1-luc at a 1:1 ratio were used to study the effect of 3 concentrations of nivolumab, ipilimumab, anti-PDL1 antibody, and bevacizumab over five days. Normalized bioluminescence served as a dependent variable to report on GBM1-luc cell death. Independent variables included 1) antibodies, 2) concentrations, 3) T cell status, and 4) observation period. All experiments were performed with three biological and three technical replicates. The dataset was subjected to multivariate, hierarchical regression using fixed and random effects (PROC MIXED, SAS 9.4). VEGF-A levels in supernatants were measured with ELISA with technical duplicates for each group. **Results:** Data analysis of post-hoc tests revealed the highest therapeutic effect of anti-PD1 and anti-VEGFA co-targeting in GBM1 cell-exhausted T cells, which was statistically significant. VEGF-A ELISA results showed significant production of VEGFA by GBM1 cells and that the addition of bevacizumab to the GBM1 cells scavenges VEGF-A from the cell culture medium. **Conclusions:** Combining PD-1 and VEGF-A blockade can be a potential treatment for glioblastoma as it enhances T cell function during exhaustion *via* consistent exposure to GBM1-luc cells.

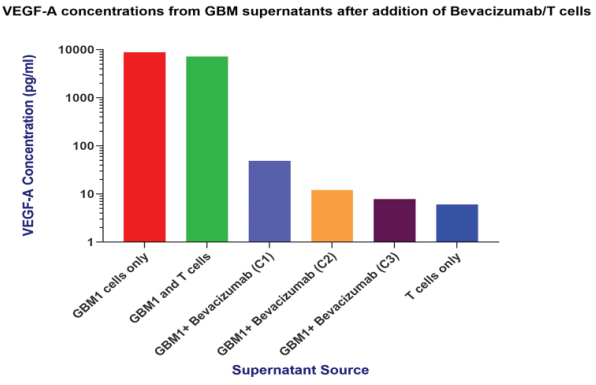


Figure 1: VEGFA measured by ELISA in supernatants of GBM cells with or without treatment of bevacizumab. NOTE: y-axis denotes a log scale.

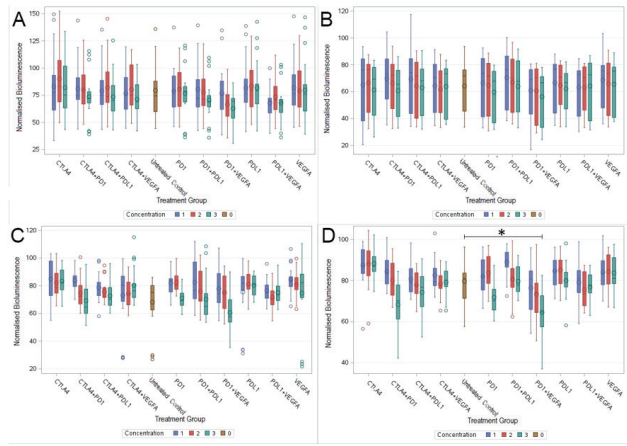


Figure 2: *In vitro* therapeutic efficacy of T cells at various stages (A - activation, B - priming, C - anti-CD3/CD28 bead-induced exhaustion, D - GBM cell-induced exhaustion) against GBM1 cells. A combination of VEGF-A and PD-1 blockade is the most effective in co-culture GBM1-luc with cell-exhausted T cells (*p-value< 0.05)

1268. Enhancing Anti-Tumorigenic Macrophage Differentiation and Potentiates PD-L1 Blockade in Melanoma through CD244 Deletion on Monocytes

Jeongsoo Kim[1], Tae-Jin Kim[2], Sehyun Chae[3], Hyojeong Ha[1], Yejin Park[1], Seon Ah Lim[4], Hyemin Lee[1], Younji Sim[1], Seyoung Oh[1], Geon Mo Koo[1], Minkyung Jo[1], Daehee Hwang[5], Kyung-Mi Lee[1]
[1]Korea University, Seoul, Korea, Republic of,[2]Korea Institute of Radiological and Medical Sciences, Seoul, Korea, Republic of,[3]Korea Brain Research Institute, Daegu, Korea, Republic of,[4]Ewha Womans University, Seoul, Korea, Republic of,[5]Seoul National University, Seoul, Korea, Republic of

Background: In the myeloid compartment of the tumor microenvironment, CD244 signaling has been implicated in immunosuppressive phenotype of monocytes. However, the precise molecular mechanism and contribution of CD244 to tumor immunity in monocytes/macrophages remains elusive due to the co-existing lymphoid cells expressing CD244. **Methods:** To directly assess the role of CD244 in tumor-associated macrophages, monocyte-lineage-specific CD244-deficient mice were generated using cre-lox recombination and challenged with B16F10 melanoma. The phenotype and function of tumor-infiltrating macrophages along with antigen-specific CD8 T cells were analyzed by flow cytometry and single cell RNA sequencing data analysis, and the molecular mechanism underlying anti-tumorigenic macrophage differentiation, antigen presentation, phagocytosis was investigated ex vivo. Finally, the clinical feasibility of CD244-negative monocytes as a therapeutic modality in melanoma was confirmed by adoptive transfer experiments. **Results:** $CD244^{fl/fl}LysM^{cre}$ mice demonstrated a significant reduction in tumor volume (61% relative to that of the $CD244^{fl/fl}$ control group) 14 days after

tumor implantation. Within tumor mass, $CD244^{fl/fl}LysM^{cre}$ mice also showed higher percentages of $Ly6C^{low}$ macrophages, along with elevated $gp100^{+}IFN\text{-}\gamma^{+}$ CD8 T cells. Flow cytometry and RNA sequencing data demonstrated that ER stress resulted in increased CD244 expression on monocytes. This, in turn, impeded the generation of anti-tumorigenic $Ly6C^{low}$ macrophages, phagocytosis and MHC-I antigen presentation by suppressing autophagy pathways. Combining anti-PD-L1 antibody with $CD244^{-/-}$ bone marrow-derived macrophages markedly improved tumor rejection compared to the anti-PD-L1 antibody alone or in combination with wild-type macrophages. Consistent with the murine data, transcriptome analysis of human melanoma tissue single-cell RNA-sequencing dataset revealed close association between CD244 and the inhibition of macrophage maturation and function. Furthermore, the presence of CD244-negative monocytes/macrophages significantly increased patient survival in primary and metastatic tumors. **Conclusion:** Our study highlights the novel role of CD244 on monocytes/macrophages in restraining anti-tumorigenic macrophage generation and tumor antigen-specific T cell response in melanoma. Importantly, our findings suggest that CD244-deficient macrophages could potentially be used as a therapeutic agent in combination with immune checkpoint inhibitors. Furthermore, CD244 expression in monocyte-lineage cells serve as a prognostic marker in cancer patients.

1269. The Discovery and Development of GT316 as a CRISPR/Cas9-Dual KO Anti-Exhaustion TIL Product for Advanced Solid Tumors

Jingwei Sun[1], Yao Sheng[1], Jingman Wang[1], Jiahui Jin[1], Yanbin Liu[1], Zhao Xu[1], Haibao Li[1], Jun Cui[1], Jing Yu[1], Pin Wang[2], Yarong Liu[1]

[1]Grit Biotechnology, Shanghai, China,[2]University of Southern California, Los Angeles, CA

Adoptive cell therapy by tumor-infiltrating lymphocytes (TILs) has demonstrated promising therapeutic effects in several types of solid tumors and significantly prolonged the survival of late-stage patients. However, the "prone-to-exhaustion" phenotype of the final TIL product after rapid expansion during manufacture and the presence of various immunosuppressive mechanisms in tumor microenvironment (TME) compromise the persistence and anti-tumor efficacy of TIL post infusion. Besides, clinical efficacy of TIL therapy is still limited to some indications. To discover potential immunoregulatory targets that could maximize the function of TIL and expand its clinical application, we established a genome-wide CRISPR/Cas9 screening platform, ImmuT Finder®, to unbiasedly identify key immunoregulatory targets for T cell product. Different *in vitro* and *in vivo* screening models were built to simulate the major challenges of T cell therapies. Comprehensive functional validations of 120+ hits were performed in TCR-T cells in a 96-well array. Then, the combinational effects of top targets validated in TCR-T were further tested in TIL by different *in vitro* and *in vivo* systems. Finally, a double KO combination of GT304 and GT312, GT316, stands out as the most potent combination, which significantly improves TIL proliferation and cytokine release, eradicates tumor growth in both CDX and PDX mouse models and promotes long-term *in vivo* persistence with low-dependence on IL-2. Bioinformatic analysis of TCGA database showed positive correlation of GT304 and GT312 mRNA expression with immune signature genes across multiple solid tumors, further indicating their clinical relevance. RNA-seq analysis of GT316 in comparison to unedited control product suggests GT316 ameliorates TIL exhaustion and promotes T cell activation, function as well as migration. Specific and potent sgRNAs targeting both targets have been selected, and manufacturing process of GT316 TIL product has been developed (KOReTIL®). First-in-human trial of GT316 in advanced solid tumor patients showed good tolerance with no AE of Grade≥3. One PR and one CR have been observed as early as 4 weeks post GT316 infusion. In summary, these data demonstrated that our ImmuT Finder® platform enables unbiased discovery of potent and novel targets for the development of T cell therapy and support further clinical assessment of GT316 as a next-generation TIL therapy.

1270. In-Depth Characterization of T-Cell Populations Utilizing a 36-Color Panel and Spectral Flow Cytometry

Gage Paul[1], Patrick J. Hanley[1,2], Christopher A. Lazarski[1,2]

[1]Children's National Medical Center, Washington, DC,[2]The George Washington University, Washington, DC

Background: Traditional polychromatic flow assays are limited in size to twenty colors due to spillover between fluorochromes. For clinical samples of small volumes, this can limit the ability to directly compare the activation and frequency of specialized T-cell subsets within the same sample. Spectral flow cytometry can however overcome this barrier by expanding the number of markers within one panel to as many as forty separate markers. Here, we developed a novel 36-color panel which combines measurement of lymphocyte subset frequency with in-depth analysis of T-cell activation, exhaustion, and intracellular cytokine production. **Objective:** Establish spectral cytometry as a service within CNRI and validate a novel 36-color fluorochrome panel utilizing spectral cytometry for in-depth characterization of T-cell subsets within research samples. **Methods:** Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats and frozen in multiple aliquots. Individual aliquots were activated using antigen-specific and non-specific stimulations to expand T-cells derived from fresh or frozen sources. Non-specific expansion was achieved using CD3/CD28 beads to proliferate cells that were not specific for any one stimulation condition. Expansion of T-cell populations under antigen-specific stimulation was accomplished utilizing a ten-day VST expansion protocol culturing cells with CMV matrix protein 65 (PP65) and immediate early protein 1 (IE-1) peptides in the presence of IL4 and IL7. Cells were stained with panels for spectral cytometry in parallel with panels for traditional cytometry to compare surface phenotype. Additionally, multiple iterations of spectral staining protocols were evaluated for median marker fluorescence intensity and fluorochrome compatibility. T-cells were analyzed for appearance of classical exhaustion and activation markers within spectral cytometry panels. **Results:** We compared the percentage of T-cells phenotyped using our 36-color stain to a validated 8-color panel. Using buffy coats from two separate donors to provide sufficient cells for both the generation of single stain controls and testing conditions, we identified CD3+CD4+ cells within ±9%, and CD56+CD3-, CD19+CD3-, and CD3+CD8+ cells all within ±4%. Additionally, we investigated whether this panel

sufficiently quantifed TNF-α and IFN-γ cytokine-producing cells from frozen PBMC samples before and after expansion with CMV peptides. The proportion of CMV reactive CD8+ IFN-γ+ TNF-α+ multi-cytokine producing cells were detectable pre-expansion and increased by two-fold after the ten-day VST expansion protocol. Furthermore, we were able to characterize the activation and exhaustion markers that are present on expanded cells. The overall CD8+ population of expanded T-cells expressed exhaustion markers TIGIT (17.79%), KLRG1 (6.29%), and PD1 (3.03%). Additionally, activation markers for CD8+ cells were identified for CD62L (6.17%) and CD40L (3.00%). Furthermore, CD8+ IFN-γ+ TNF-α+ multi-cytokine producing were characterized as TIGIT-, TIM3-, LAG3-, KLRG1low, CD45RA+, CD45RO-, CCR7-, and CD107a+. This spectral panel was used to identify differentiating T-cell subsets within heterogenous cell populations derived from multiple source conditions, including fresh blood, frozen PBMCs, and cultured cells. These investigations demonstrate our 36-color spectral panel as efficient for comprehensive analysis of reactive T-cell populations. Going forward, the Flow Core within Children's National Research Institute will work to qualify the efficiency and validity of spectral flow cytometry for research and in longitudinal studies across gene and cell therapy products.

1271. The Effect of CD9 on Natural Killer Cell Exhaustion and Dysfunction in Glioblastoma

Yuning Liu

Industrial and Molecular Pharmaceutics, Purdue University, West Lafayette, IN

In the battle against glioblastoma, the most malignant form of brain cancer, the efficacy of the immune system's natural killer (NK) cells is critical. These cells are a important component of the innate immune response, with the capacity to identify and exterminate tumor cells. However, their functionality is often compromised within the immunosuppressive microenvironment in glioblastoma. The tetraspanin CD9 has been implicated in various cellular processes, including cell adhesion, motility, and proliferation. This study aims to delineate the role of CD9 in modulating NK cell activity in glioblastoma. We began with a series of in vitro assays to determine the effects of prolonged stimulation on NK cells. Results indicated a shift towards an exhausted NK cell phenotype, as evidenced by increased CD9 and CD49a expression and decreased Granzyme B and Perforin release. To directly assess the influence of CD9, we knockout CD9 gene in NK cells via CRISPR-Cas9. These CD9-deficient NK cells demonstrated a significant increase in their ability to target and kill glioblastoma cells, suggesting that CD9 expression impairs NK cell cytotoxicity. Further investigation involved the examination of NK cells extracted from the blood of glioblastoma patients. These cells were found to have elevated levels of CD9 when compared to those from healthy controls, consistent with our in vitro findings and indicating a possible mechanism for NK cell dysfunction in vivo. Through immunohistochemical analysis of glioblastoma tissue samples, we observed that areas with higher CD9 expression coincided with regions showing NK cell infiltration. The study's findings provide evidence that CD9 plays a significant role in inducing NK cell exhaustion and dysfunction in the glioblastoma microenvironment. Our research suggests that inhibition of CD9 may offer a novel therapeutic approach to improve NK cell-mediated anti-tumor activity. Such strategies could potentially be integrated into current glioblastoma treatment regimens, offering hope for improved patient outcomes in this challenging disease.

1272. STX-003: A Novel Systemically-Delivered mRNA-Based Cancer Therapy Utilizing a Programmable Genetic Circuit Platform for Tumor-Specific Protein Expression and Reduced Toxicity

Wen Allen Tseng, Jaspreet S. Khurana, Justin H. Letendre, Gautam N. Shenoy, Rachel G. Eichman, Ashley G. Njiru, John T. Sexton, Emily Tjon, Dahyana Arias Escayola, Emily A. Clinton, Ryan A. Patenaude, Wuhbet Abraham, Zhijun Su, Madhuri Dey, Nandita Satish Menon, Isma Zarin, Breanna Burgett, Sarah O'Bryan, Mae Kingsbury, Aidan Coia, Akshay Mavani, Weiyu Zhao, Kevin Shi, Alexander C. Lemaire, Julian Quiñones, Ryan T. Sowell, Prashant R. Nambiar, Jacob R. Becraft, Tasuku Kitada

Strand Therapeutics, Boston, MA

Cancer accounts for approximately one in six deaths globally, with solid tumors representing roughly 90% of adult cancers. Significant effort has been applied toward the development of therapeutics that precisely attack tumors while sparing healthy tissue. The advent of mRNA technology, coupled with advances in LNP-mediated delivery and in silico gene expression analysis, presents new opportunities for developing precision cancer therapies. We have developed a platform in which we design programmable genetic "circuits" that detect molecular cues in a cell. These circuits are encoded as RNA and incorporated into mRNA molecules to specifically express a payload protein in cells that exhibit a particular molecular signature. Through in silico modeling and high-throughput in vitro screening, we select candidate circuits and characterize their effectiveness for classifying a particular cell type. We then evaluate a circuit's effect on payload expression in vivo, first using a luciferase reporter payload, and then using the immunomodulatory cytokine IL-12 as an anti-tumor therapeutic payload. We applied this platform to the development of STX-003, a self-replicating mRNA bearing programmable genetic circuitry that selectively expresses IL-12 in tumors. Upon systemic administration into healthy non-human primates, STX-003 demonstrates a >99% reduction in circulating IL-12 levels compared to a control without genetic circuitry, while STX-003 and the uncircuited control show similar IL-12 expression levels when transfected into patient-derived tumor organoids and the cancer cell line A549. Furthermore, intravenous injection of a murine STX-003 surrogate induces anti-tumor responses in a syngeneic mouse model and results in similar mIL-12 levels in tumors compared to an uncircuited control. However, in the spleen, which is a major target organ of LNPs formulated with mRNA, mSTX-003 induces markedly lower mIL-12 levels compared to the uncircuited control. These data demonstrate the potential of our programmable RNA circuit platform for the development of precision mRNA therapies for cancer.

1273. Bispecific T-Cell Engager Delivered through Tissue Targeting LNP Demonstrated Potent Antitumor Effects on Both Hematological Malignancies and Solid Tumors

Xin Kai, Yixin Zhang, Benjamin Wei, Daniella Tatang, Stu Angus, Caining Jin, Kun Huang, Changfeng Huang, Haishan Li, Lou Brenner, Qiaobing Xu, Kate Zhang
Hopewell Therapeutics Inc, Woburn, MA

Background The recent success of LNP-mRNA COVID vaccines has accelerated the development of lipid nanoparticles (LNP) as an attractive delivery approach for next wave of genomic medicines. Bispecific T cell engager (BiTE) therapy is an approved immunotherapy to treat hematological malignancies, such as ALL, by redirecting cytotoxic T-cells to eliminate cancer cells. However, the relatively short serum half-life of recombinant BiTE, and reported CRS and neurotoxicity have limited its applications. Here, we describe tissue targeted LNP-mRNA encoding BiTE that expressed long-lasting and therapeutic levels of BiTE protein at the targeted tumor sites. The results from the preclinical studies have demonstrated activation of T-cells to eliminate tumor cells in hematological malignancy and solid tumor mouse models and deplete circulating B-cell in a NHP model. **Methods** The LNP formulated with Hopewell's proprietary ionizable lipid HTX-L01 was selected through in vitro biophysiochemical characterization and in vivo screening. HTX-L01-003 and HTX-L01-008 encapsulated with mRNA encoding optimized single chain sequence of CD19-CD3 and GPC3-CD3 respectively, were evaluated for PK/PD, biodistribution and bio-tolerability in WT mice and NHP (dose escalating at 0.01, 0.07 and 0.10mg/kg). The tumor control activities were assessed in the Raji-xenograft non-Hodgkin lymphoma (NHL) model for HTX-L01-003 at the dose of 0.08 mg/kg and Hep3B-orthotopic hepatocellular carcinoma (HCC) model for HTX-L01-008 at the dose of 0.5 mg/kg. Similar study using HTX-L02, lung targeting LNP encapsulating GPC3-CD3, has been explored. **Results** LNP-mRNA formulated with HTX-L01 primarily targets liver, spleen, and less extent BM via intravenous injection. High levels of transfection in hepatocytes, kupffer cells and immune cells were achieved. In a hPBMC-reconstituted Raji-luc xenograft model, a much lower dosage and less frequent administration of HTX-L01-003 achieved equivalent tumor regression/elimination as recombinant BiTEs. In a NHP study, dose-dependent protein expression was observed in 3-week repeat dosing and resulted in transient T-cell activation and complete and sustained B-cell depletion. HTX-L01-008 generated the extended GPC3-CD3 BiTE protein expression, and successfully eliminated orthotopic HCC tumors in mice. Early study with lung targeting LNP HTX-L02-mRNA has demonstrated highly enriched protein expression in lung over circulation after single dose and maintain robust protein expression over 5 weeks with weekly dosing in the range of 0.03-0.3 mg/kg. **Conclusions** Systemic administration of HTX-L01-003 demonstrated in-body and "local" production of CD19/CD3 BiTE protein which resulted in highly potent and long-lasting depletion of target cells in both mouse and NHP studies. HTX-L01-008 demonstrated the ability to eliminate solid tumors in situ via i.v. injection. HTX-L02 demonstrated the potential to treat lung cancer with repeat dosing of the same mechanism. The LNP-mRNAs were well-tolerated. Overall, the studies showed the tissue targeting LNP-mRNA encoding BiTE provides a unique platform with enhanced efficacy and reduced toxicity for the treatment of both liquid and solid tumors.

Thursday Posters: Cancer - Targeted Gene and Cell Therapy

1274. AAV Based Suicide Gene Therapy with Improvised Promoters in a Murine Model of Hepatocellular Carcinoma

Vijayata Singh, Subhajit Pathak, Narendra Kumar, Jayandharan G. Rao
Indian Institute of Technology, Kanpur, India

One of the leading causes of cancer-related mortality is hepatocellular carcinoma (HCC). Suicide gene therapy is a promising approach for the potential treatment of HCC. However, the lack of specificity in delivering suicide genes to HCC cells is a major impediment in its clinical translation. In this study, we addressed this by employing Adeno-associated virus serotype 6 (AAV6) and its exosomal version (exo-AAV), containing the inducible *Caspase 9* (*iCasp9*) transgene controlled by various promoters to specifically target HCC cells. We utilized, a liver-specific promoter (LP1), and the baculoviral IAP repeat-containing protein 5 (BIRC5) promoter with E2F1 transcription factor binding site for liver and cancer cell-specific expression, respectively and tested their efficacy against the ubiquitous cytomegalovirus immediate early enhancer/chicken beta actin promoter (CAG). Subsequently, we assessed the efficacy of these vectors in inducing cell death in Huh7 cells. The *iCasp9* vectors driven by the BIRC5 and LP1 promoters exhibited superior cytotoxicity compared to those driven by the CAG promoter. Further, *in vivo* testing in a mouse model of HCC revealed that AAV6 vectors based on LP1-iCasp9 or E2F1Birc5-iCasp9 achieved tumor regression at a rate of approximately 2-fold, demonstrating comparable effectiveness to AAV6-CAG-iCasp9 vectors. Likewise, exo-AAV6 vectors also demonstrated a superior *in vivo* tumor regression of approximately 2.1-2.8-fold when compared to animals treated with a mock intervention. Our study has explored tissue/cancer specific promoters (LP1 or BIRC5) that exhibit comparable efficacy to a potent ubiquitous promoter within both the AAV6 and exo-AAV6 systems. This broadens the range of AAV vectors available, for a safe and efficacious treatment of HCC.

1275. Microbubble-Enhanced Focused Ultrasound-Assisted Delivery of Adeno-Associated Viruses in Brain Tumors with PET Imaging Monitoring

Yutong Guo, Josquin Foiret, Jai Woong Seo, Nisi Zhang, Bo Wu, Marina Nura Raie, Spencer Tumbale, Katherine Ferrara
Molecular Imaging Program, Stanford University, Stanford, CA

Introduction Improved therapeutic efficacy and enhanced survival rate are unmet needs in the treatment of brain cancers. Gene therapy utilizing adeno-associated viruses (AAVs) as a delivery vehicle presents

a promising therapeutic approach because of the high efficiency in gene transduction and low immunogenicity. However, a major obstacle to the systemic delivery of AAVs in treating brain tumors is their limited penetration across the blood-brain and blood-tumor barriers (BBB/BTB). Focused ultrasound (FUS) combined with circulating microbubbles (MB) provides a noninvasive method to locally and transiently open the BBB/BTB. Therefore, we hypothesized that MB-FUS could lead to improved and targeted delivery of AAVs into brain tumors. **Methods** To test our hypothesis, we employed positron emission tomography (PET) imaging to assess the *in vivo* spatial distribution of systemically administered ^{64}Cu-AAV9:CAG-mNeonGreen following the MB-FUS treatment of a GL26 glioma mouse model. Additionally, to verify the accumulation of AAVs in the brain tumor as observed in PET imaging, we conducted a quantitative polymerase chain reaction (qPCR) analysis of AAV9 transgene (mNeonGreen) in the tumor tissue. We performed MB-FUS experiments immediately following the AAV administration using an ultrasound-guided focused-ultrasound (USgFUS) system (1.5 MHz, 1 msec bursts, 5 Hz repetition rate, at 600 kPa) and concurrent i.v. administration of monodispersed lipid microbubbles (5 μm, 2.5×10^8 MB/kg). The USgFUS system consists of a 128-element 2D therapeutic array with a 1.5 MHz center frequency and an imaging transducer (L12-5, 38 mm aperture, Phillips/ATL) positioned in the central opening of the 2D therapeutic array to allow ultrasound guidance and treatment monitoring via passive acoustic mapping (PAM) during sonication. **Results** Firstly, to confirm the proposed protocol and FUS parameters can open the BBB/BTB and enhance the delivery of therapeutic agents, we assessed the delivery of 70 kDa fluorescently labeled dextran particles (14 nm) in the brain tumors with and without the application of MB-FUS. *Ex vivo* fluorescence imaging of the brain indicated a 2-fold increase in the accumulation of dextran particles in the MB-FUS-treated tumor as compared to the non-FUS-treated tumor (n=5, p = 0.0015). Next, we assessed the delivery of the ^{64}Cu-AAV9:CAG-mNeonGreen in brain tumors using PET imaging and qPCR. At 21 h post-injection, we observed targeted radiolabeled capsid delivery in the tumor as compared to the contralateral brain and a higher radiolabeled capsid accumulation in the MB-FUS-treated tumor as compared to the non-FUS-treated tumor in PET images (1.3-fold, n = 3, p = 0.47). Subsequently, we quantified mNeonGreen ssDNA copies in the tumor using qPCR and found a 6.4-fold increase in vector genome copies (ΔΔCt) in MB-FUS-treated tumor as compared to the non-FUS-treated tumor (n = 5, p = 0.01). **Conclusions** PET imaging and radiolabeling of AAV9 provide a method to quantitatively and longitudinally examine the spatial distribution of AAV9 after the MB-FUS treatment *in vivo*. Our results suggest that FUS combined with microbubbles provides unique opportunities to open the vascular barrier for the delivery of therapeutic agents and achieve improved targeted delivery of AAVs in the brain tumor microenvironment. A repeated PET imaging experiment with a larger sample size is warranted.

1276. Adeno-Associated Viral (AAV) Vectors Specifically Targeting the GNAQ Mutant Driver Gene Result in Uveal Melanoma Cell Death

Trace F. McCall[1,2,3], Emma J. Sawyer[1,2,3], Joshua Darnell[1,2,3], Matthew L. Hirsch[1,2,4], Jacquelyn J. Bower[1,2,3]

[1]Ophthalmology, University of North Carolina at Chapel Hill, Chapel Hill, NC,[2]Carolina Eye Research Institute, University of North Carolina at Chapel Hill, Chapel Hill, NC,[3]Lineberger Comprehensive Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC,[4]Gene Therapy Center, University of North Carolina at Chapel Hill, Chapel Hill, NC

Uveal melanoma (UM) is a rare and highly aggressive cancer arising from melanocytes within the uveal tract of the eye. Often fatal for patients, especially for metastatic cases, there is currently little success in the development and distribution of a clinically beneficial treatment. In contrast to most cancers, 80-90% of UM tumors are initiated by a unique single-point mutation in *GNAQ or GNA11* at codon 209 on a single allele, leading to constitutive activation of downstream cell proliferation pathways responsible for disease progression and metastasis. It was hypothesized that the unique properties of UM-initiating mutations could be exploited to specifically knockdown the *GNAQ*-mutant transcript, while leaving expression of the wild-type transcript in-tact, which would result in the reduction of downstream oncogenic signaling required for UM survival/progression, culminate in UM cell death, and minimize any potential off-target effects. In this study, both RNA interference and recombinant Adeno-associated virus (AAV) serotype 2 were employed to selectively target *GNAQ*-mutant transcripts in uveal melanoma in vitro. A short interfering-RNA rationally designed to target the A/T *GNAQ* mutation induced significant toxicity in multiple *GNAQ*-mutant cell lines (>50%) in clonogenic survival and alamarBlue fluorescence assays when compared to a non-targeting siRNA. Using RT-qPCR, next-generation sequencing, and western immunoblotting, selective knock-down of the mutant *GNAQ* transcript and attenuation of downstream oncogenic activity was confirmed. Towards the development of a longer lasting therapeutic, a rAAV2 viral vector was generated with a short-hairpin RNA cassette based on the successful siRNA sequence targeting the *GNAQ* A/T mutation (AAV-shGNAQ). Compared to siRNA transfection alone, delivery of AAV-shGNAQ resulted in a significant increase in death of multiple *GNAQ* A/T mutant UM cell lines. This trend was not observed upon delivery of AAV-shGNAQ to a *GNAQ*-wildtype UM cell line. Surprisingly, rAAV2-GFP transduction lacking a therapeutic gene insert also induced toxicity in *GNAQ*-mutant cell lines but not in a *GNAQ*-wildtype cell line or HEK293 cells. In summary, this work is the first to demonstrate the distinct specificity of siRNA targeting a single-point mutation in *GNAQ* that successfully discriminates between wild-type and mutant alleles differing by a single nucleotide. This ultimately resulted in significant death of multiple UM cell lines. Additionally, a rAAV2 vector expressing a shRNA cassette that can efficiently deliver our potential therapeutic was confirmed to exhibit high potency towards *GNAQ* mutant UM cells in vitro. Further studies will seek to characterize in vivo delivery of AAV-shGNAQ to treat uveal melanoma in xenograft animal models and to elucidate the mechanisms of AAV vector-induced cell death in *GNAQ* mutant UM cells.

1277. Tumor-Targeted DART-AAVs as Gene Delivery Platform for Immune-Modulators

Muhammed Burak Demircan[1], Luca Zinser[1], Florian Strassheimer[2,3], Philipp Elleringmann[2], Dirk Grimm[4], Frederic B. Thalheimer[1], Michael C. Burger[2,3], Christian J. Buchholz[1,3]

[1]Molecular Biotechnology and Gene Therapy, Paul-Ehrlich-Institut, Langen, Germany,[2]Dr. Senckenberg Institute of Neurooncology, Goethe University Hospital, Frankfurt am Main, Germany,[3]Frankfurt Cancer Institute, Goethe University, Frankfurt am Main, Germany,[4]Department of Infectious Diseases/Virology, Medical Faculty, University of Heidelberg, BioQuant, Heidelberg, Germany

In the context of cancer immunotherapy, successful administration of immunotherapeutic agents within the tumour microenvironment plays a pivotal role in maximizing therapeutic efficacy. Given the immunosuppressive characteristics of the tumour microenvironment, novel delivery approaches are currently being explored. Adeno-associated virus (AAV) vectors are the leading platform for *in vivo* gene delivery and have great potential to deliver immunotherapeutic to the tumour microenvironment. However, one of the crucial challenges associated with the delivery is off-target activities, resulting in unintended transduction of therapy-irrelevant tissues, most notably the liver. This phenomenon can lead to potential toxicity and significantly limit the applicability of the therapy. To address this issue, we have developed a rational design strategy for AAVs based on display of high-affinity binders for a tumour surface antigen, such as the HER2/neu-specific designed ankyrin repeat protein (DARPin), at the VP2 N-terminus. We have recently shown that the resulting HER2-AAV enables tumour-specific delivery of checkpoint-inhibitors. In context of glioblastoma, this resulted in substantially improved therapeutic activity in a subcutaneous tumour mouse model when combined with CAR-mediated tumour cell killing (Strecker et al., 2022). Here, we generated and analysed a second-generation targeted platform called DART-AAV, in which DARPins are inserted into the VP1 GH2/GH3 loop. The corresponding second-generation HER2-AAV selectively transduced cancer cells only if they express human HER2/neu. Compared to the first generation, they exhibited a substantial increase in gene delivery activity of more than 20-fold without being compromised in selectivity. When equipped with the coding sequence for the checkpoint-inhibitor Nivolumab, transduced HER2-positive tumour cells released functional Nivolumab at high amounts *in vitro*. When administered into nude mice subcutaneously engrafted with HER2-positive SKOV-3 cells, HER2-AAV2 delivered luciferase specifically into the tumour tissue with the absence of detectable transduction in liver. As an additional tumour cell target, we have developed DART-AAV displaying DARPins specific for programmed death-ligand 1 (PDL1). PDL1-AAV demonstrated efficient transduction of PDL1-positive tumour cells, while PDL1-negative cells did not exhibit significant reporter gene expression. This additional example confirms the high selectivity of DART-AAVs for their target cells. Ongoing investigations explore the extension of tumour-targeted DART-AAVs to other AAV serotypes as well as their therapeutic capacity in tumour mouse models. They appear especially suited for the delivery of immune checkpoint inhibitors and cytokines into the tumour microenvironment.

1278. Smart RNA Sequence Design for Onco-Selective Translation in Cancer Cells

Isaak Mueller, Kelly Brock, Leona Lee, Thomas Addison, Sadik Yildiz, Yusuf Erkul, Burak Yilmaz, Cafer Ozdemir

Kernal Biologics, Cambridge, MA

Cancer therapies must precisely target cancer cells for destruction while avoiding healthy cells, as off-target effects could present significant patient morbidity. To avoid toxicity from these therapeutics, one approach is to have an mRNA-based design where the encoded payload is preferentially expressed in cells of interest. These onco-selective sequences can be designed for multiple cancer subtypes. Here, we present the results of a novel integrated computational and experimental pipeline that has identified multiple sequence elements with significant onco-selectivity. As part of this pipeline, we employ both hypothesis-driven screenings based on machine learning on large scale transcriptomics, proteomics, and structural datasets, as well as agnostic massively parallel reporter assays, to identify onco-selectively translated sequences and their associated biomarkers. Additionally, codon optimization of complex sequence elements and process optimization enabled us to utilize our platform more broadly for complex therapeutic payloads. This iterative computational and synthetic biology approach allows us to engineer our mRNA sequences for cell-specific translation of modular therapeutic payloads with reduced immunogenicity. We have demonstrated our sequences' onco-selectivity in both murine melanoma and colorectal cancer models, and our pipeline is transferrable to other tumor types. From a broader perspective, our platform can be used to selectively express proteins for other applications, including cell-specific CRISPR gene editing or selective activation of DNA repair mechanisms.

1279. Design of Retinoid-Responsive Toxin Gene Systems for Neuroblastoma Treatment

Enzo M. Giardina[1], Stephen L. Hart[2], Andrew W. Stoker[1]

[1]Developmental Biology & Cancer, UCL Great Ormond Street Institute of Child Health, London, United Kingdom,[2]Genetics & Genomic Medicine, UCL Great Ormond Street Institute of Child Health, London, United Kingdom

Background Retinoic acid (RA) has been shown to improve clinical outcomes for neuroblastoma (NB) patients due to its ability to induce differentiation in tumour cells. However, its use has been limited by toxicity and tumour-acquired resistance after prolonged treatment, following which patients relapse and this is often fatal. **Aims** To increase the efficacy of RA, we aim to design a "toxin gene" therapeutic that is highly sensitive to transcriptional activation by RA. When tumour cells take up the DNA-based agent, they will be bathed in exogenous RA and be rapidly killed. This will effectively re-purpose RA, converting the differentiation response from a cytostatic to a cytotoxic response. Moreover, we aim to deliver the therapeutic in culture and in vivo using our tumour-targeting, liposome-based nanotechnology. **Methods** We have linked the toxin gene diphtheria toxin A (DTA) to RA-responsive promoters in plasmids and tested their cytotoxic capabilities under a range of RA concentrations in cultured cell lines. MicroRNA-response-elements (MREs) were also cloned into the plasmids to minimise

expression in off-target tissues, primarily the liver. Luciferase assays were used to quantify expression with and without the MREs, while fluorescence-based assays were used to assess cell survival. We have then used our previously documented liposomes coated with NB-targeting peptides (ref 1) to assess delivery of our plasmids to cells in culture. **Results** The RA-driven DTA vector, after transfection into NB cells, is highly sensitive to RA, requiring minimal exogenous RA (<100 nM) for maximal cytotoxic activity. Compared to RA alone, the vector shows greater toxicity at all concentrations of RA, shown by the complete loss of positively transfected NB cells following RA treatment; this is seen even in the more RA-resistant SK-N-AS cell line. Liver-specific MREs knocked down transcription by 98% in the hepatoma-derived Huh7 cell line, and also reduced cytotoxicity in this line; however, decreased activity was seen in some NB cells. The liposome-based nanocomplexes also delivered sufficient levels of DTA-expression plasmid *in vitro* to trigger efficient cell death in a range of NB-derived tumour cell lines. **Conclusion** We have developed an inducible gene therapy approach that is able to achieve greater tumour cell cytotoxicity in culture than RA alone. Liver-specific MREs provided some protection against toxicity in liver cells, while cytotoxicity in NB lines was sufficiently maintained. Next steps are to improve the tumour-selectivity of the toxin gene and to carry out proof of principle testing *in vivo* using our targeted deliver system. If successful, this novel approach could be developed as a combination treatment alongside RA in patients, as a route to more effectively remove residual tumour cells.
References 1. Tagalakis, AD et al. "Integrin-Targeted, Short Interfering RNA Nanocomplexes for Neuroblastoma Tumor-Specific Delivery Achieve *MYCN* Silencing with Improved Survival." *Advanced functional materials* vol. 31,37 (2021): 2104843. doi:10.1002/adfm.202104843

1280. miR-590-3p Inhibits Growth of Recurrent GBM by Blocking Multiple Oncogenic Nodes

Hernando Lopez-Bertoni[1], Sophie Sall[2]

[1]Neurology, Johns Hopkins School Of Medicine, Baltimore, MD,[2]Kennedy Krieger Institute Inc, Baltimore, MD

Glioblastoma (GBM) is the most lethal and aggressive form of brain cancer. Despite aggressive therapy consisting of surgery followed by radio/chemotherapy GBM recurs in almost all patients and, currently, there are no proven therapies to treat recurrent GBM (rGBM). Recent developments in nanomedicine provide new and promising opportunities to develop new targeted therapeutics to treat brain tumors. We have recently developed PBAE nanoparticle formulations optimized for in vivo miRNA delivery to brain tumor stem cells, or GSCs. Carefully selecting one miRNA to target several mRNAs dysregulated within a pathway can allow the targeting of multiple nodes using a single agent, mimicking combinatorial therapies. To test this hypothesis, we performed bioinformatic analysis of RNA sequencing from GSCs and clinical rGBM specimens combined with gene set enrichment analysis (GSEA) and identified TGFBR2 signaling as a targetable pathway in rGBM. We show that SMAD2/3 signaling, a downstream effector of TGFBR2, is enriched in rGBM and that patients with elevated TGFBR2 transcripts respond poorly to current standard of care. Utilizing patient-derived xenograft lines from rGBM, we show that blocking TGFBR2 signaling using siRNAs or ITD1, a selective TGFBR2 inhibitor, decreases the stem cell capacity, cell viability and re-sensitizes clinical rGBM cells to temozolomide (TMZ) in-vitro. In vivo, we show that ITD1 is modestly effective in subcutaneous and ineffective in orthotopic rGBM models. Not surprisingly, these tumors exhibited classical growth patterns associated with acquisition of resistance. To overcome this obstacle, we performed a miRNA-based network analysis in rGBM and identified miR-590-3p as a putative tumor suppressor that targets SMAD2/3 signaling at multiple nodes in rGBM. In-vitro, nanoparticle-mediated delivery of transgenic miR-590-3p robustly decreased the expression of 37 putative oncogenes simultaneously concurrent with decreasing stem cell capacity, cell viability and re-sensitizing rGBM PDX cells to TMZ. In vivo delivery of miR-590-3p to established PDX-derived rGBM orthotopic tumors using novel PBAE nanocarriers robustly decreased tumor sizes and increased tumor necrosis. These results show that the promiscuous nature of miRNAs, in combination with cutting-edge nucleic acid delivery vehicles, can be leveraged as advanced targeted therapeutics for rGBM bypasses the resistance that is developed to standard of care and available small molecules.

1281. *In Vivo* CRISPR Nanoformulations Targeting Castration-Resistant Prostate Cancer

Noah H. Richardson[1], Sanam Rezaei Benam[1], Samaneh Maleknia[1], Reza Shahbazi[1,2,3]

[1]Division of Hematology/Oncology, Indiana University School of Medicine, Indianapolis, IN,[2]Brown Center for Immunotherapy, Indiana University School of Medicine, Indianapolis, IN,[3]Indiana University Melvin and Bren Simon Comprehensive Cancer Center, Indianapolis, IN

Advanced metastatic prostate cancer remains an incurable disease and novel therapies are greatly needed to improve outcomes. Initially, metastatic prostate cancer responds to androgen deprivation therapy (ADT) blocking the androgen receptor (AR) signaling pathway responsible for growth and proliferation. Eventually, resistance develops and although circulating androgens remain reduced, signaling through the AR pathway continues through alternative mechanisms. This stage, known as castrate-resistant prostate cancer (CRPC), is characterized by relentless disease progression despite ongoing ADT. Changes in the AR signaling axis, such as receptor duplications, constitutively active splice variants, and point mutations, confer resistance to ADT by circumventing systemic androgen depletion. Furthermore, intratumoral androgen synthesis and receptor responsiveness to other steroid ligands contribute to the persistent activity of the AR. The continued biological significance and reliance of AR signaling in CRPC has made the definitive knockout (KO) of the gene an enticing target. We have used antibody-coated lipid nanoparticles (LNP) to specifically direct CRISPR nanoformulations to prostate-specific membrane antigen (PSMA) positive CRPC cells and knock out the AR. Our CRISPR formulation has increased safety and specificity with both extracellular and intracellular prerequisites for CRISPR activity. Intracellular activation of the CRISPR sgRNA complex is mediated by sensing cell-specific miRNA uniquely identified in malignant cells. We have screened miRNA databases and C4-2B cell lines identifying specific miRNA not expressed significantly in other healthy tissues. Using miRNA-flanked sgRNA CRISPR formulations our system once shuttled to PSMA expressing C4-2B cells allows assembly of Cas9 protein and sgRNA in a cell-specific manner causing AR knock out and

cell death. Our western blot results show 50-80% AR knockout with 40-60% gene editing efficiency analyzed by T7EI, and TIDE analysis. Our time-lapse Incucyte analysis shows 100% targeting to PSMA-positive C4-2B cells by tracking GFP mRNA uptake (Fig. 1). Currently, we are continuing our studies through in vivo analysis of LNP delivery and gene editing specificity in a CRPC mouse model utilizing our unique CRISPR LNP formulation. Our findings demonstrate the feasibility of targeted in vivo CRISPR gene editing to treat prostate cancer and overcome known resistance mechanisms of AR-based therapeutics.
References 1. Rebello, R.J., Oing, C., Knudsen, K.E. et al. Prostate cancer. Nat Rev Dis Primers 7, 9 (2021).

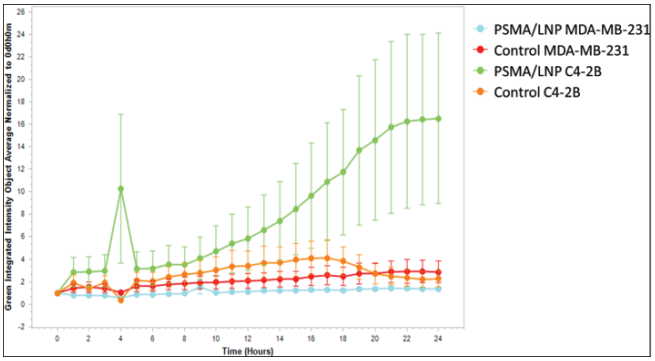


Fig. 1. Highly specific targeting of metastatic prostate cancer cells (C4-2B) by PSMA/LNP CRISPR nanoformulations. Triple negative breast cancer cells treated with the same nanoformulation was used as a control. Lines show GFP mRNA expression. All data are presented as mean ± SEM from experiments n=3.

1282. Identification the Role of Succinate and 5'-Deoxy-5'-Methylthioadenosine as Oncometabolites on Natural Killer Cell Activity Against NSCLC

Xinyu Wu

Purdue University, West Lafayette, IN

Research Summary - Background Natural killer (NK) cells are part of the innate immune system, and possess high cytolytic activity against tumor cells, along with the ability to secrete cytokines and chemokines. However, solid tumors have been largely resistant to attack by NK cells, owing to unfavorable interactions between immune cells and tumors driven by an immunosuppressive tumor microenvironment (TME). The TME of solid tumors is characterized by elevated levels of oncometabolites. However, how their presence affects the activity of NK cells against solid tumors is not known. Among oncometabolites, we have found that 5'-Deoxy-5'-methylthioadenosine (MTA), an adenosine receptor agonist, and disodium succinate, one of the metabolites of the TCA cycle, can alter NK cell behavior in the TME via different mechanisms. To understand how cancer cells respond to NK cell activity in an oncometabolite-rich environment, we studied their effects, and corresponding responses, via CRISPR screening of NK cell and solid tumor systems. - Methods and Results NK cells were isolated from lung cancer patients and healthy adult donors. To study the effect of oncometabolites, NK cells were treated with various concentrations of oncometabolites for 24 hours, then viability of NK cells was measured via the CCK8 assay. Lung adenocarcinoma (A549) cells were then cocultured with oncometabolite-treated NK cells in the absence or presence of oncometabolites to measure the cytotoxicity andIFN-γ production. Separately, oncometabolite-treated NK cells were used in a co-culture setting to identify drivers of response of cancer cells to NK-mediated killing via a genome-based CRISPR screen. A549 cells were transduced with the CRISPR pooled library and positively selected with puromycin. After co-culture with NK cells, DNA genome was isolated and sequenced. - Conclusions Our data showed that metabolites affect NK cell killing capacity. Interestingly, we found that oncometabolites heterogeneously modulates the killing ability of NK cells, suggesting that context- and receptor-specific activation - such as the continued presence of cytokines and metabolites - drives NK cell responses to metabolic factors in the TME. Among the tested oncometabolites, MTA could drastically decrease IFN-γ levels while increasing NK cell cytotoxicity, without affecting the viability. Conversely, succinic acid showed significantly detrimental effects on NK cell viability while increasing their killing ability. We also identified genetic markers of response of cancer cells to killing by NK cells in the presence of oncometabolites. Taken together, our data show divergent and heterogeneous effects of oncometabolites present in the TME and suggest possible genetic targets that can promote NK cell activation in these contexts.

1283. Characterize the Tissue Biodistribution of PD-1, PD-L1, and CD19 mRNA in Lung Carcinoma

Nirupama Chandel, Francesca Mazzoni, Kui Tian, Jack Coleman

R&D, Enzo Life Sciences Inc., Farmingdale, NY

Introduction: Cancer immunotherapy has recently been developed for effective treatments to improve the specificity and strength of the immune response against cancer. The PD-1/PD-L1 complex is known to attenuate immune response towards cancer therapy. Therefore, expression of PD-1 and PD-L1 is directly associated with the severity of cancer and insufficiency of the immune system. CD19 is a B-cell lineage marker that is also employed in CAR-T immunotherapy; the effectiveness of CAR-T cell therapy often hinges on the blockade of PD-1. Consequently, there is great value in target detection using *in situ* hybridization (ISH) to provide precise microscopic localization of nucleic acids in tissue samples. In this study, we used AMPIVIEW™ RNA probes, powered by Enzo's LoopRNA™ ISH technology, specifically targeting PD-1, PD-L1, and CD19 mRNAs to determine the gene expression level and spatial localization in lung carcinoma. **Methods:** The current study used highly sensitive and specific AMPIVIEW™ RNA probes for multiplexing ISH to detect PD-1, PD-L1, and CD19 in a single human lung carcinoma tissue section. **Results and Conclusion:** Understanding the regulatory proteins at the transcription level enables the identification, spatial localization, and quantification of transcript molecules. Deciphering the molecular mechanism of cancer cells is crucial for the development of cell therapy and personalized treatments. In this study, PD-1, PD-L1, and CD19 mRNA were detected in lung carcinoma tissue in a localized manner in stromal cells and around the tumor area. We are able to detect single targets, in addition to multiplex detection with AMPIVIEW™ RNA probes in a single tissue slide.

1284. *In Vivo* Programming of Immune Cells Using mRNA-LNP Chimeric Antigen Receptors

Shannon Argueta, Franciele K. Melber, Michael Gorgievski, Hongyun Zhao, Neha Diwangi, Edward Cochran, Thach Chu, Thomas Prod'homme, Yuxiao Wang, Robert Hofmeister, Jian Ding

Myeloid Therapeutics, Cambridge, MA

Adoptive cell therapies have provided significant clinical benefits to cancer patients. However, *ex vivo* cell therapies are costly and associated with long vein-to-vein times. To overcome these limitations, we deliver cancer-targeting chimeric antigen receptor (CAR) mRNA via lipid nanoparticles (LNPs) to reprogram immune cells directly *in vivo*. To avoid expression of the CAR in other than myeloid cells when adminsterd systemically, we created a new FcαR I (CD89) based CAR construct whose expression and function depends on the co-expression the FcR γ-chain, a signal adaptor protein that is primarily restricted to myeloid cells. Using the same design principle, we also generated CARs that are only operative in NK and T cells, respectively. Upon uptake of TROP2 CAR mRNA/LNPs *in vitro*, TROP2 CARs primarily expressed on mouse and human myeloid cells triggered CAR-dependent cytokine release and tumor cell lysis. Systemic mRNA/LNP delivery of the CAR showed robust anti-tumor efficacy against multiple tumor antigens in xenograft mouse models of human breast cancer, hepatocyte carcinoma, and ovarian cancer. Furthermore, using gp75-targeted CD89-based CAR/LNPs in the B16/F10 immunocompetent melanoma mouse tumor model, we demonstrated robust anti-tumor immune responses and profound changes in the tumor microenvironment characterized by tumoral infiltration of activated CD8+ T cells, diminished tumor-associated Tregs, systemic enhancement of dendritic cell activation and anti-tumor IgG production. Encouraged by our preclinical data, we advanced the TROP2-targeting CAR to a phase I clinical trial to assess the safety and efficacy in epithelial malignancies.

1285. Engineered IMiD Regulated Synthetic Transcriptional Switch for Controlled and Dose-Responsive Expression of Therapeutic Payload within FDA-Approved Drug Doses

Rebecca Cottman[1], Myles MacEachern[1], Michelle Hung[1], Andrew Banicki[2], Mengxi Tian[2], Chen-Ting Lee[2], Assen Roguev[1], Raghav Kannan[3], Chandra Verma[3], Chew-Li Soh[4], Conor McAuliffe[4], Mark Tomishima[4], Timothy K. Lu[2], Gordley Russell[1], Philip Lee[1]

[1]GeneFab, LLC, South San Francisco, CA,[2]Senti Biosciences, South San Francisco, CA,[3]Aplomex, Queensland, Singapore,[4]BlueRock Therapeutics, New York, NY

Background: Cell therapies have shown promise in the treatment of liquid tumors, but further engineering is required to effectively treat solid tumors. Use of potent payloads such as IL-12 could boost the efficacy of such therapies, but expression must be regulated to avoid toxicity. Transcriptional switches responsive to FDA-approved small molecules (SMs) are an attractive method for controlling payload expression, but many existing platforms are not responsive to drug concentrations typically achievable in human tissues, especially the brain. This critical limitation prevents their use in clinical applications. We set out to develop a transcriptional switch that can regulate a payload at pharmacologically relevant concentrations of an FDA-approved SM drug to enable novel cell therapies. Methods: The SM-regulated transcriptional switch is composed of a split synthetic transcription factor (SynTF) responsive to pomalidomide and an engineered inducible promoter that can be activated by the SynTF. The SynTF is composed of two parts, A and B. Part A is a zinc finger DNA binding domain (DBD) fused to del.CRBN, a modified human Cereblon that no longer complexes with ubiquitin ligases. Part B is a transcriptional activator fused to a CRBN-IMiD binding protein (CRIMP). In the presence of pomalidomide, A and B dimerize, forming a del.CRBN/CRIMP/pomalidomide complex. This dimerization brings the DBD and transcriptional activator together, forming a functional SynTF that drives payload expression from the inducible promoter. The target concentration of pomalidomide for SynTF function is 4 nM, based on estimates of pomalidomide concentration in the brain and solid tumors. The SynTF was evaluated for function at a range of pomalidomide concentrations and showed robust activity at > 100 nM, well above the 4 nM threshold. To address this, we computationally predicted mutations based on the del.CRBN-IMiD-CRIMP structure that could increase pomalidomide binding affinity. We then screened these mutants for their ability to enhance the SM sensitivity of the SynTF. Results: 112 del.CRBN mutants were evaluated, a subset of which improved the sensitivity of the SynTF to 4 nM pomalidomide. The top performing switch was used to induce expression of a therapeutic payload, IL-12, *in vitro*. This switch induced IL-12 expression at 4 nM pomalidomide from baseline of 8,650 pg/mL to 14,400 pg/mL per $1x10^6$ cells in 24h. We evaluated the engineered transcriptional switch containing our lead del.CRBN mutant for function *in vivo* by implanting NSG mice with engineered cell lines expressing the SynTF and a SynTF-inducible nanoluciferase reporter. We observed significant induction of nanoluciferase luminescence in pomalidomide-treated mice compared to the vehicle-only condition with ~5 fold activation of reporter at lowest dosage of 2 mg/kg, which is well within the dose range that can be delivered to humans. Conclusions: We have engineered a novel SynTF transcriptional gene circuit that has increased sensitivity to an FDA-approved SM drug at pharmacologically relevant concentrations for human clinical use, enabling its function in a wide range of tissues, including the brain. This circuit induces expression of therapeutic payload, IL-12, *in vitro* and functions *in vivo* in a mouse model with drug doses that simulate human dosing. This enables regulated expression of potent immune payloads for increasing the efficacy and safety of cell therapies for solid tumors and other difficult to target diseases.

1286. Versatile SpyTag-SpyCatcher System for Targeting Lentiviral Vectors

Gopal Naik Nenavath[1], Karina Krotova[1], Nandakumar Packiriswamy[1], Kah-Whye Peng[1,2], Miguel Ángel Muñoz-Alía[2], Stephen J. Russell[1,2]

[1]Imanis Life Sciences, Rochester, MN,[2]Vyriad, Rochester, MN

Several CAR-T and hematopoietic stem cell gene therapies are now FDA approved but the need for cell harvesting, ex vivo genetic modification and reimplantation brings high costs and limits these treatments to specialized centers only. Recently, the approach to modify

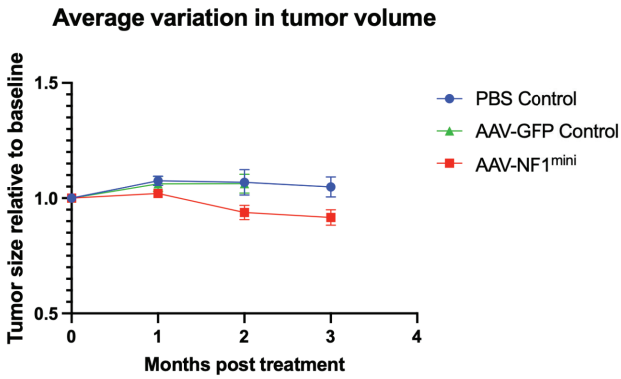
or engineer the target cells directly within the patient has become an attractive alternative but will require the development of robust cell-specific lentiviral vectors suitable for in vivo applications. Here we introduce a simple and efficient strategy for the creation of VSV-G pseudotyped lentiviral vectors specific to any desired target cell lineage. The VSV-G protein was engineered to ablate its interaction with the LDL receptor and a 13 amino acid SpyTag sequence was inserted close to its N-terminus. These modifications did not significantly impact the folding, intracellular trafficking or incorporation of the protein into lentiviral vector particles and its fusion triggering activity was well maintained. To covalently attach a receptor-targeting moiety to the virally incorporated SpyTag-modified G protein, SpyCatcher fusion proteins incorporating N-terminal cell targeting ligands were generated. Spontaneous isopeptide bond formation between the SpyTag and the SpyCatcher was subsequently shown to result in the generation of ligand-displaying lentiviral vectors and was achieved using two alternate approaches. In the first (coexpression) approach, VSV-G-SpyTag and SpyCatcher-Ligand plasmids were cotransfected into the lentivector producer cells, such that their encoded proteins could associate inside the producer cell. In the second (mixing) approach, lentivirus particles incorporating the SpyTag-modified G protein were mixed with cell supernatants containing the SpyCatcher-Ligand protein. For proof of concept, we first used this technology to display a modified EGFm123 domain on the lentivirus surface. Covalent attachment of the SpyCatcher-EGF fusion protein on the vector particles was confirmed by western blotting and they were shown to preferentially transduce EGF receptor (EGFR) positive target cells. These findings were confirmed and extended using SpyTagged lentiviral vector particles covalently bonded to SpyCatcher-aEGFRscFv, SpyCatcher-aHER2scFv or SpyCatcher-SCF (Stem Cell Factor) fusion proteins, all of which showed appropriate receptor-dependent cell entry specificity, suggesting the technology can accommodate a variety of small and larger targeting molecules. To enable the targeting of human T-lymphocytes, we generated five different SpyCatcher-aCD3scFv fusion proteins, displayed them on SpyTagged lentiviral vector particles and confirmed their ability to target CD3 receptor-positive Jurkat cells but not CD3 receptor-negative Nalm6 cells. We further showed that these retargeted lentiviruses specifically recognized and transduced T cells in a mixed population of human PBMCs. In-vivo targeting studies are ongoing. To conclude, the covalent attachment of cell targeting ligands to lentiviral vectors using the SpyTag-SpyCatcher system in conjunction with fusion-competent LDLR tropism-ablated VSV-G proteins offers a novel and versatile approach to the generation of fully retargeted lentiviral vectors.

1287. Intratumoral Gene Therapy for Plexiform Neurofibroma Utilizing AAV-NF1^{mini}

Rrita Daci[1], Simon Wentworth[2], McKenna Watson[2], Tyler Mola[2], Hannah Villeneuve[2], Pamela St Louis[2], Julie Aguiar[2], Karen Dresser[3], Thomas Smith[3], Robert King[4], Heather L. Gray-Edwards[4], Ana Rita Batista[2], Miguel Sena-Esteves[2]

[1]Neurosurgery, UMass Chan Medical School, Worcester, MA,[2]Neurology, UMass Chan Medical School, Worcester, MA,[3]Pathology, UMass Chan Medical School, Worcester, MA,[4]Radiology, UMass Chan Medical School, Worcester, MA

Introduction: Neurofibromatosis type 1 (NF1) is one of the most common human monogenetic diseases, with an incidence of 1 in 3000. The NF1 tumor suppressor gene encodes for the GTPase-activating protein, neurofibromin. Biallelic loss of *NF1* causes uninhibited GTPase RAS activity resulting in tumor proliferation. Patients with NF1 suffer from inoperable, and sometimes malignant, tumors which are only partially responsive to small molecule inhibitors. As an adjunct to surgical resection, we propose an intratumoral gene therapeutic that can initiate tumor senescence or decrease tumor growth. **Methods**: To accommodate the large size of the NF1 gene, we designed a mini-NF1 transgene (AAV9-NF1^{mini}) which encodes for the tumor suppressive GTPase-activating protein. For consistent *in vivo* vector testing, we created an induced neurofibroma model by injecting AAV1-Cre to the sciatic nerve of $Nf1^{R681*/4F}$ mice carrying an inactive ($Nf1^{R681*}$) and a floxed ($Nf1^{4F}$) allele. Precise analysis of tumor volume was done employing 7T magnetic resonance imaging (MRI). Volumetric analysis was completed by a blinded third party, utilizing ImageJ (NIH, Bethesda MD). We used western blot to assess deletion of *NF1* in the sciatic nerve plexiform neurofibroma tumor model. Since NF1 causes downstream regulation of the RAS pathway involving phosphorylation status of Erk, we also analyzed levels of phosphorylated Erk (pErk) relative to Erk in normal sciatic nerve and plexiform neurofibroma. Immunohistochemistry against Schwann cell markers S100 and Sox10 was done for tumor characterization. After establishing a reliable neurofibroma tumor model, mice were enrolled into three cohorts: treatment with AAV9-NF1^{mini} (N=12), PBS control (N=12) and AAV9-GFP control (N=5). The addition of a GFP control was done to assess for biological activity of the AAV9 capsid and any potential cross-reactivity with AAV1. **Results**: Histological evaluation of the AAV1-Cre injected sciatic nerve in $Nf1^{R681*/4F}$ mice revealed spindle cells with increased cellularity and with S100B and Sox-10 positivity, indicating tumor formation. Western blot analysis of these tumors showed deletion of NF1 and dysregulation of the pErk/Erk pathway. Initial 7T MRI evaluation showed T2 hyperintensity within the length of the AAV1-Cre injected sciatic nerve and subsequent MRIs showed slow growth of untreated tumors. Tumors treated with AAV-NF1^{mini} showed a significantly smaller difference in tumor volume as measured on MRI when compared to controls. The 1 month statistical significance (unpaired T test p=0.013) persisted at 3 months post treatment (unpaired T test p=0.025). Additionally, tumors treated with AAV-NF1^{mini} showed a reduction in tumor size at 3 months when compared to 1 month post treatment (paired T test: p=0.016)(Fig.1). **Conclusion**: We have established an induced NF1 mouse neurofibroma model in the sciatic nerve. Our data revealed that AAV-NF1^{mini} has some tumor suppressive function *in vivo*, as demonstrated by decreased tumor size in the treatment arm when compared to control. Our goal is to rapidly translate this approach for intratumoral treatment of plexiform neurofibromas that are challenging to treat surgically and/or fail to respond to Selumetinib.



1289. Suppression of PARKIN by Cigarette Smoke Through microRNA-Mediated Regulation: Implications for Impaired Mitophagy, Oxidative Stress, and Inflammation, and an Alternative Intervention Strategy

Maria Jose Santiago[1], Srinivasan Chinnapaiyan[2], Manal Imran[2], Zachary Grand[2], Kingshuk Panda[2], Suvankar Ghorai[2], Md Sohanur Rahman[2], Hoshang Unwalla[2]

[1]Chemistry & Biochemistry-Department of Cellular and Molecular Medicine, Florida International University, Miami, FL,[2]Department of Cellular and Molecular Medicine, Florida International University, Miami, FL

Cigarette smoke (CS) induces oxidative stress in airway cells, leading to disruptions in metabolic pathways and triggering inflammation. Interestingly, the mechanism by which CS leads to inflammation is not well-established. This project focuses on understanding how cigarette smoke induces inflammation to develop a therapeutic approach for mitigating its effects. Mitochondria play a pivotal role in regulating apoptotic and oxidative processes. If mitochondria are damaged or defective, they must undergo recycling or elimination through mitophagy. Improper mitophagy results in the retention of dysfunctional mitochondria, leading to the release of inflammatory molecules such as Damage-Associated Molecular Patterns (DAMPs), Senescence-Associated Secretory Phenotype (SASPs), mitochondrial DNA (mitDNA), and reactive oxygen species (ROS). Therefore, we hypothesize that CS suppresses the expression of PARKIN (mitophagy protein), culminating in defective mitophagy, senescence, and elevated ROS levels, causing inflammatory responses. To test this hypothesis, bronchial cells were exposed to CS, followed by the collection of supernatants, RNA, and proteins for comprehensive analysis. Additionally, electroporation procedures involving a microRNA, identified as a potential target for PARKIN and upregulated by CS, are currently underway. Various methodologies, including Western blotting (WB), reverse transcription-quantitative polymerase chain reaction (RT-qPCR), Amplex Red assay, staining procedures (MitoTracker Green and C12FDG dyes), and Luminex were employed to analyze factors such as PARKIN expression, H_2O_2 levels, defective mitophagy, senescence, and inflammatory profiles. Results demonstrate that CS suppresses PARKIN at the mRNA and protein levels by 70% and 40%, respectively, in bronchial epithelial cells. H_2O_2 levels increased nearly three-fold upon CS exposure. Staining procedures indicate a significant increase in defective mitophagy and senescence due to CS. A pro-inflammatory profile was established upon CS exposure by the Luminex assay. Currently, we are verifying and examining the inhibition of PARKIN facilitated by a candidate microRNA, which is upregulated by CS. We aim to confirm the regulatory role of this microRNA mediating PARKIN expression and consequently implement a therapeutic strategy that involves the synergistic combination of antioxidants with antagomirs to effectively restore PARKIN expression and alleviate the adverse effects of CS. This research provides insight into the intricate mechanisms by which CS induces an exacerbated redox state in airways, culminating in pulmonary inflammation. Furthermore, the study explores the potential of therapeutic interventions involving antagomirs and antioxidants to ameliorate the detrimental impact of cigarette smoke.

1290. Expressional & Mutational Deregulations of miRNA-146b and miRNA-181b in Papillary Thyroid Carcinoma

Rashida Khan[1], Ruqia Baig[2], Qaisar Mansoor[1]

[1]Istitute of Biomedical & Genetic Engineering Islamabad, Islamabad, Pakistan,[2]PMAS-Arid Agriculture University Rawalpindi, Rawalpindi, Pakistan

Background: Thyroid cancer is considered to exist among most common cancers and at the same time it is the most recurrent malignancy of the endocrine system. The most frequent type of thyroid cancer is papillary thyroid cancer (PTC), which contributes more than 80% globally & prevalent in females of Eastern and Western Asia, America and Iceland. The genetic elements known as MicroRNAs (miRNAs), endogenous non-coding RNAs operating as post-transcriptional regulators involved in development, proliferation and differentiation. miRNAs are gaining fame as druggable biomarkers and clinical management of neoplasm. The momentous stackeholding of miRNAs by virtue of gene expression variations in the cancer microenvironment has been witnessed in the PTC onset, amplification and apoptosis. The growing body of knowledge highlights the modifiable play at the miRNA level harbors potential in lessening the perpetuation of the disease with safe handlers. The genetic information leads to a big highway which can replace the unified yardstick to tailor PTC with the more targeted personalized disease treatment by monitoring the disease risk and aggression modalities.Objectives: The study aims to speculate the characteristic involvement of expression level changes in the miRNA genes miRNA-146b and miRNA-181b as tangible biomarkers for papillary thyroid cancer. Methodology: The present study was conducted on the PTC in Pakistan, a genetically less explored South Asian country. Specimen of cancer tissue, normal samples and multi nodular goiter (MNG) samples from patients were collected. The anthropometric and clinical parameters of patients were recorded after informed consent. Total RNA was isolated and cDNA was synthesized. Gene expression profile for miRNA-146b and miRNA-181b was done by quantitative Real-Time PCR. Relative gene expression was identified as fold change and mutational deregulations were checked through DNA sequencing showing the involvement of these miRNAs in PTC. Results: The statistically significant relative expression of genes miRNA-

146b; 5 to 20 folds and miRNA-181b; 4-60 folds were observed in PTC in comparison to MNG and healthy tissue specimens. Conclusion: The boosted gene expression of the miRNA genes miRNA-146b and miRNA-181b manifests the plausible misregulations in deployment of these molecular musketeers as foes in PTC. This forged maladaptation of the miRNA-146b and miRNA-181b in the cancer microenvironment may warrant analytically, therapeutically and genetically surmountable miRNA targets for PTC clinical management prevention

Thursday Posters: Challenges to Immunological Responses to Therapeutic Interventions

1291. Peptide-Mediated Activation of PI3K Class III Complex Containing Beclin1 and ATG14L Boosts Gene Delivery by Lentiviral Vector at the Stage of Cellular Entry

Saliha Majdoul, Scarlett Shi, Kazi Rahman, Alex A. Compton

HIV Dynamics and Replication Program, National Cancer Institute, Frederick, MD

Cell-intrinsic barriers to enveloped virus infection constitute a major limitation to the effectiveness of lentivector-mediated gene therapy ex vivo and in vivo. We previously identified that triggering autophagy through mTOR inhibition resulted in increased cellular susceptibility to lentivector transduction at the stage of virus-cell membrane fusion. Here, we determined if and how autophagy induction by a Beclin1-derived peptide (Tat-Beclin1) impacted lentivector entry into hematopoietic and nonhematopoietic cells. We found that Tat-Beclin1 peptide boosted lentivector entry into cells, a process driven by vesicular stomatitis virus (VSV) glycoprotein, by more than an order of magnitude. In contrast, peptide mutated at phenylalanine-270 had no effect. Mechanistically, we found that lentivector entry enhancement by Tat-Beclin1 requires it to bind to and activate cellular Beclin1 protein in a F270-dependent manner. Beclin1 forms a complex with PI3K class III and ATG14L, and this complex produces phosphatidylinositol-3-phosphate (PI3P), a lipid essential for macroautophagy induction. Indeed, we found that Tat-Beclin1 promoted PI3P production in endosomal and autophagosomal membranes. By using a specific inhibitor of PI3K class III, we found that PI3P production is necessary for the enhancement of lentivector entry by Tat-Beclin1. Furthermore, specific knockdown of Beclin1 or ATG14L abrogated lentivector entry enhancement by the peptide, and a specific inhibitor of the Beclin1-ATG14L interaction blocked lentivector entry as well. Therefore, Tat-Beclin1 boosts lentivector transduction by activating the PI3K class III-Beclin1-ATG14L complex. In accordance with the lentivector entry step being affected, we also found that authentic VSV infection is dramatically boosted by Tat-Beclin1. Our findings can be leveraged to not only significantly improve the efficiency of lentivector-mediated gene therapy, but also immunotherapies that depend upon deployment of VSV, such as oncolytic virotherapy for cancer.

1292. Co-Stimulatory Blockade Regimen Prevents Anti-Transgene and Anti-Vector Immune Responses in Hemophilia a Mice After *In Vivo* LV Gene Therapy

Andrea Annoni[1], Michela Milani[1], Cesare Canepari[1], Silvia Iaia[1], Fabio Russo[1], Mauro Biffi[1], Luigi M. Naldini[1,2], Alessio Cantore[1,2]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), IRCCS San Raffaele Scientific Institute, Milan, Italy,[2]"Vita-Salute San Raffaele" University, Milan, Italy

Lentiviral vector (LV)-based in vivo gene therapy is attractive for its potential applicability to permanently correct monogenic diseases, even in childhood, with negligible limitations derived from pre-existing immunity towards the parental virus. Although stable transgene expression by hepatocytes have been achieved in mice, dogs and non-human primates by a single systemic LV administration, immunity towards transgene and vector may limit the therapeutic outcome and LV redosing. In a mouse model of Hemophilia A (HA), LV-mediated factor VIII (FVIII) expression by hepatocytes revealed that multiple factors may contribute to its immunogenicity, such as the protein structure, the proportion of transduced hepatocytes, the translation rate and the protein half-life. High dose of LV encoding for human (hu)FVIII (5E10 TU/kg) in adult HA mice established stable expression of huFVIII (50% of normal) in absence of anti-FVIII immune responses in 80% of treated mice, by a combination of fasting and type-I interferon (IFN) signaling blockade (anti-IFNa receptor, IFNAR) which allowed maximizing LV transduction efficiency and dampening innate immunity. Conversely, administration of LV alone, did not result in detectable huFVIII and induced anti-FVIII immunity. Comparable doses of LV encoding for half-life extended huFVIII. XTEN resulted in minimal and only transient FVIII expression in vivo, again abrogated by anti-FVIII immunity. In order to improve FVIII output in vivo at lower LV doses, the transgene sequence was codon-optimized (coFVIII) enabling 10-fold increased expression compared to huFVIII. This strategy allowed to obtain high levels of circulating FVIII produced by a limited proportion of hepatocytes but resulted in the induction of a strong anti-FVIII humoral and cellular immune response leading to clearance of LV-corrected hepatocytes. Therefore, we tested T cell targeted immunotherapies to control anti-transgene immunity. We found that the administration of anti-IFNAR immediately before coFVIII-LV or coFVIII-XTEN-LV administration combined with co-stimulation blockade regimen allowed stable huFVIII expression at 110% and 500% of normal, respectively, in HA adult mice by lower LV doses (2E10 TU/kg). This immunotherapeutic regimen abrogated the induction of anti-FVIII antibodies and CD8+ T cell allowing persistence of LV-modified hepatocytes releasing FVIII in absence of antibody-mediated neutralization, largely beyond the time of its administration. Furthermore, we did not detect any neutralizing anti-VSV.G humoral response, which is typically induced by in vivo administration of VSV.G-pseudotyped LV. Overall, these data indicate that FVIII immunogenicity can be controlled by i) increasing the proportion of transduced hepatocytes and lowering the amount of huFVIII produced *per* cell and ii) applying a co-stimulation blockade regimen to achieve stable FVIII expression at therapeutic levels even increasing the rate of transgene translation in a reduced fraction of

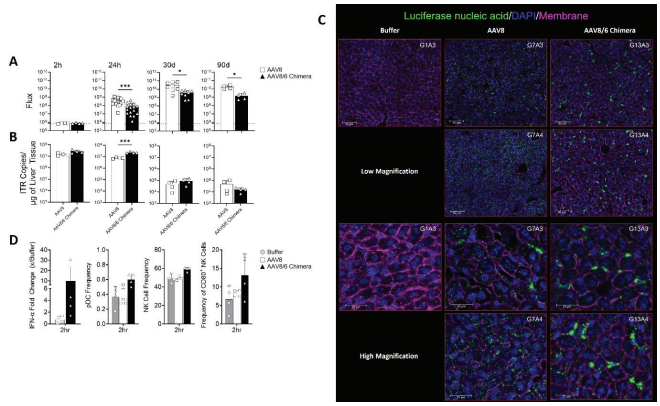
hepatocytes. Moreover, the use of the latter strategy allows to adjust transgene output by administering a second LV infusion thanks to the absence of immunity to FVIII or LV particles.

1293. Preclinical Modeling of Immunity in Murine Liver Following Adeno-Associated Virus Delivery: A Cross-Sectional Comparison of AAV8 and an AAV8/6 Chimeric Capsid

Alan D. Curtis[1], Rachel Hedinger[1], Kelsey Higgins[1], Youjun Chen[1], Abbie Svancarek[1], Charles Krause[1], Joshua Hatfield[1], Layth Awartani[1], Dylan Southers[1], Erik Wielechowski[1], Rachel Reynolds[1], Audry Fernandez[1], Angela Mote[1], Scott Hammond[1], Sherif Gabriel[1], Anna Tretiakova[1], Shari Gordon[1], Xiao Xiao[2], R. Jude Samulski[1,3]

[1]Asklepios BioPharmaceutical, Inc., Durham, NC,[2]Belief Biomed, Shanghai, China,[3]University of North Carolina at Chapel Hill, Chapel Hill, NC

Adeno-associated virus (AAV) vectors are leading candidates for gene therapy product delivery. However, the developing capsid specific immune response has been associated with waning transgene expression and immune-related adverse events. AAV8 is a well-described liver tropic capsid with strong transduction efficiency. However, liver inflammation and in rare cases liver toxicity has been observed after high-dose AAV8 administration (Konkle, 2021; Wilson & Flotte, 2020). Conversely, a recently described liver tropic AAV8/6 chimera capsid demonstrated safety, minimal liver inflammation, and sustained transgene expression (median 58 weeks) in a clinical trial in hemophilia B patients (Xue, 2022). Better understanding of innate and adaptive immune pathways induced by AAV in the liver has the potential to inform capsid design, immunomodulatory regimens, and enhance clinical trial outcomes. The current cross-sectional study aimed to directly compare the immunogenicity, transgene expression and biology of AAV8 and an AAV8/6 chimera. We assigned groups (n=4) of adult female B6(Cg)-Tyrc-2J/J albino mice to receive $1E^{11}$ vg (~$5E^{12}$ vg/kg) of AAV8 (AAV8-Luc) or AAV8/6 chimera (AAV8/6 chimera-Luc) carrying a luciferase reporter. Control mice received formulation buffer. All dosing was conducted by tail vein injection and mice were sequentially culled at 2, 5, 8, 16, 24, 72 hours (h) or 7, 30, or 90 days (d) post-dose. Both whole body IVIS imaging and ex-vivo imaging of the liver showed significantly higher transgene expression from AAV8 compared to AAV8/6 chimera (Figure A). Intriguingly, AAV8/6 chimera exhibited higher (~0.5 log) viral DNA copies in liver compared to AAV8 at early timepoints (Figure B). In situ hybridization of luciferase DNA/RNA in liver revealed distinct vector distribution patterns 2h post dose. AAV8 nucleic acids were as small, punctate foci that were uniformly distributed. In contrast, AAV8/6 chimera was organized into large areas of intense staining with an increased number of membrane-associated aggregates, suggesting vector amalgamation (Figure C). AST and ALT liver enzyme levels in serum were similar across all groups and time points and apoptotic hepatocytes were not observed by TUNEL staining, implying normal liver function despite AAV aggregates at early timepoints. We used flow cytometric techniques and qPCR to quantify the fraction of infiltrating immune cells in liver and changes in gene expression of specific cytokines at the various timepoints post AAV administration. At 2h post-dose, AAV8/6 chimera transiently elicited an upregulation of select innate genes including interferon alpha (~10 fold), increased plasmacytoid dendritic cell frequency and natural killer cell activation relative to AAV8 and mock controls (Figure D). At later timepoints we observed similar levels of activated T cells in the liver and circulating AAV binding antibodies induced by both capsids. Overall, this study suggests capsid specific differences in the early host response may impact transgene expression.



1294. iqDNA is an Engineered DNA Cargo That Avoids Innate Immune Activation While Retaining Durable Transgene Expression

Russell Monds, Kelly Miller, Andrew O'Hagan, Nicholas Parsonnet, Joris Cipi, Nick Hanovice, Christian Slubowski, Luis Agosto, Anastasia Lymar, Matthew Stanton

Generation Bio, Cambridge, MA

Non-viral delivery of large DNA molecules offers the potential for re-dosable gene therapy and may broaden the scope of in vivo gene editing to support full gene correction or insertion. One of the central challenges to realizing this enormous potential is the acute toxicity of DNA-mediated activation of the innate immune system. Immune quiet DNA (iqDNA) is a derivative of closed ended DNA (ceDNA) that was engineered to avoid recognition and activation of Pattern Recognition Receptor (PRR) systems, including cGAS/ STING, TLR9 and inflammasome pathways. When iqDNA is administered systemically using lipid nanoparticle delivery, mice show baseline levels for a broad range of cytokines across a dose range (Fig. 1). This profile is comparable to chemically-modified mRNA and a stark contrast to existing DNA formats, including ceDNA which elicits cytokines when dosed above 0.1 mg/kg. The immune-quiet profile of iqDNA is robust across species, avoiding innate immune activation in non-human primates and human PBMCs. Importantly, despite being designed to avoid immune recognition, iqDNA retains the capacity for robust and stable episomal transgene expression. iqDNA encoding a luciferase reporter showed similar expression to that of ceDNA (~ 10^8 total flux, @ 0.25 mg/kg) when delivered to mice by LNP. Furthermore, expression of luciferase was durable, showing no change over the course of a 30-day study. To support the therapeutic potential of iqDNA, a FVIII transgene cassette was synthesized in an iqDNA format and tested for innate immune activation and expression. Similar to the results with luciferase, little to no elevation of cytokines was observed

in mice or monkeys treated with 2 mg/kg and 0.5 mg/kg, respectively. The immune-quiet profile of DNA was consistent across five different LNP compositions, reinforcing the generality of the iqDNA's ability to avoid innate immune system detection. FVIII expression was detected in mice treated with iqDNA at a level of 0.06 IU/ml (6% normal levels). Potency of FVIII iqDNA is being optimized to facilitate robust translation to NHP. Progress towards this goal is presented as well as insights into the mechanisms by which iqDNA avoids immune system recognition. The toxicity of exogenous DNA severely limits the safety and efficacy of DNA gene therapy technologies. iqDNA represents a pivotal step forward, and sets a framework to enable functional, durable, and re-dosable non-viral gene therapies.

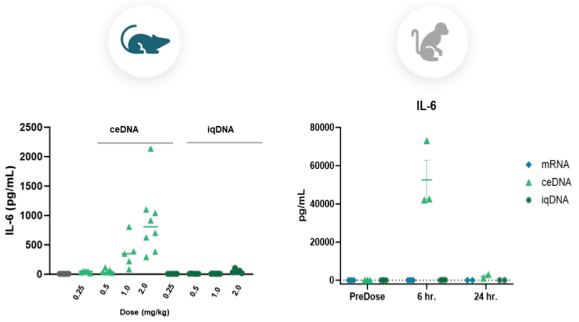


Figure 1. iqDNA does not activate the innate immune system in mice or NHP. Left) Systemic IL-6 concentration 6 hours after dosing CD-1 mice intravenously with formulated ceDNA or iqDNA. Right) Systemic IL-6 concentration before and after dosing cynomolgus monkeys intravenously with formulated mRNA, ceDNA or iqDNA at 1.0 mg/kg.

1295. B cell Production of the eCD4-Ig-Knob-in Hole-Reversed HIV-1 Immunoadhesin in Humanized NBSGW Mice

Chet Ojha[1], Craig Schindewolf[1], Anna Helmers[1], Amalia Icreverzi[1], Neele Thom[1], Gregory Asher[1], Meagan Neu[1], Andrea Repele[1], Richard James[2], David J. Rawlings[2], Bruce E. Torbett[2]

[1]Center for Immunity and Immunotherapies, Seattle Children's Research Institute, Seattle, WA,[2]Department of Pediatrics, University of Washington School of Medicine, Seattle, WA

B cells have the capacity to produce high levels of IgG proteins and may persist for years as long-lived plasma cells. Moreover, B cell-produced exogenous proteins have been reported to be immune system tolerant. These properties support the concept that B cells are an attractive cellular target for gene therapy. The HIV-1 immunoadhesin molecule, eCD4-Ig-Knob-in-Hole-Reversed (KiHR), is a CD4- and CCR5/CXCR4-mimetic antibody-like protein which we have engineered so as not to interact with B cell immunoglobulin Fc domains, and it has been shown to have nanomolar HIV-1 neutralizing potency against primary isolates. We have previously shown that the B cell-specific enhancer and promoter, EμB29, promotes eCD4-Ig-KiHR expression in B cells *ex vivo* (PMID-36879849). We hypothesized that the presence of the eCD4-Ig-KiHR transgene in CD34$^+$ HSPCs could provide for long-term generation of eCD4-Ig-KiHR protein-expressing B cells. To evaluate whether eCD4-Ig-KiHR could be produced long-term from B cells in humanized NBSGW mice, we transduced adult CD34$^+$ hematopoietic stem and progenitor cells (HSPCs) with a VSV-G-pseudotyped EμB29-GFP-P2A-eCD4-Ig-KiHR lentiviral (LV) vector. CD34$^+$ HSPCs were transduced to achieve viral copy numbers (VCN) of 1, 3 or 8 and then HSPCs were adoptively transferred to busulfan-treated NBSGW mice. Mouse peripheral blood human CD45$^+$/GFP$^+$ cells and serum eCD4-Ig-KiHR levels varied depending on the transduction levels in CD34$^+$ HPSCs. VCNs of 1, 3 and 8 resulted in 10 - 15%, 25 - 35% and 50 - 70% human CD45$^+$/GFP$^+$ cells in peripheral blood of humanized mice and 1 - 2 ng/mL, 3 - 4 ng/mL, and 15 - 20 ng/mL of eCD4-Ig-KiHR in sera, respectively. eCD4-IgKiHR levels were maintained until week 24 post-transfer. Since B cell maturation is restricted in humanized mice to IgM-producing B cells, we evaluated whether differentiation *ex vivo* of IgM$^+$ B cells isolated from spleens of humanized mice would result in greater eCD4-Ig-KiHR protein production. Indeed, differentiation *ex vivo* of IgM$^+$ B cells resulted in IgG$^+$ class-switched CD138$^+$ plasmablasts after 10 days. Importantly, differentiated B cells produced higher amounts of eCD4-Ig-KiHR (up to 100 ng/million cells/day). We also quantified eCD4-Ig-KiHR production at the single cell level by eCD4-Ig enzyme-linked immunosorbent spot (ELISpot), and we detected a 90-fold increase in the proportion of eCD4-Ig-KiHR-expressing B cells during culture. These data suggest that the B cell population derived from transferred eCD4Ig-KiHR-transduced CD34$^+$ HSPCs is capable of expressing high amounts of transgene from a B cell-specific enhancer/promoter when allowed to mature. Our findings indicate that at all levels of B cell GFP-marking we observed, eCD4-Ig-KiHR could be detected in humanized mouse sera at levels sufficient for HIV neutralization (> 1 ng/mL). The findings support the promising strategy that LV-mediated delivery of the HIV-1 immunoadhesin eCD4-Ig-KiHR under the control of a B-cell specific enhancer/promoter to CD34$^+$ HSPCs may lead to the production of high levels of eCD4-Ig-KiHR by mature B cells *in vivo*.

1296. Generation of Highly Potent HIV/SIV Neutralizing Molecules after *In Vivo* HSC Transduction of Rhesus Macaques with a Novel HDAd Strategy

John K. Bui[1], Chang Li[1], Anne-Sophie Kuhlmann[2], Anna K. Anderson[1], Hongjie Wang[1], Donna J. Palmer[3], Philip Ng[3], Andrew Riker[2], Michael Farzan[4], Jasmin Martinez-Reyes[2], Michael Alpert[5], Brandon Keele[6], Hans-Peter Kiem[7], Andre Lieber[1]

[1]University of Washington, Seattle, WA,[2]Fred Hutchinson, Seattle, WA,[3]Baylor College of Medicine, Houston, TX,[4]Boston Children's Hospital, Boston, MA,[5]Emmune, Inc., Juno Beach, FL,[6]NIH, Frederick National Laboratory for Cancer Research, Frederick, MD,[7]Fred Hutch, Seattle, WA

In vivo gene delivery using helper-dependent adenovirus vectors (HDAd) promises accessible and versatile treatment for various diseases. HDAd vectors offer the potential for sustained therapeutic delivery, addressing challenges posed by chronic diseases, notably HIV, that require lifelong treatment. We are pioneering *in vivo* transduction approaches using HDAd vectors to transduce hematopoietic stem cells (HSCs) for the *in vivo* delivery of HIV therapeutics, including eCD4-Ig. This broadly neutralizing molecule possesses exceptional breadth against diverse HIV isolates and has a high barrier to HIV resistance. *In vivo* delivery of eCD4-Ig eliminates the need for repeated passive eCD4-Ig infusions, offering a scalable and accessible approach for

middle and low-income countries that face a disproportionate burden of HIV/AIDS. We have previously shown that our HDAd platform can achieve stable therapeutic levels of eCD4-Ig in non-human primate (NHP) models. Here, we used an improved HDAd (HDAd6/3+-cxcr4) to deliver an enhanced eCD4-Ig variant ("eCD4-Ig-Emm006") that was modified for improved potency, *in vivo* stability, and immunogenicity. eCD4-Ig-Emm006 vectors also contained a TPST2 cassette to sulfonate the eCD4-Ig-Emm006 molecule for increased binding affinity to HIV/SIV. Both the eCD4Ig-Emm006 and TPST2 cassettes were under the control of ubiquitously active promoters, thus allowing for expression in all HSC progeny cells that could be targets for SIV/HIV infection. To test the ability of *in vivo*-delivered eCD4-Ig-Emm006 to protect against SIV infection (an HIV-like virus), we compared two groups of rhesus macaques that received the eCD4-Ig-Emm006 vector (N=4) or a control vector (N=3). HSCs were mobilized using AMD3100 and G-CSF followed by intravenous infusion of integrating HDAd vectors. HDAd vectors were well-tolerated. Using our enhanced HDAd6/3+-cxcr4 vector platform, we achieved stable vector copy numbers (VCN) in the range of 0.1 vector copies in bone marrow mononuclear cells and PBMCs after an in vivo enrichment step, reflecting ~10-fold improvement compared to our prior HDAd vectors. The eCD4-Ig-Emm006 variant had exceptional SIV neutralization potency: *in vivo* serum-derived eCD4-Ig-Emm006 IC50s ranged from 10.6 to 34.7 ng/mL. eCD4-Ig-Emm006 serum levels stabilized at 2.1 μg/mL to 38.3 μg/mL, which are ~1,000x fold higher than the measured IC50. We are currently challenging animals with SIV to assess the protective efficacy of our approach. Pending analyses include determinations of SIV serum titers, gene-marking in hematopoietic subsets and systemic tissues, as well as flow-based immunophenotypic analyses. These data demonstrate the ability of our *in vivo* approach to achieve stable, long-term *in vivo* levels of eCD4-Ig for the prevention and treatment of HIV. The results of this proof-of-concept study demonstrate the capacity of our HDAd platform to deliver therapeutic proteins with the prospect of addressing infectious diseases, including HIV and COVID-19. Our HDAd platform could emerge as a viable alternative for vaccines, particular benefitting immunocompromised hosts who are unable to mount protective endogenous immune responses. This evidence not only underscores the platform's potential but also positions it as a promising therapeutic modality for infectious disease prevention and management.

1297. A Deep Learning Model for Predicting Patient Response to Virus-Specific T Cell Therapy

Pamela A. Chansky[1], Wei Li[1], Michael D. Keller[2], Catherine M. Bollard[2]

[1]Center for Genetic Medicine, Children's National Hospital, Washington, DC,[2]Center for Cancer & Immunology Research, Children's National Hospital, Washington, DC

Viral infections are a significant risk for many immunocompromised patients, including recipients of bone marrow transplantation. Virus-specific T cell (VST) therapy allows rapid reconstitution of antiviral T cell immunity, with 70-90% response rates following HLA-mismatched VST therapy. However, failure of VST therapy to control viral infections remains a frequent problem. Aside from immunosuppressive agents, there is limited understanding of why patients fail VST therapy. VST response is defined as the viral load reaching an undetectable level via PCR or a noticeable reduction in viral load combined with other clinical indicators of improved health. We hypothesized that a variety of commonly collected clinical variables from the period prior to VST therapy, such as pre-treatment viral load, prior drugs received, and diagnostic category, could be used in a deep learning model to predict the likelihood of response to VST therapy. We used a clinical dataset comprised of patient records from prior VST studies as input to a variational autoencoder (VAE) to overcome the limited sample size (n=79). The VAE generated tens of thousands of unique records with high similarity to the real infusion records, as determined by correlation tests, thus improving the ability to draw statistically meaningful conclusions and generate accurate predictions. A combination of the real and synthetic data was then used to train a deep learning model of VST response. The deep learning model was tested on a subset of the real records which were withheld from the data generation and training processes and was found to accurately predict the likelihood of response given a patient's pre-treatment clinical variables. Averaging over 100 iterations, the model achieved over 80% accuracy, recall and precision, indicating model's usefulness in predicting patient response to VST therapy. The in-silico model will help identify the best VST therapy candidates to optimize treatment planning.

1298. Elucidating Complement Activation Mechanisms in AAV Gene Therapy: Development of a Novel ELISA-Based Assay for Enhanced Safety and Efficacy

Klaudia Kulak[1], Viktoria Kozma[1], Cecilia Klint[1], Camilla Nilsson[1], Michael Tovey[2], Michael Schwenkert[1], Therese Segerstein[1], Jordi Rodo[3]

[1]Svar Life Science, Malmö, Sweden,[2]Svar Life Science, Paris, Sweden,[3]Svar Life Science, Cambridge, MA

Gene therapy utilizing adeno-associated virus (AAV) vectors demonstrates excellent safety profiles and remarkable clinical efficacy in treating diverse diseases. Despite its relative safety, evidence from clinical trials has shown that AAV can trigger severe life-threatening inflammatory responses. Many of these adverse reactions are associated with the secondary activation of the complement system, leading to complications such as acute kidney injury due to atypical hemolytic uremic syndrome-like (aHUS-like) complement activation, thrombotic microangiopathy (TMA), thrombocytopenia, and immune-mediated myocardial inflammation. Though AAV-mediated complement activation is a major safety concern, we still lack understanding of the activation mechanism and a predictive method to prevent or mitigate activation in administered patients. In this context, our research has been focused on the development and evaluation of an ELISA-based AAV-mediated Complement Activation assay. The technique combines an AAV-coated plate with a tailored buffer composition, selectively allowing evaluation of the distinct classical, lectin or alternative complement system pathways. The assay detects *ex vivo*-generated terminal complement complex (TCC) deposition, making it a robust tool for testing and predicting complement reactions to AAV in analyzed sera. Utilizing this assay, we have explored the impact of each pathway on AAV-mediated activation and investigated the potential of anti-AAV antibodies to initiate the classical pathway. The platform

is also designed to assess the potential presence and impact of other AAV-specific motifs capable of activating the lectin pathway. Given the complexity of serum interactions with anti-AAV antibodies and the complement system, our assay holds promise in shedding light on pivotal immune interactions and offers valuable insights to enhance the safety and efficacy of AAV-based gene therapies, thus, advancing the field.

1299. Pre-Existing Immunity in the Human Population to Cas Proteins Derived from Bacteriophage, Casϕ, and Acidaminococcus sp., Cas12a

Pratima Bajgain, Vijaya L. Simhadri, Atul Rawal, Zuben E. Sauna

Center for Biologics Evaluation and Research, Food and Drug Adminstration, Silver Spring, MD

Introduction: The clinical use of the CRISPR/Cas system requires in vivo delivery of Cas proteins, and it could induce immune responses which would limit the utility of the technology. Pre-existing immunity to Cas9 which is of microbial origin has been demonstrated. Here, we evaluate pre-existing T- and B-cell immunity to two alternatives to Cas9: AsCas12a (derived from *Acidaminococcus* sp bacteria, normal fecal flora) and Casφ (derived from a huge bacteriophage). Methods: The study was performed using blood from 40 healthy donors obtained from the National Institute of Health blood bank. Pre-existing antibodies to SaCas9, AsCas12a and Casφ were identified using ELISA while memory T-cell responses were measured using the ELISpot assay. Results: We determined the percentage of donors who exhibited pre-existing antibodies to Cas9 in the serum using an ELISA assay. Compared to 90% of donors with pre-existing antibodies to Cas9, preexisting antibodies to Casφ and Cas12a were identified in 97.5% and 82.5% of donors respectively. We also measured the memory T-cell response to Cas proteins in an ELISpot assay. PBMCs from 95%, 72.5% and 97.5% of donors showed memory T-cell responses to Cas9, Casφ and Cas12a respectively. Conclusion: It has been speculated that pre-existing immunity to AsCas12a and Casφ may be lower than that found in Cas9 which is derived from human pathogens. Our study finds that this is not the case and that immune responses may affect the safety and efficacy to alternatives to Cas9 when used in the clinic.

1300. Utilizing Platelet-Targeted Gene Therapy to Induce Immune Tolerance in Food Allergy

Saurabh Kumar[1], Jocelyn A. Schroeder[1,2], Hongyin Yu[1,2], Talal Chatila[3], Calvin B. Williams[2], Qizhen Shi[1,2]

[1]Blood Research Institute, Versiti Wisconsin, Milwaukee, WI,[2]Dept of Pediatrics, Medical College of Wisconsin, Milwaukee, WI,[3]Harvard Medical School, Boston, MA

Background: Food allergy is a serious public health and economic issue which is an increasing concern for families and clinicians. Food allergy emerges in the context of oral immune tolerance failure, with no cure currently. Our previous studies have demonstrated that platelet-specific gene therapy can effectively induce antigen-specific IgG-mediated immune tolerance in hemophilia A and B models. We hypothesize that targeting allergen expression specifically to platelets will induce IgE-mediated immune tolerance in food allergies. *Objective*: The goal of this project is to develop a novel approach to induce immune tolerance to the allergen. *Methods*: Here, we used OVA as a surrogate allergen for this study. Two mouse models were used in our studies. One was the OVA-specific OT-II model, in which 98% of CD4 T cells are OVA-specific. The other was IL4ra^{F709} mutant mice, a model prone to allergic response. Platelet-targeted OVA expression was introduced via 2bOVA lentivirus transduction of hematopoietic stem cells (HSC) followed by HSC transplantation (HSCT). After HSCT, mice were assessed by PCR for proviral DNA expression and ELISA for platelet-OVA expression levels to ensure successful gene transfer. After bone marrow was fully reconstituted, animals were sensitized by gavage treatment with OVA (10 mg/kg) plus staphylococcal enterotoxin B (SEB, 0.4 mg/kg) weekly for 8 weeks, followed by a high dose of OVA (6 g/kg) challenge. After the challenge, the anaphylaxis reaction was monitored by measuring the changes in body temperature, and tissue samples were collected to investigate if immune tolerance was established in 2bOVA-transduced recipients. *Results:* PCR analysis showed that 2bOVA proviral DNA was detected in all 2bOVA-transduced recipients. Average platelet-OVA expression levels were 11.49 ± 7.49 ng/10^8 platelets in 2bOVA-transduced OT-II HSCs in wild-type recipients (the OT-II model) and 6.82 ± 6.89 ng/10^8 platelets in 2bOVA-transduced IL4ra^{F709} recipients (the IL4ra^{F709} model). After the OVA challenge, within 20 min, the body temperature dropped 1.89 ± 1.29 ^oC and 2.05 ± 0.59 ^oC in the 2bGFP-transduced control mice in the OT-II model and the IL4ra^{F709} model, respectively, but did not change in the 2bOVA-transduced animals. The anti-OVA IgE titers in the 2bGFP group significantly increased, but not in the 2bOVA group. Flow cytometry analysis showed that Th2 cells, a subset of T helper cells that mediate IgE immune responses, in the control group (3.18 ± 2.11% and 2.89 ± 1.39% in the OT-II and IL4ra^{F709} model, respectively) were significantly higher than in the 2bOVA group (0.87 ± 0.60% and 0.40 ± 0.33%). Of note, both Treg and Tr1 cells significantly increased in the 2bOVA-transduced group compared to the control group in both OT-II and IL4ra^{F409} models. Furthermore, Helios$^+$ Treg cells were significantly increased in the 2bOVA-transduced recipients compared to the control group, indicating that platelet-targeted gene therapy can induce Treg cell expansion. In summary, data from our study demonstrated that platelet-targeted gene therapy has the ability to induce immune tolerance through the Th2/IgE pathway. This study proved that ectopic expression of a neo-allergen in platelets is effective in preventing anaphylaxis in allergy models, even when under intense immune challenges. Our findings suggest that platelet-targeted gene therapy is a promising strategy for inducing immune tolerance in food allergy models.

1301. Gene Delivery Using Double Stranded DNA Vectors Triggers a RIG-I Mediated Intracellular Innate Immune Response in CD34+CD90+ Hematopoietic Stem and Progenitor Cells (HSPCs)

Christi Salisbury-Ruf[1], Richard H. Smith[1], Sudipto Das[2], Thorkell Andresson[2], Oladele Oluwayiose[3], Neelam Redekar[3], Pradeep K. Dagur[4], Andre Larochelle[1]

[1]Cellular and Molecular Therapeutics Branch, NHLBI, Bethesda, MD,[2]Protein Characterization Laboratory, Cancer Research Technology Program, Frederick National Laboratory, Frederick, MD,[3]Integrated Data Science Section, NIAID, Bethesda, MD,[4]Flow Cytometry Core Facility, NHLBI, Bethesda, MD

The innate ability of mammalian cells to detect and respond to foreign proteins and nucleic acids poses a significant challenge to many gene therapy applications. This obstacle is especially pertinent in the context of hematopoietic stem and progenitor cells (HSPCs), where such innate defenses can markedly impede the efficiency of successful gene editing. Overcoming these cellular barriers is crucial for the advancement of gene therapy strategies targeting HSPCs. Previous studies have explored various aspects of innate immune responses in HSPCs, but a comprehensive analysis, particularly in CD34+CD90+ phenotypically enriched hematopoietic stem cells, is lacking. To provide a broader understanding of the innate intracellular immune response to DNA and RNA delivery vehicles, we implemented multiplex tandem-mass-tag (TMT) proteomics in sorted CD34+CD90- and CD34+CD90+ HSPC populations exposed to plasmid DNA, the double stranded DNA (dsDNA) virus baculovirus (BV), and the single stranded DNA (ssDNA) virus AAV6. Additionally, as a comparative measure, we included treatments with an RNA-based vector, specifically an integrase-deficient lentivirus (IDLV). Each vector was constructed using the same reporter expression cassette encoding EGFP. Following treatment, sorted cells were harvested at 24 hours for global protein analysis. A total of 4,779 and 5,962 proteins were identified in CD90- and CD90+ cells across all treatments, respectively. Treatment with double stranded plasmid DNA induced 90 and 57 differentially expressed proteins ($p<0.01$, $FC>1.5$) in CD90+ and CD90- cells, respectively, when compared to an electroporation control. The most prominent proteins in the CD90+ group were well-characterized markers of the intracellular innate immune response ISG15, IFIT2, and MX2. We also noted a significant upregulation of the RNA sensor DDX58 (RIG-I) by 7.5-fold in CD90+ cells and 6.1-fold in CD90- cells, along with other RIG-I regulator proteins, namely DHX58 (FC=5.9 in CD90+ cells) and DDX60 ($FC\geq4.1$ in both CD90+ and CD90- cells). Likewise, transduction with a dsDNA vector, baculovirus, elicited a pronounced interferon response, with IFIT3, an enhancer of RIG-I mediated signaling, as the most prominently upregulated protein (FC=6.2). Contrasting with the response elicited by dsDNA vectors, ssDNA vectors, specifically AAV6, did not activate the RIG-I pathway. Among the 52 and 51 differentially expressed proteins ($p<0.01$, $FC>1.5$) in CD90- and CD90+ cell populations, respectively, the top enriched pathway in both groups was p53 signaling, aligning with existing AAV6 literature. Furthermore, our proteomic investigations did not indicate the RIG-I pathway as a major response mechanism to the RNA-based IDLV in either CD90+ or CD90- cells. The overall response to IDLV was less robust compared with other vectors, with only 17 and 15 differentially expressed proteins identified in CD90+ and CD90- cells, respectively, relative to untreated controls. These findings collectively indicate a heightened sensitivity of CD34+CD90+ HSPCs to foreign dsDNA, leading to an inflammatory response mediated through the RIG-I pathway. This unexpected intersection between RNA sensing and exposure to dsDNA has important implications not only for our understanding of how HSPCs sense foreign nucleic acids, but also reveals potential therapeutic targets which can be leveraged to improve HSPC gene editing.

Thursday Posters: Immune Targeting and Approaches with Genetically-Modified Cells and Cell Therapies

1302. Efficient Ggeneration of CD8$^+$ T_{SCM} CAR-Ts by Treatment with Novel Cytokine-Based Scaffolds

Erin Cole[1], Moriah Rabin[1], Niraj Shrestha[2], Jian Hua Zheng[1], Rimas J. Orentas[3], Boro Dropulic[3], Hing Wong[2], Harris Goldstein[1]

[1]Microbiology & Immunology, Albert Einstein College of Medicine, New York, NY,[2]HCW Biologics, Miramar, FL,[3]Caring Cross, Inc., Gaithersburg, MD

Despite the success of CAR-T therapy in treating B-cell malignancies, >40% of patients experience relapse in part due to poor persistence of circulating CAR-T cells. Activation of T-cells with αCD3/αCD28-based reagents prior to transduction with CAR encoding gene vectors is associated with production of a more differentiated phenotype and is associated with reduced functional persistence. T_{SCM}-cells are the least differentiated T-cell population and have a self-renewal capacity that facilitates the diversification of immune memory. We hypothesize that treatment of T-cells with specific cytokines will both drive cell-cycle transitioning to enable lentiviral transduction and allow expansion of T_{SCM}-like CAR-T cells. Using cytokine scaffolds, we evaluated the impact that IL-7-, IL-15-, and IL-21-based activation have on the generation of T_{SCM}-like CAR-T cells specific for either CD19$^+$ B-cell malignancies or HIV-1. HCW Biologics has developed a fusion molecule, which incorporates IL-7, IL-15 and IL-21 cytokine motifs into a tissue factor scaffold (HCW9206) in addition to a scaffold comprised of only the IL-15 motif (HCW9238). To generate CAR-T cells, donor-derived CD8$^+$ T-cells are treated with HCW scaffolds prior to transduction with an αHIV-duoCAR lentivector targeting two epitopes of gp120 or a vector expressing an αCD19-CAR. 5-7 days post transduction we compared T-memory differentiation of HCW-treated to αCD3/αCD28-treated CAR-T cells by flow cytometry. The functional activity of HCW-treated duoCAR-Ts was determined by an HIV suppression assay against autologous CD4$^+$ T-cells infected with an HIV-Renilla luciferase (HIV-LucR) virus expressing either BaL **(Fig. 1D)** or JRCSF **(Fig. 1E)** HIV-1 Env glycoprotein. Cytotoxicity of CD19 CAR-T cells was assessed in a PKH26 cytotoxicity assay against a NALM6 tumor cell line. In addition, cytokine secretion was evaluated following a 16hr co-culture with cell lines expressing the target antigen

using intracellular cytokine staining **(Fig. 2)**. After stimulation with HCW scaffolds, the resulting population of T-cells were enriched in a T_{SCM}-like phenotype, allowing the generation of CAR-T_{SCM}s defined by a $CD45RO^{-}CCR7^{+}CD45RA^{+}CD95^{+}CD27^{+}$ phenotype **(Fig. 1A-B)**. HCW-stimulated CD19 CAR-Ts demonstrated more potent cytotoxic function towards NALM6 than CAR-Ts generated using αCD3/αCD28 crosslinking-based activation **(Fig. 1C)**. Both HCW-stimulated and αCD3/αCD28 activated duoCAR-Ts displayed potent activity against autologous HIV-infected $CD4^{+}$ cells **(Fig. 1D-E)**. Finally, we demonstrated that cytokine stimulation with HCW proteins led to increased expression of pro-inflammatory cytokines by CAR-T cells following co-culture with the target antigen **(Fig. 2)**. Our results indicate that treatment with cytokine scaffolds generates a large population of T_{SCM}-like CAR-Ts which will likely enhance long-term *in vivo* survival of CAR-T therapy across multiple disease types and enable sustained suppression of both cancer and viral malignancies.

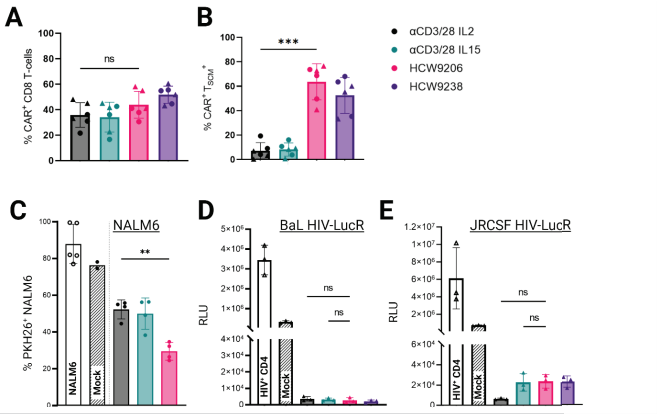


Figure 1: **(A)** CAR expression in $CD8^{+}$ T-cells (triangle: duoCAR; circle: CD19 CAR). **(B)** T_{SCM} enrichment of CAR-Ts (n = 3 donors). **(C)** CD19 CAR-T cytotoxicity of NALM6 at 1:1 E:T. **(D-E)** duoCAR-T suppression of HIV-1^{+} $CD4^{+}$ T-cells at 1:1 E:T (RLU, relative light units) (n = 1 donor). Data is shown as mean ± SEM and significance is calculated by 1-way ANOVA. ***P < 0.001, **P < 0.01, and *P < 0.05.

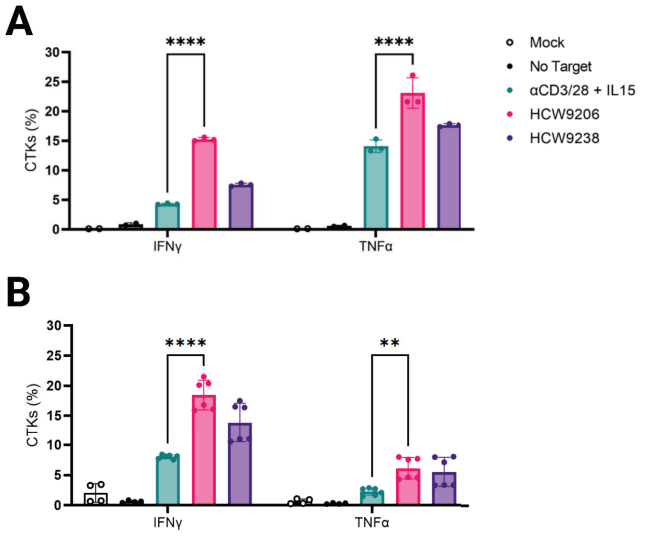


Figure 2: Cytokine (IFNγ, TNFα) secretion by CD19 CAR-T cells following co-culture with NALM6 **(A)** or duoCAR-T cells co-cultured with 293T cells expressing HIV-1 gp120 **(B)** at a 1:1 E:T.Gated on CD8+ T-cells. (n = 2 donors). Data is presented as mean ± SEM and statistical significance is calculated by 2-way ANOVA. ****P < 0.0001, ***P < 0.001, **P < 0.01, and *P < 0.05.

1303. Follicular Targeting CAR-T Cells with PD-1 and CCR5 Knocked Out to Treat HIV Infections

Liliana Kopeck Thron[1], Pongthorn Pumtang-on[1], Jae Woong Chang[1], Joshua B. Krueger[1], Max Cantor[1], Mary Pampusch[1], Sofia Casares[2], Vaiva Vezys[1], Branden Moriarity[1], Pamela J. Skinner[1]

[1]University of Minnesota, Minneapolis, MN,[2]US Navy, Silver Spring, MD

As of 2021, at least 38.4 million people are living with HIV globally. Although ART therapy has dramatically improved patients' survival rate and quality of life, these drugs impose great financial and practical burdens and cannot cure patients of the disease. Recent research has focused on cell therapy as an alternative to small-molecule antiretrovirals. Engineered cell therapies that target viral-producing cells are promising candidates, however, they are not yet being used to treat humans with HIV. One barrier that these treatments face is their inability to access infected cells in the immune-privileged B-cell follicle. To overcome this obstacle, we have developed HIV-specific chimeric antigen receptor (CAR) T cell therapies that employ the CXCR5 receptor to direct CAR cells to the lymphoid follicles. We further engineered the CAR/CXCR5 T cells with CCR5 KO to prevent HIV infection, as well as PD-1 KO to prevent exhaustion. We hypothesize that these optimized CAR/CXCR5 T cells will lead to the suppression of HIV in vitro and in vivo in the absence of ART. To test this hypothesis, we have designed cell constructs and tested them using transposon engineering and in vitro assays. Results from the in vitro studies showed successful dual expression of CAR and CXCR5, KO of CCR5 and PD-1 expression. Engineered cells migrated to the CXCR5 ligand CXCL13 in Transwell assays. When exposed to antigen expressing cells the engineered cells secreted cytokines. When mixed with HIV-infected cells, they reduced the spreading of HIV infections. Compared to mock-engineered cells, the engineered cells were resistant to HIV infection. These studies demonstrate that PD-1KOCCR5KO CAR/CXCR5 T cells are functional in vitro. We next plan to evaluate the efficacy of these optimized CAR T cells in vivo using HIV-infected-humanized mice. These studies may further the development of cell-based interventions for the durable remission of HIV in the absence of antiretroviral drugs.

1304. Follicular Targeting CAR-NK Cells Armored with IL-15 and with PD-1 Knocked out to Treat HIV Infections

Pongthorn Pumtang-on[1], Jae Woong Chang[2], Liliana Kopeck Thron[3], Joshua B. Krueger[3], Max Cantor[3], Mary S. Pampusch[3], Sofia Casares[4], Vaiva Vezys[3], Branden S. Moriarity[3], Pamela J. Skinner[3]

[1]Veterinary and Biomedical Sciences, University of Minnesota, Minneapolis, MN,[2]Department of Pediatrics, University of Minnesota, Minneapolis, MN,[3]University of Minnesota, Minneapolis, MN,[4]US Navy, Silver Spring, MD

Currently there are over 39 million people living with HIV. Although antiretroviral therapy (ART) regimens have produced substantial reductions in HIV-1-related morbidity and mortality, they are not a cure, requiring life-long and strict ART adherence to maintain viral

loads at undetectable levels. Alarmingly, it is estimated that 35% of people living with HIV in the US are not virally suppressed with ART. Moreover, non-adherence to ART can lead to the emergence of ART-resistant HIV. Thus, novel HIV therapies that do not involve ART are critically needed. HIV replication is concentrated in lymph node follicles, where low in vivo effector T cell to target cell ratios permit ongoing replication. Given this, we are developing engineered CAR-NK cells that target lymphoid follicles. We hypothesize that follicular targeted HIV-specific CAR-NK cells will eliminate HIV-producing cells in follicles and lead to the sustained remission of HIV. We have engineered NK cells that express an HIV-targeting CAR as well as the lymphoid follicle-homing molecule CXCR5. In addition, we knocked out the inhibitory PD-1 molecule (95% reduction) to prevent exhaustion and armored the cells with constitutive IL-15 expression to promote replication and persistence. These engineered cells secreted cytokines when exposed to antigen expressing cells, and killed antigen expressing cells. Thus, the PD-1KO IL-15 armored CAR/CXCR5-NK cells were functional in vitro. We are now proceeding to evaluate these engineered cells in vivo in HIV-infected-humanized mice. Humanized-DRAGA mice were infected with HIV and are being treated with ART. Engineered cells or control cells will be infused into the mice to evaluate safety and efficacy. These studies aid in the advancement of novel cell therapies for HIV to achieve durable remission of HIV without ART.

1305. Rapamycin Modifies Transcriptome and Improves Functions of HSCs Based CAR T Cells in HIV Infected Humanized Mice

Wenli Mu[1,2], Shallu Tomer[1,2], Jeffrey Harding[1,2], Nandita Kedia[1,2], Valerie Rezek[1,2], Vaibahavi Patankar[1,2], Ethan Cook[1,2], Heather Martin[1,2], Mayra Carrillo[1,2], Matthew Marsden[3], Scott G. Kitchen[1,2], Anjie Zhen[1,2]

[1]Division of Hematology/Oncology, Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA,[2]UCLA AIDS Institute, University of California, Los Angeles, Los Angeles, CA,[3]Department of Microbiology & Molecular Genetics, School of Medicine, University of California, Irvine, Los Angeles, CA

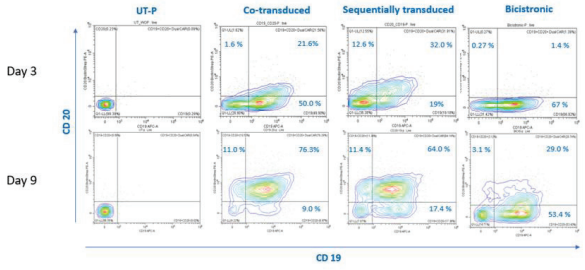
Chimeric Antigen Receptor (CAR) T cell therapy has emerged as a powerful immune therapy for various cancer. Recent studies demonstrated that anti-HIV CAR T cell therapy also have the potential to improve anti-HIV immunity for functional cure. We showed that CAR modified hematopoietic stem cells (HSCs) are capable of lifelong engraftment and allow development of functional CAR-T cells in vivo. Our studies in humanized mice and non-human primates (NHPs) showed that CAR-HSCs transplanted animals have significantly reduced viral rebound after ART withdrawal. However, driven by chronic immune activation, T cell exhaustion remains a major challenge for CAR T therapy and additional measures are needed to achieve functional cure. Our recent studies demonstrated that in vivo treatment of low dose autophagy inducer rapamycin reduced chronic inflammation and improved anti-HIV T cell function in HIV infected humanized mice. Combined treatment of rapamycin and anti-retroviral therapy (ART) resulted in accelerated viral suppression and reduced viral rebound after ART withdrawal. Here we report that rapamycin significantly improved CAR-T cell function both in vitro and in vivo. In vitro treatment of rapamycin improved CAR-T cell mitochondria respiration and cytotoxicity. In vivo treatment of low dose rapamycin in HIV infected, CAR-HSCs treated mice reduced chronic inflammation, prevented exhaustion of CAR-T cells and further lowered viral load compared to CAR-HSCs treatment alone. We also observed significant delayed viral rebound after ART withdrawal and smaller reservoir in mice that were treated with rapamycin and CAR-HSCs as compared to CAR-HSCs treatment alone. RNAseq analysis of sorted CAR-T cells from humanized mice showed that rapamycin significantly modified CAR-T cell transcriptome, including down regulation of multiple check point inhibitors and upregulation of genes related to cell survival. Taken together, our data suggest that HSCs-based anti-HIV CAR-T combined with rapamycin treatment is a promising approach for treating persistent inflammation and improving immune control of HIV replication.

1306. Preclinical Development of Humanized CD19.CD20 Dual CAR Ts for B Cell Malignancies

Hascitha J[1], Dhivya Bharathi[2], Melina Soares[1], Lakshmikanth Satya Gandikota[3]

[1]Research and Development, Immuneel Therapeutics, Bengaluru, India,[2]R&D, Dhivya Bharathi, Bengaluru, India,[3]Immuneel Therapeutics, Bengaluru, India

Background: CD19 CAR T therapies have radically improved the treatment outcomes for patients with B cell malignancies. However, downregulation or loss of CD19 expression remains a challenge and accounts for 27-100% of the relapses seen in B cell malignancies. To address this need, We are developing humanized CD19-CD20 dual CAR Ts. We describe and compare here the preclinical phenotypic and functional characterization of humanized CD19-CD20 dual CAR Ts generated *in vitro* using a single bicistronic lentiviral vector expressing both CD19 and CD20 CARs or generated by co-transduction with two different lentiviral vectors expressing CD19 and CD20 respectively. **Methods:** CD19, CD20 and bicistronic CD19-CD20 CAR lentiviral vectors were designed and synthesized. Different co-transduction conditions were evaluated using our in-house platform to generate CAR T cells expressing > 20% of both CD19-CD20 CARs on the surface. Dual CAR Ts expressing >20% of both CD19-CD20 were also generated using bicistronic CD19-CD20 lentiviral vectors. The dual CAR T cells have been characterized both phenotypically and functionally against tumors cells expressing the target CD19 and CD20 antigens. **Results:** Our results indicate that the CAR Ts had a higher % CAR when generated by sequential transduction with CD19 and CD20 LV as compared to those generated by co-transduction at Day 3. The % CAR in both are comparable at later time points during the process. The current design of the humanized bicistronic CD19-CD20 construct resulted in >20% CAR only after day 7 of the process. We will also present 1) the impact of bicistronic design changed on dual CAR expression at earlier time points in the process and 2) the phenotypic and functional characterization of the CAR Ts. **Conclusion:** Our results indicate that the method of transduction (co vs sequential) and the Bicistronic CAR design affect dual CAR expression. This has implications on the CAR T manufacturing process and design of bicistronic CAR constructs. **Figure 1:** % CD19-CD20 dual CAR Expression on CAR T cells at days 3 and 9. CD19 and CD20 CARs were detected using CD19 and CD20 recombinant proteins respectively.

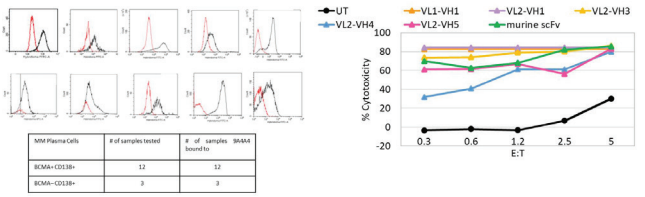


1307. A Novel Membrane-Proximal Humanized CD307e-Specific CAR T (IMN-005) for Multiple Myeloma: Preclinical Development and Potency

Dhivya Bharathi[1], Xing Du[2], Hascitha J[1], Rohan Kamat[1], Giridhar Chenji[3], Lakshmikanth Satya Gandikota[1], Abdullah Ali[2], Melina Soares[1], Siddhartha Mukherjee[2]

[1]Research and Development, Immuneel Therapeutics, Bengaluru, India,[2]Medicine, Columbia University, New York, NY,[3]Manufacturing, Immuneel Therapeutics, Bengaluru, India

Background: Multiple Myeloma (MM) remains an incurable disease. CAR-T cell therapy, targeting antigens such as BCMA have shown proven therapeutic efficacy in MM. Consistent with previous reports, our analysis of bulk and single cell transcriptomes from normal and cancer tissues identified CD307e as an attractive immunotherapeutic target on Multiple myeloma. We are developing IMN-005, a novel anti-CD307e humanized CAR T cell product. This comprises a humanized scFv with a 4-1BB co-stimulatory domain that recognizes a membrane proximal region of the CD307e molecule. We describe here the identification and characterization of anti-CD307e scFv's. We also demonstrate its binding specificity for MM patient samples. In vitro functional potency of several humanized IMN-005 CAR T cells (having different scFv modifications) against MM cells is also presented. These studies are the first to demonstrate the anti-myeloma potency of humanized anti-CD307e CAR T cells targeting a membrane proximal domain of CD307e. **Methods:** Hybridoma supernatants were generated after immunization of mice with peptides selected from the membrane proximal domain of CD307e and were screened *in vitro*. Clones were screened for cross-reactivity with FCRL proteins by ELISA and binding with CD307e expressing cell lines by flow cytometry. They were also analysed for binding with bone marrow plasma samples from MM patients. The murine and humanized version of scFv from the lead clone was used to make various CAR constructs. These constructs of IMN-005 CAR T cells were tested for their functional potency against CD307-expressing MM tumor cells. **Results:** A total of 44 hybridoma clones showing strong binding to the immunizing peptides were chosen for further screening. They differed in terms of their cross-reactivity profiles with other FCRL proteins within the FCRL family. Around 10% clones showed binding to CD307e-expressing cell lines. Of those clones, Clone 25 showed consistent binding to all the multiple myeloma patient bone marrow samples evaluated. SPR-based binding studies studies suggest that the K_D of clone 25 is in the nanomolar range. Humanized IMN-005 CAR T cells with different variations of scFv had significant cytotoxicity against CD307-expressing tumors *in vitro*. The cytotoxicity of the humanized CAR T cells against MM patient samples is currently being evaluated. **Conclusion:** Clone 25 binds to plasma cells in all the multiple myeloma bone marrow samples evaluated. Binding data from a limited number of multiple myeloma patient samples suggests that Clone 25 binds to both BCMA+ and BCMA- plasma cells. Humanized clone 25-derived IMN-005 CAR T cells have potent cytotoxic activity against CD307e-expressing multiple myeloma tumor cell lines *in vitro*. Both the binding data and the *in vitro* anti-tumor activity suggest that IMN-005 may have therapeutic efficacy in all multiple myeloma patients. This is the first report of the development of a membrane-proximal CD307e-directed humanized CAR T cell product. **Figure 1:** Binding of clone 25 to Multiple Myeloma patient plasma cells; Red = secondary alone, Black = clone 25 **Figure 2:** Cytotoxicity of clone 25 scFv-derived humanized CAR T cells (black = untransduced control T cells, colored lines = multiple humanized CAR T constructs, green = murine scFv CAR T cells))



MM Plasma Cells	# of samples tested	# of samples 9A4A4 bound to
BCMA+CD138+	12	12
BCMA−CD138+	3	3

1308. Immunosuppressive Monocytes Suppress CART19 Functions Through Modulation of the IL-1 Pathway

Kun Yun, Reona L. Sakemura, Ismail Can, Omar Gutierrez Ruiz, Ekene J. Ogbodo, Truc N. Huynh, Claudia Manriquez Roman, Olivia Sirpilla, Carli M. Stewart, James H. Girsch, Jennifer M. Feigin, Long Mai, Hong Xia, Brooke Kimball, Lionel A. Kankeu Fonkoua, Mehrdad Hefazi, Michael W. Ruff, Elizabeth L. Siegler, Saad S. Kenderian

Mayo Clinic, Rochester, MN

CD19-directed chimeric antigen receptor T (CART19) cell therapy has shown remarkable outcomes in B cell malignancies, but durable remissions are limited to 40%. Inhibitory myeloid cells have been found to contribute to CART19 failure. Here, we aimed to unravel interactions between monocytes, CART19 and tumor cells to understand how monocyte-CART19 interactions impact CART19 effector functions. First, CD28-costimulated CART19 cells generated from healthy donors were cocultured with JeKo-1, a $CD19^+$ mantle cell lymphoma (MCL) cell line, and freshly isolated monocytes or *ex vivo* differentiated M2-like macrophages. CART19 antigen-specific proliferation was significantly inhibited by M2-like macrophages but not fresh monocytes (*JeKo-1+CART19 vs JeKo-1+M2+CART19, p=0.00503*). Transwell assays indicated that suppression by M2-like macrophages was contact independent. Cytokine profile analysis of coculture supernatants showed significant elevation of interleukin-1 receptor antagonist (IL-1ra) in M2-like macrophage cocultures (*JeKo-1+Mono+CART19 vs JeKo-1+M2+CART19, p=0.0292*), which was further confirmed by IL-1ra intracellular staining in M2-like macrophages after coculture. This led us to hypothesize that M2-like macrophages inhibit CART19

by secreting IL-1ra to block IL-1 signaling in CART19. To test this hypothesis, we interrogated the role of IL-1ra in CART19. CART19 + JeKo-1 cocultures were supplemented with IL-1β + IL-1ra or IL-1β + IL-1ra + IL-1ra neutralizing antibody (neuAb). CART19 proliferation was improved by IL-1β (*PBS vs IL-1β, p<0.0001*). IL-1ra inhibited IL-1β-dependent CART19 proliferation (*IL-1β vs IL-1β+IL1ra, p=0.0006*), which was restored by IL-1ra neutralization (*IL-1β+IL-1ra vs IL-1β+IL-1ra+IL-1ra neuAb, p=0.0215*). Next, we assessed the impact of IL-1ra on CART functions in mouse MCL xenografts in presence of M2-like macrophages. NOD-SCID-γ-/- mice were subcutaneously injected with human macrophages and JeKo-1. Mice were then treated with CART19 with IL-1ra neuAb or control IgG for 3 weeks. Combination of CART19 cells and IL-1ra neuAb led to improved antitumor activity based on BLI measurements compared to control IgG (*IL-1ra neuAb vs Control IgG, p=0.0033*). Additionally, expression of IL-1RI, receptor of IL-1β and IL-1ra, was measured on T cells after coculturing CART19, JeKo-1, and M2-like macrophages. M2-like macrophages downregulated IL-1RI expression on CART cells, which might potentially desensitize CART response to IL-1β, resulting in further suppression in CART proliferation (*JeKo-1+M2+CART19 vs JeKo-1+CART19, p=0.001*). Overall, our study revealed a role of IL-1ra in macrophage-induced CART inhibition by blocking IL-1β—IL-1RI signaling.

1309. Harnessing Minicircle Vector to Engineer Potent CD19 Specific CAR T Cells for Targeted Leukemia Treatment

Ali Moghimi-Khorasgani, Naeimeh Rezaei, Abbas Kiani-Isfahani, Kianoush Dormiani

Department of Animal Biotechnology, Cell Science Research Center, Royan Institute, Royan Institute for Biotechnology, Isfahan, Iran, Islamic Republic of

Introduction: Chimeric antigen receptors (CARs) are designed receptor proteins that provide T-cells additional targeting abilities. Chimeric receptors combine antigen-binding and T-cell-activation processes. This technology for producing CAR T-cells targeting cancer-specific antigens has been safe and effective for treating hematological malignancies. For manipulating T cells, minicircle vectors to seem ideal due to long and high-level transgenic expression without viral vector side effects. Minicircle DNA is produced from a parental plasmid and is created by deleting prokaryotic sequences from parental plasmid DNA during enterobacterial recombination. Uncontrolled production of bacterial backbone sequences and antibiotic-resistance genes in classical plasmids may harm target cells. Practically, minicircles, as a minimal vector is, smaller and safer than the common plasmids because they lack the bacterial backbone. The outstanding features of minicircle make it a new nonviral vector with strong potential for various therapeutic applications including cancer immunotherapy. In the present study, we used minicircle as a safe nonviral vector to create CD19-specific CAR T-cells for leukemia treatment. **Material and Methods:** This study aimed to construct and clone the coding sequence of the second-generation CAR into a replicative minicircle (CAR-MC) that encodes CD19-specific CAR. By using electroporation, we could transfer this CAR encoding minicircle (CD19CAR-MC) into human T-cells isolated from peripheral blood. The resultant CAR T-cells were then co-cultured with the cancer cell line containing CD19 antigen and another cell line that did not include CD19 as the control. *In vitro* testing was carried out to examine the activities of the generated CAR T-cells including cytolytic effects, cytokine release, and cell proliferation. **Results:** Our results indicated that CAR-MC-based CD19-specific CAR T-cells preferentially targeted CD19-positive cancer cells with substantial lethal effects compared to CD19-negative cells. In addition, compared to the control T-cells, these CAR T-cells were able to secrete higher levels of some inflammatory cytokines compared to the control group, which confirmed the functionality of these cells for cancer immunotherapy. **Conclusion:** The current research demonstrated that the CAR-MC as a nonviral vector has the potential to induce the antitumor activity of T-cells, which was the primary objective of the study. The encoding of a functional CAR receptor by the minicircle confers upon the T-cells the ability to selectively target CD19 as a tumor-associated antigen and specifically eliminate CD19- containing leukemia cells. The successful generation of the functional CAR-MC-mediated CAR T-cells can be attributed to some of the advantages of the vector. These advantages include a higher transfection efficiency due to the smaller size, the capability of long and high-level transgene expression due to the absence of bacterial sequences, and the lack of immunogenicity and random integration into the genome of the host cells. **Keywords:** CAR T-cell, CD19, Cancer immunotherapy, Leukemia, Minicircle, Nonviral vector

1310. Disproportionality Analysis of T-Cell Malignancy Incidence Following CAR-T Therapy: Potential Causal Association

Nancy Lunney[1,2], Kausik Maiti[3]

[1]Cell and Gene Center of Excellence, Parexel, Newton, MA,[2]Cell and Gene Therapy Center of Excellence, Parexel, Newton, MA,[3]Global Safety Services, Parexel, Newton, MA

The incidence of T-cell malignancies after chimeric antigen receptor T-cell (CAR-T) therapy has warranted increased review following a U.S. FDA report in November of 2023 announcing potential risks following BCMA-directed or CD19-directed CAR-T administration. Herein, we describe a disproportionality analysis (DA) conducted to assess the relative reporting rates of T-cell malignancies as an adverse event in patients receiving FDA approved CAR-T therapy. DA using Bayesian and Frequentist methods was performed on all applicable data within the FDA Adverse Events Reporting System (FAERS). Analysis yielded mixed results in quantitative signal thresholds based on the therapeutic agent employed for the six FDA-approved CAR-Ts evaluated. Results showed increased reporting of T-cell malignancies in patients treated with some CAR-Ts relative to others. FAERS data indicated a total of 8 reports of T-cell malignancies, 6 for Kymriah and 2 for Yescarta. Kymriah demonstrated an Empirical Bayes Geometric Mean (EBGM) score (Lower bound) of 7.109 as compared to Yescarta with an EBGM (lower bound) of 0.987. Reporting Odds Ratio (ROR) was 52.44 for Kymriah and 12.266 for Yescarta, with a Proportional Reporting Ratio (PRR)/Chi Square scores of 52.639/301.954 for Kymriah and 12.259/20.637 for Yescarta. Limitations exist in FAERS data due to lack of harmonized reporting, data quality and under-reporting. Due to these limitations, a large Real World (RW) dataset, TriNetX, was further mined to explore the association of T-cell malignancies with CAR-Ts. RW data from 206,054,910 patients including 78,350 diagnosed with T-cell malignancies revealed 366,540 patients diagnosed with acute lymphoblastic leukemia (ALL), diffuse large B-cell lymphoma (DLBCL)

or follicular lymphoma (FL). Of these 366,540 patients, 14,290 were also diagnosed with T-cell lymphoma. Hence, the background incidence rate of T-cell lymphoma in patients diagnosed with ALL or DLBCL or FL was estimated to be 3.9%. The total number of patients who received Kymriah according to RW data was 960 for which lifetime incidence of T-Cell lymphoma was 2.08% (20 patients out of 960). Furthermore, only 10 out of these 20 patients developed T-cell lymphoma after Kymriah, yielding an incidence rate of 1.04%. This finding indicates that the incidence of T-cell malignancy temporally associated with Kymriah is not greater than the background incidence of T-cell malignancies in patients with ALL, DLBCL and FL. The higher incidence of T-cell malignancies may explain the higher disproportionality scores in the FAERS dataset and indicates this association may be due to the background incidence rate rather than being attributable to Kymriah therapy. Results of this DA, while identifying a quantitative signal for one FDA-approved CAR-Ts in the FAERS database, were refuted by subsequent analysis of RW data indicating that the background incidence contributed to disproportionality. Heightened vigilance in monitoring and reporting adverse events associated with CAR-T is therefore supported by the FAERS data.

1311. Multi-Epitope Specific Cytotoxic CD4+ T Cells for Adoptive Immunotherapy of Myelodysplastic Syndrome

Mithil Soni[1], Prabesh Khatiwada[1], Abdullah Ali[2], Azra Raza[2], Pawel J. Muranski[1]

[1]Columbia Center for Translational Immunology, Columbia University, New York, NY,[2]Department of Hematology & Oncology, Columbia University, New York, NY

Myelodysplastic Syndromes (MDS) are complex hematological disorders with limited treatment options, often progressing to acute myeloid leukemia (AML). Curative allogeneic hematopoietic stem cell transplant (HSCT) is reserved only for younger patients and relapses after HSCT remains common. Hypomethylating agents offer modest benefit to HSCT-ineligible patients. Thus, novel more effective therapies are needed. Several studies have shown safety and efficacy of multi-epitope specific T cell-based adoptive immunotherapy in subjects with high-risk AML or post-HSCT relapse. However, these are T cell population containing a variable mixture of $CD8^+$ and $CD4^+$ T cells with limited potency. $CD4^+$ T cells are often present in tumor infiltrating lymphocytes (TILs) and TIL-like products, displaying antigen-specific activity but have not been formally studied despite their central role in orchestrating the the immune response. In our realistic animal model of adoptive immunotherapy we have previously discovered that antigen-specific $CD4^+$ T cells have a capacity to completely eradicate advanced tumors. Here, we studied the feasibility of generating autologous TIL-like multi-TAA-specific $CD4^+$ T cells with enhanced potency for high-risk patients with MDS and AML.We evaluated a novel *ex vivo* priming strategy to generate highly active $CD4^+$ cytotoxic T cells ($CD4^+$ CTLs) simultaneously targeting multiple TAAs. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors or MDS patients collected under the IRB-approved protocol (AAAF2693). Total $CD4^+$ T cells or naïve $CD4^+$ T cells were purified and cocultured with autologous monocyte derived dendritic cells (MoDCs) pulsed with overlapping peptide libraries spanning PRAME, WT1, NYESO-1 and MAGE-A3 and expanded with conventional cytokines or in the presence of pro-inflammatory cytokines for 10-14 days. Two rounds of stimulation were performed. The reactivity, expansion and proliferative capacity, phenotyping upon repetitive stimulation were assessed. The ability of the final $CD4^+$ CTLs to recognize naturally processed targets was tested by loading autologous antigen presenting cells with full length recombinant proteins.T cells cultured under Type 1/17 conditions displayed significantly increased reactivity against TAAs compared to T cells cultured under conventional conditions (defined here as Th1; Fig. 1A). Furthermore, Th1/17 antigen-specific reactivity was markedly enhanced by repetitive stimulation while Th1 cells showed gradual reduction in the secretion of effector cytokines. Th1/17 cells also expanded significantly better than Th1 cells (displayed enhanced polyfunctionality evidenced by increased IL-2 and Granzyme B secretion indicative of self-renewal and cytotoxicity, respectively (Fig. 1B/C). In CFSE assay Th1/17 cells showed marked proliferative capacity despite long term culture and repetitive stimulation (Fig. 1D). These cells also recognized naturally processed antigen indicating these cells are clinically relevant (Fig 1E). Next, we successfully generated multi-TAA CTLs cells from the PBMCs of multiple MDS patients where Th1/17 cells displayed superior reactivity, polyfunctionality and proliferative capacity, as seen in healthy subjects (Fig 1F/G). In conclusion, e*x vivo* priming with TAAs in the presence of pro-inflammatory cytokines reliably induces multi-TAA $CD4^+$ CTLs

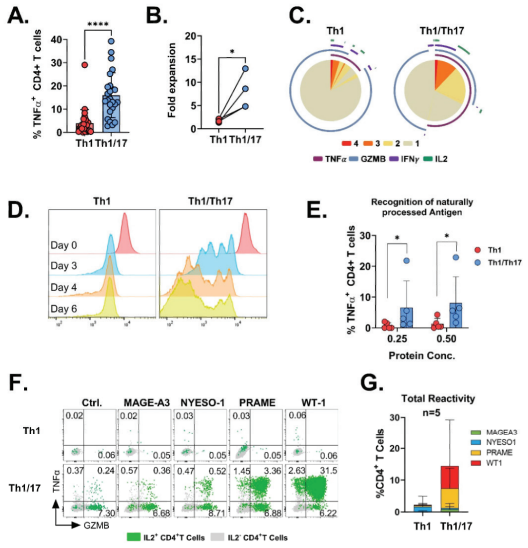


Fig. 1: Generation of autologous multi-epitope specific $CD4^+$ T cells from healthy donors and MDS patient samples. A-E. Generation of PRAME-specific $CD4^+$ T cells from PBMCs of healthy donors under conventional cytokines (Th1) or proinflammatory cytokines (Th1/17). **A.** Frequency of antigen-specific $CD4^+$ T cells after two rounds of stimulation (n=25). **B.** Fold expansion under both conditions after two rounds of stimulation (n=4). **C.** Comparison of Polyfunctionality between Th1 cytokine condition and Th1/17 cytokine condition. **D.** CFSE-based proliferation assay after two stimulations. CFSE labeled cells were re-challenged with antigen after two rounds of stimulation followed by flow cytometric analysis to measure antigen specific proliferation. **E.** Recognition of naturally processed full-length antigen. Antigen specific $CD4^+$T cells were co-cultured with recombinant full-length antigen pulsed autologous dendritic cells followed by intracellular cytokines release assay. **F-G.** Generation of multi-epitope specific $CD4^+$ T cells from PBMCs of MDS patients. **F.** Representative dot plot demonstrating successful generation of multi-epitope specific, polyfunctional, cytotoxic T cells from an MDS patient. **G.** Comparison of overall reactivity of $CD4^+$ T cells from five MDS patients against four TAAs between both conditions.

1312. Characterization of Short Production Cycle and Non-Genotoxic mRNA-Based Anti-CD19 and Anti-CD22 CAR-T Cells

Chenyun Zhang[1,2], Haizhou Liu[2], Hrithik Sangani[2], Yen-Michael S Hsu[2,3]

[1]School of Medicine, University of Tsinghua, Beijing, China,[2]Department of Medicine, Division of Hematology and Oncology, University of Pittsburgh, Pittsburgh, PA,[3]UPMC Hillman Cancer Center, Pittsburgh, PA

The chimeric antigen receptor (CAR) T cell therapy targeting CD19-positive malignancies has shown considerable efficacy in clinical settings. However, the potential genotoxicity of viral-based CAR-T therapy, their considerable manufacturing cycle and high costs, prompts questions about its safety and leads to limitations in clinical application. Aiming to explore a safe, efficient and low-cost mRNA-based CAR-T therapy, we characterize a non-viral, mRNA-based approach utilizing mRNA CAR and electroporation to generate CD19 and CD22-targeting CAR-T cells within only 48 hours with 75% CAR expression. We isolated fresh peripheral blood mononuclear cells from healthy donors and activate them for 30 hours before electroporating with mRNA constructs encoding anti-CD19 and anti-CD22 CAR on the 2nd generation CAR backbone via Maxcyte ExPERT GTx™ GMP Electroporator. Flow analysis indicated that approximately 78% of T cells expressed CD19-CAR eighteen hours post-electroporation, and this transient expression decreased to below 42% after 72 hours (p<0.034, ANOVA). To validate the *in vitro* tumor-killing ability of mRNA-based CAR-T cells, we co-cultured anti-CD19 and anti-CD22 CAR-T cells with ZsGreen-labeled NALM6 expressing CD19 and CD22 antigen. Flow results revealed that T cells electroporated with two mRNA CAR constructs demonstrate robust *in vitro* tumor-killing abilities (Figure 1A). At an E:T ratio of 1:1, 1:3, 1:5 and 1:10, anti-CD19 CAR-T cells can kill 97.24% (p<0.01, t-test), 83.52% (p<0.0001, t-test), 61.75% (p<0.01, t-test), 50.48% (p<0.01, t-test) of NALM6 cells within 24 hours compared to the control T cells. Anti-CD22 CAR-T cells also exhibit similar tumor-killing capability. At the E:T ratio of 1:3, CAR-T cells exhibited a nearly five-fold proliferation (p<0.01, t-test) compared to the MOCK-EP control within 48 hours (Figure 1B). When focusing on the CAR-positive and CAR-negative populations within total T cells, we observed that these two cell populations proliferated at different rates during co-culture. In the presence of target cells, the CAR expression ratio significantly decreased from initial value of 4.48 to 0.47 (p=0.0814, ANOVA) and 1.92 to 0.35 (p<0.05, ANOVA), respectively, for anti-CD19 and anti-CD22 CAR expression detected (Figure 1C). When co-cultured with NALM6 cells, mRNA CAR-T showed a significantly higher intracellular TNFα, IFNγ and IL-2 production (p<0.001, p<0.01, p<0.05, by t-test respectively) compared to MOCK-EP control. Additionally, mRNA CAR-T with higher proliferation index were noted to have a higher Ki67 expression (p<0.01, t-test). In this study, we also focus on the application of CAR-T manufacture in clinical settings which often involves cryopreserving the cells. We find out that short-term cell cryopreservation before and after electroporation does not significantly impact cell viability, CAR expression and anti-tumor functionality. We believe this approach holds promise as a viable alternative that addresses the limitations of current CAR-T therapies, offering a potential solution for future non-viral CAR-T clinical application.

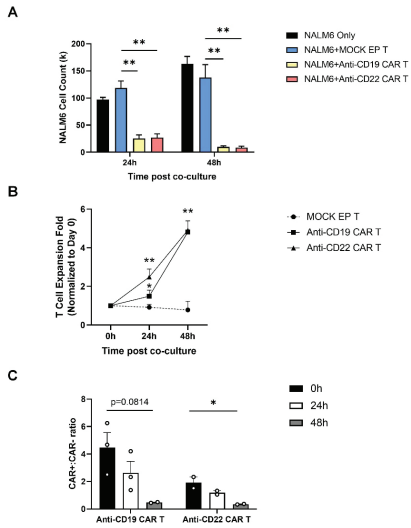


Figure 1. T cells electroporated with mRNA CAR showed in-vitro tumor killing capacity during NALM6 co-culture. Activated T cells electroporated with anti-CD19 and anti-CD22 were co-cultured with ZsGreen NALM6 at an E:T ratio of 1:3 (25k+75k). NALM6 cell count (A), T cell count (B) and CAR-T cell count (C) were measured by flow cytometry at 24h and 48h post co-culture. *p<0.05, **p<0.01, ***p<0.001 by t-test and ANOVA.

1313. Enhanced CAR-T Transduction with Lentiviral Vector (LVV) in Medium Supplemented with Poloxamers P 188 and P 338

Ying Xiong[1], Yanping Xie[1], Zhongyu Zhu[1], Meike-Maria Roskamp[2], Nadya Morales-Cummings[3], Mahsa Mohiti-Asli[3], Nadine Löw[2], Rimas J. Orentas[1], Boro Dropulic[1]

[1]Caring Cross, Inc., Gaithersburg, MD,[2]BASF SE, Ludwigshafen, Germany,[3]BASF Corporation, Tarrytown, NY

Poloxamers are nonionic triblock copolymers with amphiphilic structures. Different poloxamers have been widely applied in pharmaceuticals. By changing the hydrophilic/hydrophobic molar ratio of the subunits of the molecule, unique properties emerge, facilitating delivery of drugs into different cell types or physiological compartments. In combination with polybrene, Kolliphor® P 338 (Synperonic® F 108) has a demonstrated positive effect on LVV transduction of adherent and suspension cell lines, CD34+ hematopoietic stem cells, and primary immune cells. Meanwhile, poloxamer P 188 (F 68) has been broadly applied in the treatment of numerous diseases, taking advantage of its ability to increase cell membrane stability. To further investigate the application of poloxamers in CAR-T cell generation, we explored the combination of these two poloxamers in the transduction and cell-expansion conditions used in the workflow. Purified T cells were activated with activation reagents (anti-CD3, -CD28) on Day 0. LVV were added on Day 1 of activation at different MOIs, followed by a complete medium replacement on Day 3. The transduced cells then were analyzed on Day 8, 10, 13 for CAR-T expression. X-vivo15 (Lonza) with 2% knockout serum replacement (Thermo Fisher) and 30IU/ml IL-2 (Miltenyi Biotec) were used as the basal culture medium. Our studies showed that supplementing both poloxamers in the medium in the transduction phase, and only P188 in the cell expansion phase,

significantly improved CAR T transduction efficiency. In addition, the transduction enhancement demonstrated with different activation reagents at different dilutions was explored. There were no negative effects on T cell expansion and viability throughout the whole process due to the inclusion of poloxamers. In conclusion, our study provides a new strategy to enhance CAR-T transduction with poloxamer supplementation in the culture medium. Improved transduction allowed for the use of less LVV in order to achieve clinically relevant transduction efficiency. This strategy not only helps lower the cost of CAR-T cell generation (as less vector is used), but also provides a novel way to raise transduction efficiency for some LVV applications with complex payloads that are more difficult to employ. Further testing with different culture media and the application of poloxamers in LVV production and stabilization are under further investigation. MarComm-2024-00032

1314. Changes in Glycosylation on the Cell Surface Result in Highly Potent and Long-Lasting Allogeneic CAR-T Therapy in Patients

Zeguang Wu[1], Pengfei Yuan[2], Feifei Liang[2], Yang Liu[3], Jinhong Shi[3], Qiezhong Lamao[1], Jinxin Yang[1], Yuanyuan Qiu[1], Wei Tang[1], Haixia Zhang[2], Zhonghan Yang[2], Ling Yang[2], Yongjian Zhang[2], Ming Jin[2], Weidong Han[3], Wensheng Wei[1]

[1]Peking University, Beijing, China,[2]EdiGene Inc., Beijing, China,[3]Chinese PLA General Hospital, Beijing, China

Adoptive CAR T-cell therapy, a breakthrough in treating aggressive B-cell malignancies, faces logistical and scalability challenges. Allogeneic CAR T-cell therapy, which utilizes donor T cells, offers a viable alternative. However, these cells encounter obstacles such as limited persistence due to host-versus-graft rejections and activation-induced cell death (AICD). Ongoing research aims to enhance the viability of allogeneic CAR T-cells by modifying B2M and HLA molecules, thereby reducing the risk of T-cell mediate rejection. However, this approach introduces new challenges, including the targeting of modified HLA-deficient cells by natural killer (NK) cells. Overcoming these obstacles, including host rejection and AICD, remains a crucial area of focus in T cell-based therapies. In our study, we employed a "bottom up" approach to identify gene targets that could enhance the persistence of primary T cells, counteracting attacks from allogeneic NK and T cells, and the detrimental effects of AICD. Through a combination of whole-genome CRISPR screenings, we have made a significant discovery regarding the role of GeneX in primary T cells. Our findings demonstrate its active inhibitory function on glycan-processing enzymes and its consequential influence on the glycan composition of immune molecules. This influence is pivotal in several immune processes, including potent rejection responses by allogeneic NK and T cells, antibody binding, and the initiation of AICD. Both *in vitro* and *in vivo* testing, using GeneX^{KO}/TCR^{KO}/anti-CD19 CAR T cells, demonstrated that these engineered cells were able to resist the killing of by allogeneic effectors while retaining their anti-tumor activity, without inducing graft-versus-host disease (GvHD). In an on-going related clinical trial (NCT06014073), GeneX^{KO}/TCR^{KO}/anti-CD19 allogeneic CART has demonstrated high anti-tumor activity and persistence for more than 3 months in the first 5 evaluable R/R NHL patients (Data cut-off date is January 20, 2024), with 100% ORR and 60% CR, while maintaining a manageable safety profile. Continuous observation is ongoing. Our research enhances the understanding of the complex interactions among immune effectors and positions GeneX engineering as a promising strategy for creating universal therapeutic cells.

1315. Efficient Genome Editing of Human T Cells with Innovative Transformer Base Editor (tBE) for Construction of Next Generation CAR-T Therapies

Peixue Li[1], Qing Su[1], Ya Xu[1], Jing He[1], Lijie Wang[1], Yichuan Wang[1], Huanyu Li[1], Keiwei Lan[1], Huiming Zheng[1], Di Zhu[1], Lang Zhang[1], Yuchen Zhang[1], Jia Chen[2], Xiaodun Mou[1]

[1]CorrectSequence Therapeutics Co., Ltd, Shanghai, China,[2]ShanghaiTech University, Shanghai, China

Background: Autologous chimeric antigen receptor T (CAR-T) cell therapy has achieved great success for antitumor treatments especially against hematological malignancies, and several autologous CAR-T therapies have been approved across the world. However, autologous CAR-T therapies are highly personalized as the T cells are derived from individual patient and thus also involve complex manufacturing process. This gives rise to some limitations that hinder the broader application of autologous CAR-T cell therapy, such as the long manufacturing periods, the high cost, and the occasional manufacturing failure due to suboptimal cell quality from the patient after earlier lines of cancer treatment.Regarding to the above problems currently faced by the autologous CAR-T therapies, the development of off-the-shelf universal CAR-T cell therapy with donor derived healthy T cells has been proposed to be a promising solution by many companies globally, in which case allogenic T cells need to be genome modified to avoid immune rejection between the T cells and the host. **Methods:** The transformer base editor (tBE) exclusively licensed to CorrectSequence Therapeutics is a recently developed innovative base editing system with high editing efficiency and significantly reduced off-target activity. Here we evaluated the feasibility of tBE system for multiplex editing in primary T cells to construct off-the-shelf CAR-T cell therapy. The simultaneous knockout (KO) efficiency for *TRAC*, *CD52* and *PDCD1* in primary T cells was tested then CAR-T cells were constructed with *TRAC*, *CD52* and *PDCD1* triple KO. The risk for off-target mutations and chromosome translocation was verified and the cytotoxicity efficacy against tumor cells was tested. **Results:** With optimized electroporation conditions for tBE system, *TRAC*, *CD52* and *PDCD1* could be knocked out simultaneously in primary T cells with KO efficiency reaching ~90% for each target gene. None off-target mutation was detected at the potential off-target sites predicted by Cas-OFFinder and Digenome-Seq. Moreover, multiplex editing with tBE doesn't cause chromosome translocations as it won't induce double strand breaks while CRISPR/Cas9 does. CD19 directed CAR-T cells constructed with triplex base editing exhibits comparable antitumor efficacy in *in vitro* and *in vivo* efficacy models compared with unedited CAR-T cells. **Conclusion:** Our results proved that, with comparable editing efficiency and lower safety risk, tBE system might work better for construction of next generation universal or enhanced CAR-T therapies than CRISPR/Cas9 system.

1316. Preclinical Development of TRX103 as an Off-the-Shelf IL-10 Engineered CD4+T Cell Product for the Prevention of Graft-versus-Host Disease

Molly J. Uyeda, Audrey Re, Chunyi Tang, Daryl Humes, Matthew Breton, Michelle Nguyen, Maximilian Richter, Ryan Bjordahl, James Adams, Maria Grazia Roncarolo, Xavier Paliard

Tr1X, San Diego, CA

Hematopoietic stem cell transplantation (HSCT) is a curative therapeutic approach for many hematological malignancies, but its therapeutic potential is limited by a high incidence rate of graft-versus-host disease (GvHD) following transplantation. Type 1 regulatory T (Tr1) cells, an IL-10 secreting regulatory T cell subset, are an appealing therapeutic modality for GvHD prevention as clinical studies have demonstrated that the infusion of cellular products containing Tr1 cells were well tolerated and resulted in a lower GvHD incidence. Here we describe the preclinical characterization of TRX103, an engineered, allogenic cellular therapy product that recapitulates the major regulatory functions of Tr1 cells. TRX103 is designed to halt pathogenic immune responses and (re)-establish immune tolerance and is being developed to prevent the onset of GvHD in patients undergoing HSCT. TRX103 is engineered by separately transducing $CD4^+$ T cells from three donors with a bidirectional lentiviral vector encoding human IL-10 and a non-signaling form of the nerve growth factor receptor (NGFR, CD271). Transduced $CD4^+$ T cells from the three donors are then pooled at an even ratio (1:1:1) to yield the drug product. The TRX103 GMP-compatible manufacturing process consistently yields > 85% $CD4^+CD271^+$ transduced cells. TRX103 did not show activation-independent and IL-2 independent growth properties, demonstrating that its manufacturing process does not induce cellular or molecular alterations. TRX103 secreted IL-10 at rest and secreted high amounts of IL-10, low amounts of IL-4, and variable amounts of IFN-γ and TGF-β following TCR activation. TRX103 displayed a broad polyclonal TCR repertoire and a unique transcriptome profile. The regulatory activity of TRX103 was demonstrated in vitro by its ability to suppress the proliferation of effector $CD4^+$ and $CD8^+$ T cells and the production of pro-inflammatory cytokines by activated monocytes. In addition, TRX103 suppressed the expansion of human effector T cells and prevented the onset of GvHD in a humanized mouse model. Furthermore, the administration of high doses of TRX103 alone or in combination with allogeneic effector T cells in this humanized mouse model did not result in any remarkable pathological findings in the target organs.Together, these data describe the preclinical efficacy and safety of a novel off-the-shelf IL-10 engineered, Tr1-based cell therapy and support a first-in-human Phase 1 clinical study for the prevention of GvHD.

1317. Efficient Lentiviral Transduction of Genetically Barcoded AntiCD20-CAR-IL15 Cassette into Peripheral Blood-Derived Rhesus Macaque NK Cells Using Poloxamer 407

Taha Bartu Hayal[1], Chuanfeng Wu[1], David S. J. Allan[2], Brynn B. Duncan[1], Sogun Hong[1], Cynthia E. Dunbar[1]

[1]TSCBB NHLBI, Bethesda, MD,[2]LTI/CMTB NHLBI, Bethesda, MD

Natural killer (NK) cells can detect and eliminate virally infected and tumor cells, contributing to immunosurveillance and antitumor immunity. NK cell-based immunotherapies are emerging as promising treatments due to their specific killing capacity and lower risk of graft versus host disease in allogenic adoptive transfer settings. NK cell killing activity is optimized by approaches enhancing *in vivo* expansion and persistence of cells as well as genetic modifications improving specificity. Lentiviruses, with an excellent safety and efficiency profile for engineering of immune cells, have been widely used, particularly in engineering T cells with chimeric antigen receptors (CARs) for clinical testing against liquid tumors, notably targeting CD19. However, VSV-G pseudo-typed lentiviruses have proven inefficient in transducing NK cells, leading to the exploration of Baboon endogenous virus envelope (BaEV) lentiviruses. While BaEV can efficiently transduce NK cells, the low virus titer and lack of a clinical track record are hurdles to utilization.The rhesus macaque (RM) model offers a robust pre-clinical platform to evaluate the efficiency and safety of NK-based cell therapies. Our focus is on investigating the clonal dynamics and persistence of CAR-NK cells post adoptive transfer in this model, with and without inclusion of an IL15 transgene in the CAR vector. We inserted an antiCD20-CAR with or without the cDNA for RM sIL15 and linked to a high diversity genetic barcode library to generate VSV-G pseudo-typed lentiviruses and worked to improve primary RM NK cell transduction efficiency. We tested whether inclusion of 100μg/ml of Poloxamer 407 (P407), a non-ionic amphiphilic molecule previously proven to enhance transduction of RM HSPCs (Uchida, et al. 2019), would improve transduction of RM NK cells. We used magnetic cell separation to enrich for RM PB $CD3^{neg}NKG2A^{pos}$ NK cells. These cells were expanded *ex vivo* in 10% heat-inactivated RM serum, 500IU/ml rhIL2 and 2mM Glutamax supplemented X-Vivo20 media together with irradiated (100Gy) universal antigen-presenting (uAPC) feeder cells (Liu et al. 2021), for 5 days before transduction (Fig. A).Stable high transduction efficiencies of 40-60% for RM NK cells (n=4) were achieved with an MOI 20-50 (Fig. B) for a variety of vectors, including with control barcode-GFP, barcoded and non-barcoded CAR-CD20 and CAR-CD20/IL15 constructs. Notably, IL15 "armored" CAR-NK cells exhibited superior growth, even without IL2 supplementation, compared to cells transduced with the non-IL15 CAR vector (Fig .C). The functionality of *in vitro* expanded CAR-NK cells was assessed against ffluc-K562 ($CD20^{neg}$) and ffluc-Raji tumor cells ($CD20^{pos}$) on days 3, 7, and 14 post-transduction. The results indicated better maintenance of CAR-NK function after 14 days of culture for those cultures containing IL15 armored CAR-NK cells (Fig. D).These findings suggest that P407 serves as an efficient transduction enhancer for PB-derived NK cells, resulting in high efficiency transduction even with VSV-G pseudo-typed lentiviral vectors, even those with titer limitations due to the large size of complex CAR cassettes. Currently, we are generating IL15 armored and unarmored CAR-CD20 NK cells

with genetic barcode tags to analyze in both a xenograft tumor murine model and in a RM autologous model to track the function, persistence, and clonal dynamics of these cells *in vivo*.

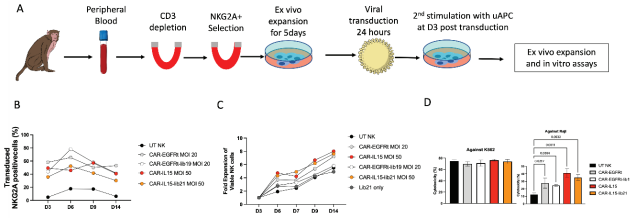


Figure. A) Experimental design. Rhesus macaque peripheral blood derived CD3^{pos}NKG2A^{pos} NK cells were isolated by magnetic cell sorting, and NK cells were expanded and activated *ex vivo* for 5 days. Activated NK cells were transduced with VSV-G enveloped lentivirus in NK growth media supplemented with 100μg/ml P407. After second stimulation with irradiated uAPC, NK cells were further expanded followed with and *in vitro* assays. B) NK transduction efficiency, and transgene expression levels over time compared to untransduced NK (UT NK) cells. C) NK expansion folds over time. D) The cytotoxicity of NK cells against K562 and Raji at 1:1 ratio was measured 2 hours after co-culturing on day 14 post-transduction.

1318. The Direct Addition of RetroNectin® Solutions in the Liquid Phase Improves the Efficiency of Lentiviral Gene Transduction

Yu Okubo, Yoko Kudo, Sachiko Okamoto

Takara Bio Inc., Kusatsu, Shiga, Japan

RetroNectin is a recombinant human fibronectin fragment CH-296 that contains three functional domains: the cell-binding domain (C-domain), the heparin-binding domain(H-domain), and the CS-1 sequence. RetroNectin can enhance lentiviral- and retroviral-mediated gene transduction by facilitating the colocalization of target cells and viral particles. This is achieved through specific interactions between RetroNectin and various cell surface integrin receptors. The H-domain of RetroNectin binds to viral particles, on the other hand, target cells primarily bind to RetroNectin through the interaction of their integrin receptors VLA-5 and VLA-4 with the fibronectin C-domain and CS-1, respectively. By promoting close physical proximity between viral particles and target cells, RetroNectin significantly enhances the efficiency of viral-mediated gene transfer. RetroNectin is now considered the gold standard for enhancing viral transduction in various clinical applications involving gene-engineered cells, including T cells and hematopoietic cells. There are several RetroNectin-mediated transduction protocols, such as the supernatant (SN) method and the RetroNectin-bound virus (RBV) method. In the SN method, cells are mixed with viruses and loaded onto a vessel coated with RetroNectin. In the RBV method, the viruses are first bound to the RetroNectin-coated vessel, and after removing the supernatant, cells are added to the vessel with the virus. The addition of centrifugation in these protocols can further increase the transduction efficiencies. Furthermore, we have also developed a method that combines the RBV method with low-temperature shaking (RBV-LTS method) for large-scale closed cell processing systems. This method has been successfully applied in the manufacturing of CAR/TCR-T cells to enhance transduction efficiency. CAR/TCR-T cell therapy holds great promise as an adoptive immunotherapy for cancer treatment. However, there are several challenges in manufacturing high-quality, clinical-grade gene-engineered T-cell products that must be addressed in order to standardize manufacturing methods and achieve consistent results. To simplify and streamline the gene transduction process, it is feasible to incorporate RetroNectin as a transduction enhancer directly into the liquid phase instead of using RetroNectin-coated vessels. This modification would reduce the number of steps involved and can be easily implemented in automated cell processing systems. In this study, we evaluated the efficiency of enhancing gene transduction by directly adding RetroNectin into the liquid phase during the transduction process of T cells using lentiviral vectors. The results showed that the direct addition of RetroNectin and lentiviruses to the activated PBMCs significantly improved transduction efficiency in T cells, with a 1.5 to 3-fold increase compared to viral infection without RetroNectin. This improvement was similar to the transduction efficiency achieved using the static SN infection method on RetroNectin-coated plate. Importantly, the addition of RetroNectin did not have any adverse effects on cell viability, cell growth, or cell phenotype. In summary, the direct addition of RetroNectin solutions without vessel coating can enhance viral transduction efficiencies. This approach simplifies the gene-engineered cell manufacturing process, including CAR-T and TCR-T production, by reducing the number of steps involved. Moreover, it can be easily integrated into automated cell processing systems.

1319. ML Guided High-Throughput Discovery Pipeline Identifies Best-in-Class CAR-T Candidates

Danielle L. Fairbrass, Arif Kirmizitas, Rui Rodrigues, Alice Godson, Edward King, Jake Leigh Watson, Jennie A. Drew, Jake Burton, Jonathan Lees, Annabel Ling, Benedikt Bornschein, Nils Y. Hammerla, Yulia Lampi, Simon Bornschein

coding.bio, Oxford, United Kingdom

Chimeric antigen receptor (CAR)-T cell therapy, a promising cancer treatment, is expanding its applications to target autoimmune disorders, neurobiology, and ageing. Industry standard CAR-T development relies heavily on i) selecting an antibody based on affinity screening and ii) selecting high-affinity binders in the context of a CAR. However, recent data challenge the notion that the strongest binder always leads to the best CAR, revealing that low-affinity binders can enhance CAR-T activity and improve patient responses (Ghorashian *et al.*, Nature Medicine 2019). We have developed a platform for holistic engineering of 5 key CAR domains, integrating *in vitro* library-based screening with ML-based generative modelling. Through high-throughput testing of 10,000s of CARs / binders we generate biologically relevant datasets at scale enabling the fine-tuning of large transformer-based sequence models (Vaswani *et al.*, NIPS 2017). Beyond merely identifying optimal CARs, our platform facilitates the design and functional exploration of novel CARs with best-in-class performance. Further, our data confirms that the strongest binder is not always the best CAR, and we observe that even subtle modifications within coding regions have a profound impact on performance. Our collaborative wet-lab and *in silico* approach has demonstrated efficacy in identifying best-in-class CARs and dual CARs for clinically relevant targets. We are developing the platform further to expand its applicability to multiple cell types, targets, and modalities, promising a versatile and impactful tool for advancing CAR-T therapy. References: Ghorashian, S., Kramer, A.M., Onuoha, S. et al. Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR. Nat Med 25, 1408-1414 (2019). Vaswani, A., Shazeer,

N., Parmar, N., Uszkoreit, J., Jones, L., Gomez, A.N., Kaiser, Ł. and Polosukhin, I., 2017. Attention is all you need. Advances in neural information processing systems, 30.

1320. Examining the Reciprocal Effects of Natural Killer and Acute Myeloid Leukemia Cell Interactions on Immune Checkpoint Receptor Expression

Danielle G. Jones[1], Natalie Holl[1], Jun Choe[2], Ruyan Rahnama[1], Megan Zinsky[1], Arjun Modi[1], Challice Bonifant[1]

[1]Johns Hopkins University School of Medicine, Baltimore, MD,[2]Baltimore, MD

Background: Recent developments have shown that engineered cell immunotherapies are a promising anti-cancer treatment option for hematopoietic malignancies, namely Acute Myeloid Leukemia (AML). The process of developing cell immunotherapies requires the expansion of a selected lymphocyte from donor peripheral mononuclear blood cells (PBMCs). We utilize Natural Killer (NK) cells with AML antigen-specific chimeric antigen receptors (CARs) to target and kill AML blasts *in vitro* and *in vivo*. NK cell activity is carefully mediated by a balance of signals integrated from activating and inhibitory receptors present on the NK cell surface. One group of these regulatory receptors is commonly referred to as the Poliovirus Receptor (PVR) family, which includes the inhibitory checkpoint molecule T cell immunoglobulin and ITIM domain (TIGIT). We are also interested in TIGIT's complementary activating receptor DNAM-1 present on NK cells, and the ligands that bind these checkpoint receptors on AML blasts: CD112 and CD155. Monoclonal antibody blockade of TIGIT is a promising strategy that has been pursued by numerous pharmaceutical companies to enhance effector cell function. Therefore, we endeavored to investigate the surface expression of these immune checkpoint receptors, competing activating receptors, and binding ligands using flow cytometry. This work provides insight into the dynamic receptor expression levels on NK cells and AML blasts in disease-relevant conditions and provides a basis for pursuing a CAR-NK cell inhibitory receptor modifications. **Methods:** We have designed and optimized fluorescent antibody staining panels to monitor relevant PVR family receptors on both AML blasts and NK cells. Molm-13, MV4-11, KG-1a, and OCI-AML3 AML cell lines were used. Qualitative histogram analysis was performed to confirm that NK cell and AML blast populations could be differentiated when the two sample types were mixed. Next, NK and AML cells were mixed at a 1:5 E:T ratio for 24 hours, then stained with our optimized NK and AML antibody panels. The resulting histogram and median fluorescence intensity (MFI) data was compared to that of NK and AML cells stained individually to measure differences in PVR receptor expression after interactions between effector and target cells. **Results:** NK and AML cells were resolvable when mixed at a 1:5 ratio with distinct populations of cells separated by CD123 or NKp46 expression. Flow cytometry data from 24-hour coculture samples indicated that while there are no significant changes in the percentage of TIGIT$^+$ NK cells (Figure 1A), there are significant decreases in the percentage of DNAM-1$^+$ NK cells across all four cell lines used for coculture when compared to NK cells alone ($p<0.0001$ vs NK alone, Figure 1B). **Conclusions:** We have successfully characterized the composition of NK cell populations in terms of TIGIT and DNAM-1 receptor levels when cocultured with four AML cell lines and found that there is a significance decrease in the DNAM-1$^+$:TIGIT$^+$ NK cell ratio. These results show that in the presence of AML blasts, NK cells are more likely to be inhibited via TIGIT than activated via DNAM-1. With this information, we intend to test TIGIT blockage using CAR-NK cells, with a hypothesis that CAR-NK cell function can be enhanced by preventing TIGIT inhibition. We believe this will ultimately both increase NK cell AML-targeted cytotoxicity and prevent NK cell exhaustion.

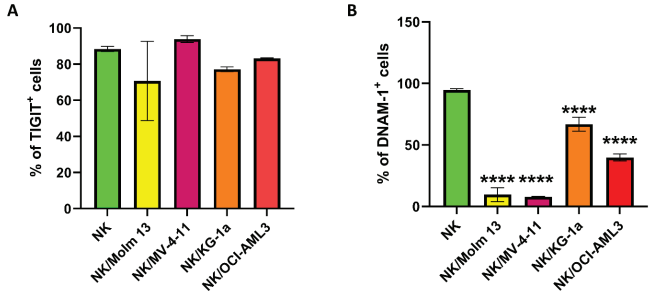


Figure 1. The effects of AML/NK coculture on TIGIT and DNAM-1 surface receptor expression

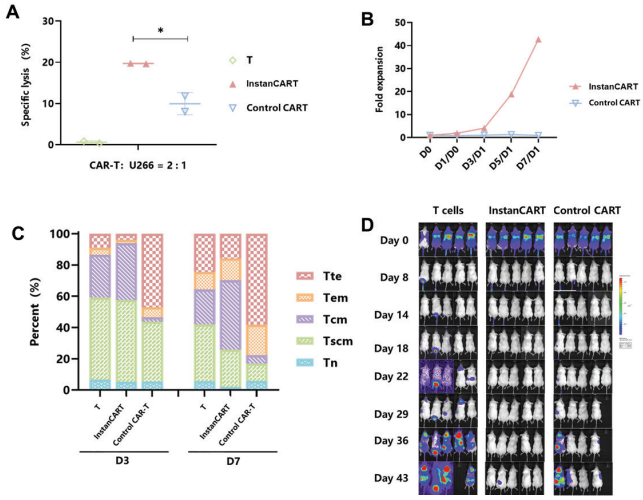
1321. A Rapid CAR-T Preparation Process InstanCART Improves Cell Stemness and Tumor-Killing Capabilities

Yujing Liu[1], Guanghua Liu[2], Yinkang Liu[1], Ting He[2], Xin'an Lu[1]

[1]Beijing Cygenta BioTechnology Co., Ltd, Beijing, China,[2]Beijing Imunopharm Technology Co., Ltd, Beijing, China

CAR-T therapy effectively targets refractory tumors, notably in hematological malignancies, by engineering patients' immune cells into Chimeric Antigen Receptor T-cells (CAR-T). These specialized cells aim to eliminate cancer cells upon reinfusion into the patient. Despite its efficacy, CAR-T therapy poses challenges, including a lengthy production cycle, individual variability, and complex manufacturing. The average timeline spans one to two weeks, with an extra week for quality release. Research has shown that extended *ex vivo* cultivation of T cells can compromise the presence of original cell subpopulations, including naive and stem cell memory T cells (Tscm) and central memory T cells (Tcm), in the final products of CAR-T cells. These subpopulations are closely linked to the therapy's therapeutic potential. To overcome the shortcomings of traditional CAR-T preparation, we produced InstanCART cells which preserves the stemness of T cells, do not require long-term *in vitro* culture. The production cycle can be completed in 2-3 days. Initially, T cells are enriched from the patient's leukocytes, cultured for 24 hours following lentiviral transfection for the expression of Chimeric Antigen Receptor. After collection and washing, the cells are formulated into the final product. InstanCART cells demonstrate increased CAR-T cell expansion potential with reduced exhaustion levels, and heightened anti-tumor activity. Compared to traditional CAR-T cell products, the InstanCART rapid production process allow for a substantial reduction in reinfusion dosage, holding the promise of providing patients with more effective and durable responses, enhancing long-term outcomes, and mitigating the risk of severe adverse events. In our

cytotoxicity experiments, we assessed the targeted killing efficiency of InstanCART cells in comparison to traditional CAR-T cells. The results reveal that InstanCART cells demonstrate specific cytotoxicity against BCMA-positive target cells (U266), showcasing a significantly elevated killing capacity in comparison to traditional CAR-T cells (Fig. A). Furthermore, when exposed to stimulation from BCMA-positive target cells, InstanCART cells exhibit a notably higher proliferation level than traditional CAR-T (Fig. B). Additionally, our investigation into the differentiation level of InstanCART cells affirms that the shorten *ex vivo* cultivation of CAR-T cells produces T cells with a reduced degree of differentiation, thereby significantly amplifying their effector functions (Fig. C). To further evaluate the therapeutic impact, we employed NCG mice to establish a multiple myeloma model through tail vein injection. The ensuing comparison of anti-tumor effects between InstanCART cells and traditional CAR-T cells, as illustrated in Fig. D, underscores that traditional CAR-T cells can inhibit the proliferation of tumor cells in mice. Moreover, a halved dosage of InstanCART cells significantly inhibits the proliferation of tumor cells in mice. This suggests that InstanCART cells wield potent tumor-inhibiting functions under reduced dosage conditions. Our initial data suggests that the current InstanCART rapid preparation process is proficient in generating low-differentiation CAR-T cells, showcasing favorable therapeutic efficacy and safety in both *in vitro* and *in vivo* experiments. The BCMA targeted InstanCART therapy, holds promising clinical prospects for effectively treating multiple myeloma.



Figure, A, Comparison of Cytotoxicity Levels between InstanCART and Traditional CAR-T (*, $P<0.05$). B, Comparison of expansion Levels between InstanCART and Traditional CAR-T. C, Comparison of T Cell Subtyping between InstanCART and Traditional CAR-T. D, InstanCART cells targeting BCMA significantly inhibit the growth of tumors in mice.

1322. Assessing the Influence of Non-Signaling Domains on Anti-TIM3 CAR T Cell Development for the Treatment of AML

Kristine Cate S. Pe[1], Sirirut Jewmoung[2], Ali Hosseini Rad S.M.[3], Koramit Suppipat[2,4], Supannikar Tawinwung[1,4]

[1]Department of Pharmacology and Physiology, Chulalongkorn University, Bangkok, Thailand,[2]Chulalongkorn Comprehensive Cancer Center, King Chulalongkorn Memorial Hospital, Bangkok, Thailand,[3]Kite Pharma, Santa Monica, CA,[4]Cellular Immunotherapy Research Unit, Chulalongkorn University, Bangkok, Thailand

TIM3 is overexpressed on AML cells and is absent in majority of normal hematopoietic cells. CAR T cells expressing the anti-TIM3 antigen-binding domain acquire specificity for AML. However, the effect of the non-signaling domain remains largely undefined with anti-TIM3 CAR. To investigate the effect of the spacer and transmembrane (TM) non-signaling domain, we generated TIM3-targeting CAR with CD8α spacer-CD8α TM (CD8/CD8) or IgG2-CH3 spacer-CD28 TM (CH3/CD28) bearing 4-1BB and CD3ζ endodomain. T cells transduced with CD8/CD8 yielded higher anti-TIM3 CAR positivity than with CH3/CD28 (CD8/CD8=82.8± 3.9 and CH3/CD28=36.7± 3, p=0.0001). The same phenomenon was observed even when the scFv was substituted with anti-CD19, CD8/CD8 construct exhibited higher CAR positivity than CH3/CD28. As TIM3 was expressed on activated T cells, we screened for its expression on CAR-T and determined the extent of fratricide by frequency of apoptotic cells. Compared with CH3/CD28, CD8/CD8 exhibited higher TIM3 expression and enhanced fratricide. Nonetheless, the absolute number of T cells in both constructs continuously expanded as fratricide was transient and CH3/CD28 proliferated more than CD8/CD8. Despite the significant difference in expression level, both constructs exhibited dose-dependent killing of $TIM3^+$ AML cell lines while sparing the $TIM3^-$ target cell line, wherein CD8/CD8 exhibited superior cytotoxic function. Together, our results demonstrate that non-signaling domain contributes to anti-TIM3 CAR T cell functionality. Anti-TIM3 CAR with CD8/CD8 construct has potential clinical advantages due to its higher CAR expression and anti-tumor activity. Therefore, this construct was used to further develop anti-TIM3 CAR with different costimulatory moiety to obtain the optimal CAR design.

1323. An Envelope-Modified Lentiviral Vector for Efficient Gene Delivery to Human Natural Killer Cells

Jing Huang

OBiO Tech, North Hollywood, CA

Natural Killer (NK) cells play a crucial role in tumor immunotherapy. The cytotoxicity of NK cells depends on a delicate balance between activated and inhibitory receptors, and it is not restricted by MHC. Allogeneic NK cell-based clinical therapies have demonstrated *in vivo* expansion without inducing graft-versus-host disease (GvHD). While Chimeric Antigen Receptor T cells (CAR-T) have shown remarkable success in hematologic tumors, CAR-NK holds tremendous potential for treating various tumors However, gene delivery for NK cells has posed a challenge. Reports indicate that BAEV-TR and BAVE-less pseudotyped lentiviruses effectively infect NK cells. Yet, we observed

that the titers of transducible lentiviral particles were exceptionally low when packaged with these two mutated envelopes. To identify the most effective method for modifying human NK cells and achieving industrialization, we optimized viral envelopes to allow for the highest levels of transduction (up to 90%) and a significant increase in viral yield (titer up to 5E+06 TU/mL before concentration). Lentiviral virus-transduced primary human NK cells did not show significant alterations compared to untransduced NK cells in aspects such as receptors and markers expression, K562 tumor-killing activity, cell proliferation, etc. Transduction of NK cells with lentiviral vectors containing the Nectin4 CAR expression cassette selectively enhances the cytotoxicity of NK cells against Nectin4-expressing tumor cells. In conclusion, the envelope-modified lentiviral vector proves to be a potent tool for NK cell-mediated cancer immunotherapy.

1324. Assessing Individual Variability in Autologous CD19 CAR T Therapy with PBMC-Humanized Mice

Won Lee, Destanie Rose, James Keck, Jiwon Yang
The Jackson Laboratory, Sacramento, CA

Chimeric antigen receptor T-cell (CAR-T) therapy targeting CD19 represents a significant advancement in the treatment of hematological malignancies. However, its broader application is challenged by inter-patient variability in response and associated toxicities. This study leverages a peripheral blood mononuclear cell (PBMC)-humanized mouse model to capture individual variability in responses to CD19 CAR-T therapy, focusing on efficacy, cellular expansion, and cytokine profiles. Autologous CD19 CAR-T cells, engineered with a CD28 costimulatory domain and CD3z chain, were derived from three different human PBMC donors (A, B, C), exhibiting varying transduction rates of 92%, 52%, and 95%, respectively. This study was structured into two distinct experiments to comprehensively evaluate the therapy's efficacy and toxicity. In our first experiment, NSG™-MHC I/II double knock-out (DKO) mice were humanized with human PBMCs from Donors A, B, or C. Six days post humanization, we treated the mice autologous CAR-T cells. All three CAR-T preparations showed efficacy in eradicating CD19+ cells from the peripheral blood. Notably, CAR-T cell expansion varied among the donors and did not correspond with the transduction purity. Interestingly, Donor B, with the lowest CAR-T purity, showed comparable expansion to others, suggesting factors beyond purity influencing CAR-T behavior. Despite moderate weight loss observed in mice treated with Donor B's CAR-Ts, the anticipated correlation between cytokine profiles and CAR-T expansion was not straightforward. Elevated levels of cytokines typically associated with CAR-T expansion, such as IL-2, IL-6, IL-10, IFNγ, and TNFα, were not seen in Donor B's cohort. In the second experiment, we engrafted DKO mice with Raji-luc B cell lymphoma to increase CD19 targets. Seven days post tumor engraftment, the mice were humanized with PBMCs from Donors A, B, or C, followed by treatment with autologous CAR-T cells. Donor A's CAR-Ts led to the most efficient clearance of Raji-luc, as measured by IVIS bioluminescence imaging. Consistent with Experiment 1, Donor B's CAR-T cells showed the highest level of expansion. However, this did not correlate directly with treatment efficacy, adding complexity to the relationship between CAR-T expansion and therapeutic success. Serum cytokine profiling further illustrated this complexity, with Donor A's efficacious CAR-Ts inducing higher levels of key cytokines like Granzyme A, Granzyme B, IL-9, MIP-1A, and MIP-1B.This study underscores the PBMC-humanized mouse model's effectiveness in mirroring patient response variability in CD19 CAR-T therapy. Our analysis of CAR-T expansion, efficacy, and cytokine profiles sheds light on the mechanisms driving therapy variability. These insights can inform the refinement of CAR-T cell therapies, advancing personalized treatment approaches in hematological malignancies.

1325. Developing a First-in-Class Universal Allogeneic SNAP-CAR NK Cell Therapy

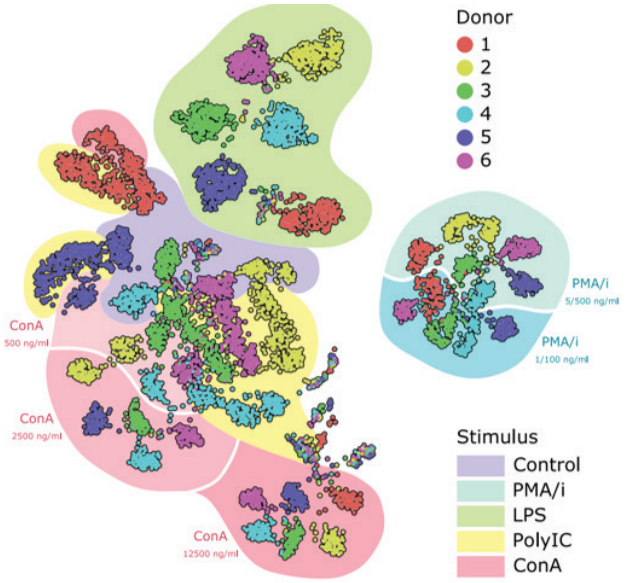
Carrie Stoltzman[1], Erika von Euw[1], Braxton Jamison[1], Emily Hsieh[1], Kevin Green[1], Lara Ionescu Silverman[2], Dan Yerace[2], Dave Mehalick[2], Colleen Delaney[1,2]
[1]Deverra Therapeutics, Seattle, WA,[2]Coeptis Therapeutics, Wexford, PA

Introduction: Natural killer (NK) cells can kill tumor cells without prior activation through their complement of activating and inhibitory surface molecules. Chimeric antigen receptor (CAR) expression by engineered NK cells can improve their innate anti-tumor functions by specifically activating NK cells in the presence of tumor antigen. Compared to autologous CAR therapies, allogeneic CAR NK cells can be generated and cryopreserved for future use, do not require HLA matching and do not cause graft versus host disease or cytokine release syndrome, offering the potential for a safer, more clinically accessible and cost effective cell therapy. Based on demonstrated successful use of a novel SNAP-CAR technology in T cells, we are developing a universal allogeneic SNAP-CAR NK cell. This first-in-class product replaces the extracellular antigen binding domain of a CAR with a SNAPtag enzyme that carries out a self-labeling reaction to covalently attach any antibody conjugated to a benzylguanine (BG) tag to create a functional antigen-specific CAR. **Methods & Results:** SNAP-CAR NK cells were generated from viral vector-transduced cord blood-derived CD34+ cells that were expanded and differentiated to NK cells using a proprietary *ex vivo* cell culture process that utilizes cell activation by a Notch ligand. SNAP-CAR NK cells were assessed for SNAP-CAR expression, NK cell phenotype, and *in vitro* anti-tumor cytotoxicity using BG-conjugated antibody. The SNAP-CAR NK cells displayed viability and phenotyping comparable to the untransduced control NK cells generated in parallel. Anti-tumor cytotoxicity against a CD20+ acute lymphoblastic leukemia (ALL) cell line was enhanced when SNAP-CAR NK cells were co-cultured with a BG-conjugated anti-CD20 antibody in an *in vitro* assay. **Conclusions:** Our off-the-shelf allogeneic NK cell generation platform pairs exquisitely with the highly versatile nature of the novel universal SNAP-CAR platform. Tumor targeting of the SNAP-CAR NK cell therapy can be modulated by the specificity and concentration of the BG-conjugated antibodies used, allowing for one bank of SNAP-CAR NK products to be used across multiple patients and multiple tumor types. Furthermore, for a single patient, multiparametric targeting of cancer can be achieved by infusing SNAP-CAR NK cells functionalized with multiple different antibodies, used concurrently or sequentially to target the tumor microenvironment and different antigens on the same tumor, to impede tumor escape resulting from variation or loss of tumor antigens (*e.g.*, patients who relapse after CD19 CAR T cell treatment due to tumor loss of CD19 antigen could receive follow-up treatment with CD20 and/or CD22 SNAP-CAR NK cells without additional risk of GvHD or CRS).

1326. nELISA High-Throughput Protein Profiling Captures Variability Across Cell Donors and Perturbations - Implications for Cell Therapy

Nathaniel Robichaud, Kevin Hernandez, Milad Dagher
Nomic Bio, Montreal, QC, Canada

The intra- and extra-cellular cues regulating cellular responses are complex, posing a significant challenge for cell engineering. Unfortunately, proteomics tools to capture cellular phenotypes compromise either content or throughput/cost, limiting their use. To overcome this issue, we developed the nELISA: a high-throughput miniaturized ELISA capable of quantifying hundreds of cytokines, chemokines, proteases, and growth factors, at 10x-reduced cost compared to previous tools. Here, we applied the nELISA to donor-derived cell models and demonstrated its ability to capture donor-specific and treatment-specific changes in the proteome. We ran the largest PBMC secretome screen to date, in which ~10,000 PBMC samples were profiled in 1 week, at a throughput of 1536 samples/day. Cells were treated with inflammatory stimuli, and a selected library of 80 recombinant cytokine perturbagens; 191 secreted proteins were profiled in all samples, resulting in ~2M datapoints. The broad nELISA content provided critical resolution power to distinguish between different cell donors, as well as between similar perturbagens, helping to select the most appropriate conditions. For example, IFN gamma was potently induced by >12 perturbagens: IL-23 had almost no impact other than to induce IFN gamma; in contrast, IL-15 also induced IL-1 beta, TNF alpha, CXCL9, CXCL10, and CCL5, whereas IFN beta impacted the expression of >20 other cytokines. Furthermore, the nELISA revealed that IL-1 Receptor antagonist shared all of the immunosuppressive effects of IFN beta, without the latter's pro-inflammatory effects, which can cause adverse events in patients receiving treatment for multiple sclerosis. These findings highlight the ability of the nELISA to capture a wide range of phenotypes across cell donors and perturbations, at a throughput and cost amenable to large-scale efforts. We will expand on these findings and discuss the applicability of the nELISA to selecting ideal cell donors to generate cell therapies, identifying ideal gene edits and culture conditions, as well as strategies for predicting response to therapy.



1327. CasPlus Effectively Prevents On-Target Chromosome Loss and Translocations in Multiplex Gene-Edited Human Primary T Cells

Qiaoyan Yang[1], Michelle Mai[1], Jonathan Abebe[1], Colin Konishi[1], Chengzu Long[2]
[1]NYU School of Medicine, New York, NY,[2]New York University, New York, NY

CRISPR-Cas9 mediated ex vivo T cell engineering enables precise and targeted modification to the T cell genome, facilitating enhanced functionality in autologous and allogenic T cell therapies. Despite these advancements, safety concerns persist regarding the potential complete or partial loss of targeted chromosome and translocations, especially in multiplex gene-edited t cells.Our previous work introduced the CasPlus gene editing platform which includes Cas9, guide RNA and engineered T4 DNA polymerase. This platform successfully alleviated on-target large deletions and chromosomal translocations in cultured HEK293T cells and iPSCs. To evaluate its translational potential, we conducted a comprehensive analysis of on-target large deletions, chromosome loss and translocations in human primary T cells induced by Cas9 and CasPlus editing, utilizing the same guide RNA pairs employed in ongoing clinical trials. Employing PacBio long-read sequencing, we discovered that CasPlus significantly mitigates on-target large deletions when components for Cas9 editing are delivered in the form of both mRNA and RNP. Additionally, through digital droplet PCR, we quantified the on-target chromosome loss, revealing a strong correlation between rates of chromosome loss measured by ddPCR and those estimated by PacBio long-read sequencing. Remarkably, our findings unveiled a negative correlation between rates of chromosome loss and the frequencies of insertions induced by distinct guide RNAs. Furthermore, we compared the translocations induced by the guide RNA pairs with lower rates of chromosome loss and the original ones deployed in the clinical trial. Guide RNA pairs with lower rates of chromosome loss also resulted in a reduced rate of translocations

between targeted chromosomes. The combination of CasPlus with guide RNA pairs exhibiting lower rates of chromosome loss demonstrated the lowest rate of translocations, suggesting that CasPlus can effectively prevent on-target chromosome loss and translocations in multiplex gene-edited human primary T cells.

1328. Revolutionizing Gene and Cell Therapy: The Power of Precision Mutant Libraries

Elaine Lou[1], Xing Gao[2], Andrew Tsao[1], Kevin Chen[2], Cedric Wu[1], Qingwei Meng[2], Jing Min[2]

[1]GenScript USA Inc., Piscataway, NJ,[2]GenScript Biotech Corporation, Nanjing, Nanjing, China

Variant libraries play a crucial role in the early-stage development of Gene and Cell Therapies (GCT). One key application is the modification of Adeno-Associated Virus (AAV) capsids to circumvent immune detection and improve targeting specific tissues. In CAR T-cell therapy, novel Chimeric Antigen Receptor (CAR) constructions are enhancing cell targeting and efficiency, with VHH DNA variant library screenings playing a significant role in improving affinity. Furthermore, the integration of artificial intelligence in computational design is revolutionizing the exploration of a large library of novel genetic sequences, thereby boosting the efficacy of gene and cell therapies. The conventional techniques for creating mutant libraries, reliant on degenerate oligonucleotides and error-prone PCR, often suffer from limited control over the codons introduced, and variants can incur significant codon bias and early termination. This results in a large library with over- or underrepresented variants and incomplete variants, causing a large screening effort on the researcher's part to ensure they are capturing the entire variation of the library. Our proprietary semiconductor-based electrochemical oligonucleotide synthesis technology, known for its high-throughput, precise, and accurate synthesis capabilities, allows customizable codon distribution at each mutated position, preventing the introduction of unwanted or stop codons and limiting codon frequency bias. This technology offers a uniform distribution of amino acids, reduces bias, and excludes stop codons, ensuring the generation of functional proteins. Moreover, its customizable nature facilitates targeted genetic investigations, thereby accelerating the creation of tailored, effective therapies. With approvals of groundbreaking therapies and the integration of artificial intelligence in genetic design, precision mutant libraries are not only enhancing current GCT research but also laying the foundation for a new era of sophisticated, efficacious gene and cell-based therapies.

1329. Next Generation Protein Characterization of CD19-CAR Signaling Cascades

Francisco Ramírez[1], G. Dalton Smedley[2], Charles Haitjema[1], Amrik Singh[3], David Hermanson[2], Chris Heger[1]

[1]Bio-Techne, San Jose, CA,[2]Bio-Techne, Minneapolis, MN,[3]Cell Signaling Technology, Danvers, MA

Chimeric antigen receptor (CAR)-T cell therapy is a revolutionary new pillar in cancer treatment with success in treating specific subsets of B cell leukemia, lymphoma, and multiple myeloma [1]. Much attention is focused on expanding CAR-T cell therapy to other hematologic malignancies and solid tumors. Central to engineering effective CAR-T cell therapies is a detailed understanding of the signaling cascades that regulate CAR-T cells, including trafficking and tumor infiltration, preventing antigen escape, resisting immunosuppressive responses, and ameliorating potentially fatal toxicity [1]. Detailed characterization of extracellular and intracellular signaling molecules is required, often with limited and complex sample types. To study CAR-T signaling, we leveraged a non-viral gene editing platform (TcBuster) to introduce a CD19-CAR into T-cells. Chemically selected cells were isolated and CAR surface expression was verified by flow cytometric analysis using an antibody raised against the peptide linker within the CAR scFv. We then stimulated the CAR-T cells with immobilized CD19 and implemented automated solutions to characterize IFN-γ secretion using automated ELISA (Simple Plex) and intracellular signaling events using a capillary immunoassay platform (Simple Western). The capillary immunoassay provided multiplex protein expression measurements of multiple signaling molecules, including phosphorylated and total protein isoforms, with specific molecular weight characterization, and required only 3 µL of lysate for analysis. Together, these data show the importance of understanding the delicate interplay between signaling molecules that regulate CAR-T function.

[1] Sterner, R.C., Sterner, R.M. CAR-T cell therapy: current limitations and potential strategies. *Blood Cancer J.* 11, 69 (2021).

1331. CD33 Targeted gd T cells for Treatment of Acute Myeloid Leukemia

Justin C. Boucher[1], Anna L. Austin[1], Elena Kostenko[1], Kayla Reid[2], Mate Z. Nagy[1], Marco L. Davila[3], Jose A. Guevara-Patino[1], Nelli Bejanyan[2]

[1]Department of Immunology, Moffitt Cancer Center, Tampa, FL,[2]Department of Blood & Marrow Transplant and Cellular Immunotherapy, Moffitt Cancer Center, Tampa, FL,[3]Department of Medicine and Immunology, Roswell Park Comprehensive Cancer Center, Buffalo, NY

Acute myeloid leukemia (AML) is the most common acute leukemia in adults. Allogeneic hematopoietic cell transplantation from healthy donor stem cells is the only curative therapy for most adults with AML who achieve remission with chemotherapy. However, in close to 40% of patients AML is either chemotherapy refractory or relapses after initial remission, leading to poor survival due to lack of effective treatment options. Prior reports suggest better survival with higher blood or marrow γδ T cell counts in patients with relapsed or refractory AML. However, γδ T cells are rare in the blood and functionally impaired in patients with malignancies. We previously reported an efficient expansion of an allogeneic healthy donor-derived γδ T cells to achieve cell doses sufficient for clinical use. Therefore, we hypothesized that incorporating a chimeric antigen receptor (CAR) into healthy donor γδ T cells would further enhance its efficacy. We used a CAR targeted against CD33 with 41BB co-stimulation and CD3ζ signaling domains in a retroviral vector. When we tested the cytotoxicity of these cells *in vitro* we found that γδ T cells transduced with the CAR had significantly enhanced killing compared to untransduced (UT) γδ T cells (**Figure 1A**). We then examined the effect of γδ CAR T cells on NSG mice given the AML tumor cell line MV411. We found a significant reduction of peripheral blood circulating AML cells at week 4 in the CAR treated group compared to the UT group. We also found that at week 7, 50% (4 out of 8) of mice in the CAR treated group survived, whereas in the UT

and tumor only groups only 12.5% (1 out of 8) survived (**Figure 1B**). This suggests treatment with γδ CAR T cells can reduce tumor burden and increase survival *in vivo*. We subsequently compared γδ CAR T cell and αβ CAR T cell *in vitro* killing. γδ CAR T cells had quicker and longer lasting cytotoxicity compared to αβ CAR T cells at lower effector to target ratios, suggesting that γδ CAR T cells may be more effective in higher stress environments. Additionally, both γδ CAR and UT T cells produced significantly higher levels of IL2 compared to αβ CAR T cells. In contrast, IL6, IFNγ, and TNFα were undetectable in the γδ CAR and UT T cells. This suggests that immunotherapy with γδ CAR T cells could potentially reduce toxicities such as cytokine release syndrome while retaining beneficial autocrine IL2 signaling. Together our data show that a CD33 directed CAR can enhance γδ T cell cytotoxicity against AML both *in vitro* and *in vivo*. This has potential to offer novel allogenic healthy donor derived γδ CAR T cell therapy to patients with relapsed and refractory AML.

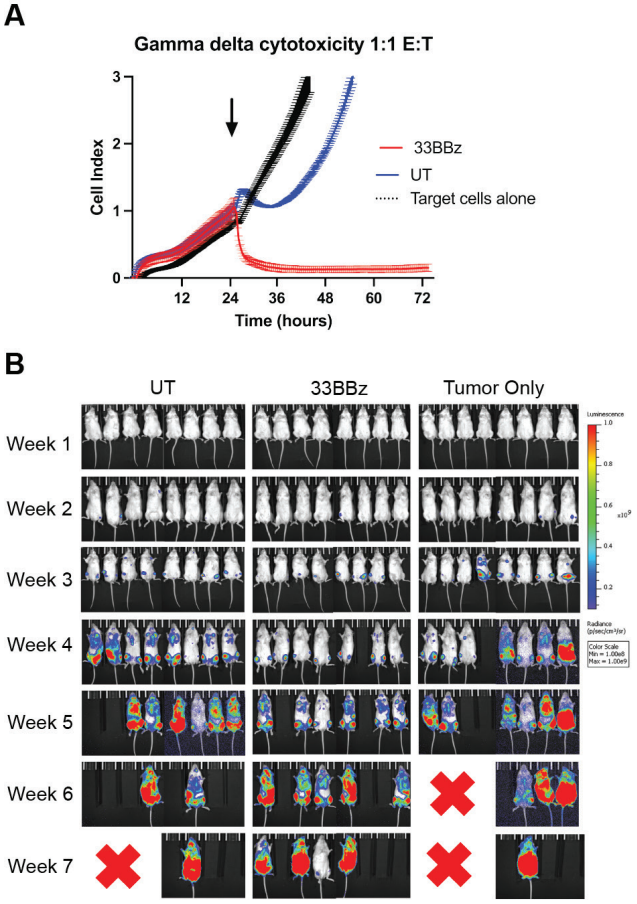


Figure 1. CAR enhances γδ T cell anti-tumor affect. **(A)** γδ CAR T cells (33BBz) were co-cultured with target cells expressing CD33 at a 1:1 effector to target (E:T) ratio. Arrow indicates when cells were added. **(B)** NSG mice were injected IV with $1x10^6$ AML tumor cell line MV411-FFL at week -1. UT or CAR γδ T cells were administered by IV at week 0. BLI was measured weekly. (N=8).

1332. Development of *In-Vitro* Potency Assays to Evaluate Early Activation, Granzyme B Secretion, and Cytotoxicity of CAR-T Cells

Jiu Jiang, Lisa Yu, Imelda Mema, Rachana Patel, Wayne Zhou, Sicco Popma, Bhoopathy Sid

Pharmaron (Exton) Lab Services LLC, Exton, PA

Purpose. The purpose of this study was to develop *in-vitro* potency assays for determining early activation and cytotoxicity of CAR-T cells using flow cytometry and measuring Granzyme B (GrB), a cytotoxic serine protease secreted by CAR-T cells, using ELISA. **Methods.** CAR-T cells recognizing the CD19 molecule on B cells were used as effector cells and Toledo cells from human B cell lymphoma were used as target cells. Toledo cells were incubated and re-suspended in complete medium, then plated in a 96-well round-bottom plate (5,000 cells/well). In parallel, a frozen vial containing anti-CD19 CAR-T cells was thawed and the cells were washed and re-suspended into the medium supplemented with rhIL-2. As effectors, the CAR-T cells were added to the wells in appropriate dilutions to obtain different effector-to-target (E: T) ratios (0.1:1, 1:1, 4:1, and 8:1). Negative-control wells contained the target cells with non-CAR-T cells. The cells were co-cultured at 37°C for 1 h, 2 h, 5 h, and 24 h, and supernatant was collected to measure GrB by ELISA. In addition, harvested co-cultures were stained with various antibodies for flow cytometric analysis determining early activation and cytotoxicity of the CAR-T cells at different times post co-culture. **Results.** Flow cytometry data showed that the percentage of tumor cells ($CD19^+$) was substantially lower when co-cultured with CAR-T cells at the 8:1 of E:T ratio for all time points. 77%, 91%, and 96% of tumor cells were killed at 24 h when E:T ratio was 1:1, 4:1, and 8:1, respectively. Significant upregulation of CD69, an early activation marker, was observed on the CAR-T cells at 5 h. Up to 70% of the CAR-T cells was activated when co-cultured for 24 h at 8:1 ratio while only marginal activation was observed in control T cells. Most remaining tumor cells were dead and/or apoptotic ($7\text{-}AAD^+$ and/or Annexin V^+) in the wells containing CAR-T cells. To investigate whether the expression of GrB, a cytotoxic molecule, was upregulated in the CAR-T cells, the supernatant of co-culture was examined using ELISA. GrB was detected at the earliest timepoint measured (1 h) in co-cultures containing CAR T cells. After co-culture for 24 h, the GrB level of the CAR-T cell group was approximately 20-fold higher than non-CAR-T cells at both 0.1:1 and 8:1 ratio. The data shows that GrB expression coincides with T cell activation and tumor cell death. Therefore, GrB may lend itself as a potency measure for CAR T cells. **Conclusions.** Early activation and a profound cytotoxic effect of CAR-T cells, coinciding with the secretion of GrB in co-cultures, was determined by flow cytometry and ELISA. Upregulation of CD69 and secretion of GrB demonstrate potential value as an *in-vitro* potency assay for CAR-T cells.

1333. Enhancing Transduction Efficiency and Efficacy of Non-Activated CAR T Cells by Modulating IFN1 Signaling

Joseph Durgin[1], Ashwin Sannecy[2], Andre Kelly[2], Roddy O'Connor[2], Saba Ghassemi[2]

[1]Department of Dermatology, University of Michigan, Ann Arbor, MI,[2]Center for Cellular Immunotherapies, University of Pennsylvania, Philadelphia, PA

Chimeric antigen receptor (CAR) T cells have demonstrated their efficacy in treating blood-based cancers. However, the durability of responses is often hindered by challenges related to long-term T-cell persistence and engraftment. The success of CAR T cell immunotherapy relies on the differentiation status and overall fitness of the CAR T cell product. Current protocols involve the activation and ex vivo expansion of patient T cells, however, this activation results in irreversible differentiation, compromising their therapeutic potency.Our recent work showed that employing a manufacturing protocol utilizing non-activated T-cells results in superior differentiation characteristics and reduced exhaustion, thereby promoting long-term tumor control. Despite these advantages, the use of non-activated T cells poses a significant challenge in CAR T manufacturing yield due to the high resistance of quiescent T cells to lentiviral infection.Quiescent T-cells initiate an IFN1-mediated innate response upon lentiviral vector transduction, which limits CAR T-cell transduction efficiency. Our data indicate that pre-treatment of non-activated T-cells with an IFN1-binding protein enhances CAR T-cell transduction efficiency and promotes a more naïve and central memory phenotype. We further evaluated the effect of sustained IFN1 blockade by constructing lentiviral transfer plasmids encoding both a CAR and a secreted anti-IFN1 binding protein. The anti-IFN1-secreting CAR T-cells showed greater transduction efficiency as well as enriched central memory differentiation and reduced TIM3 expression. They also exhibited enhanced cytolytic activity and cytokine production against target cells. In NOD-SCID γc−/− (NSG) mice bearing Nalm6 tumor xenografts, anti-IFN1-secreting CAR T-cells showed sustained persistence and anti-tumor control. These results suggested that the anti-IFN1 protein secretion enhances therapeutic activity against tumors employing sustained type I IFN signaling for immune escape.These studies represent a significant step toward enhancing T-cell fitness by countering anti-viral defenses triggered during manufacturing. Targeting the IFN1 response during non-activated CAR T-cell manufacturing offers a potential solution to improve transduction efficiency and enhance CAR T-cell functionality. Given the therapeutic promise of non-activated CAR-T cells, our findings have immediate translational relevance for broadening their appeal against cancer.

1334. Engineering Gate CAR-T for AML Using Non-Viral Delivery Methods

Rachel Bito, Samy Leon Jambon, Shawn Barman, Babak Moghimi

Cancer and Blood Disease Institute, Children's Hospital Los Angeles, Los Angeles, CA

Survival outcomes for pediatric acute myeloid leukemia (AML) lag significantly behind other pediatric leukemia and lymphomas. Given the unprecedented success of Chimeric Antigen Receptor T cells (CAR-T) for lymphoblastic leukemias, there is a dire need to develop AML-targeted CAR-T cells. However, its success has been limited due to the need for tumor-specific antigen targets in AML and the risk of off-tumor toxicities in the absence of such antigens. Recently, a novel combinatorial strategy using a synthetic Notch receptor called SynNotch has been developed. SynNotch gated CAR-T cells, designed to target a combination of antigens, are programmed to express CAR proteins in response to the presence of specific gated antigens. This logical circuit ensures safe and precise tumor targeting, significantly limiting the risk of off-tumor toxicities. Our team was the first to show clinically relevant application of SynNotch technology in murine models. We have further engineered gated CAR-T cells targeting the CD33-CD123 combination in AML using lentiviral-based engineering. Our preliminary work has demonstrated that these gated CAR-T cells are effective against AML cell lines and patient-derived xenografts in cell culture and in animal models. More importantly, these circuit CARs exhibit a superior T cell phenotype, characterized by a higher percentage of memory phenotype, enhanced metabolic state, and lower expression of exhaustion markers. Despite the advantages of these CAR-T cells over conventional ones, the complexity of engineering them, requiring large cargo and multiple lentiviral vectors, poses a barrier to the clinical translation of these novel immunotherapies. To address this issue, we have engineered these CAR-T cells using a non-viral PiggyBac transposon-based method aiming to facilitate the manufacturing of these novel treatments for AML. We demonstrate that CD123 CAR, expressed either constitutively or through a synthetic Notch receptor engineered using the PiggyBac system, was effective against the MOLM13 AML cell line both in vitro and in a mouse model of AML transplanted with MOLM13 cells. We have successfully engineered these gated CAR-T cells using a larger cargo incorporated into a single frame of the transposon cassette, which has streamlined the process of CAR-T cell selection. The implementation of non-viral methods to create complex CAR-T modules is novel and could significantly contribute to advancing future clinical trials more rapidly and at a lower cost. This approach could translate into improved access and equity of these life-changing treatments for all patients.

1335. Advancing Cancer Immunotherapy: Large-Scale Non-Viral Engineering and Feeder-Free Production of CAR-NK Cells

Deepak Kumar[1], Anna Liu[1], Erica Heipertz[2], Namritha Ravinder[3]

[1]Cell Biology, Thermo Fisher Scientific, Carlsbad, CA,[2]Thermo Fisher Scientific, Frederick, MD,[3]ThermoFisher Scientific, Carlsbad, CA

The use of engineered primary human natural killer (NK) cells holds great promise in immuno-oncology due to their demonstrated safety and their potent anti-tumor properties, especially in the case of solid tumors. Unlike primary T cells, NK cells do not rely on a matching human leukocyte antigen to function, reducing the risk of graft-versus-host diseases in allogeneic therapies. Gene delivery into NK cells is a crucial tool for studying NK cell biology and developing NK cell-based immunotherapies. However, efficient, safe, non-viral gene delivery methods for primary NK cells remain limited. To date, there is very limited published data demonstrating high-efficiency (>20%) non-viral gene knock-in for primary NK cells. In this study, we optimized a scalable, non-viral, closed-system gene delivery method for production of CAR-NK cells. To optimize the gene delivery protocol, we first investigated the effects of different electroporation parameters, including voltage, pulse length, and number of pulses, on cell viability and gene delivery efficiency. We then assessed the impact of various electroporation buffers and DNA concentrations on gene delivery efficiency and cell viability. Here, we present a fully non-viral workflow for achieving permanent knock-in of both a green fluorescent protein (GFP) and a chimeric antigen receptor (CAR) transgene in primary NK cells using the optimized electroporation protocol. We isolated PBMCs

using a closed counterflow centrifugation system followed by negative selection of CD56+ NK cells. Isolated NK cells were then expanded in feeder-free culture using CTS™ NK-Xpander™ Medium for 6 days before proceeding to gene engineering using CRISP/Cas9 RNP and relevant donor DNA delivered via electroporation. We conducted optimization of the electroporation conditions at a small scale (100 µl) using the Neon™ NxT Electroporation System, and successfully translated the optimized conditions to a clinically relevant scale using the CTS™ Xenon Electroporation System. Our optimized non-viral workflow achieves permanent transgene expression of GFP at the Rab11a locus in a single electroporation step by co-delivering a CRISPR-Cas9 RNP and a single-stranded DNA (ssDNA) template for homology-direct repair. We utilized this optimized workflow for non-viral permanent transgene expression of anti-meso3 CAR at the AAVS1 locus in primary NK cells. We also demonstrate the applicability of our electroporation optimizations for combined viral/non-viral workflows by performing non-viral knockout of the AAVS1 locus using electroporation followed by targeted viral knock-in of anti-meso3 CAR using an AAV6 virus. The genome edited NK cells were expanded after electroporation, maintaining high cell viability, and achieving clinically relevant levels of feeder-free cell expansion. To assess the impact of gene delivery on NK cell function, we performed functional cytotoxicity assays. We observed that gene delivery using the CTS™ Xenon Electroporation System did not impair the cytotoxicity of primary NK cells against target cells. The addition of anti-meso3 CAR was shown to enhance targeted killing compared to unmodified NK cells. In conclusion, this study presents closed automated cell processing and scalable, non-viral gene delivery methods for primary NK cells that enable efficient and permanent transgene expression without compromising NK cell viability or function. The ability to genetically modify primary NK cells will facilitate further studies on NK cell biology and enable the development of NK cell-based immunotherapies.

1336. Differential Susceptibility to Senescence in CART Cells Based on Co-Stimulatory Signaling

Ismail Can[1], Leo R. Sakemura[1], Claudia Manriquez Roman[1], Olivia Sirpilla[2], Carli Stewart[3], Kun Yun[3], Truc Huynh[1], James Girsch[3], Ekene Newton Ogbodo[1], Lionel Kankeu Fonkoua[1], Jennifer M. Feigin[3], Long Mai[1], Hong Xia[1], Brooke Kimball[1], Mehrdad Hefazi Torghabeh[4], Michael Ruff[1], Justin Boysen[1], Neil Kay[1], Elizabeth Siegler[1], Berke Alkan[5], Fatih Sezer[5], Atakan Ekiz[5], Saad Kenderian[1]

[1]Division of Hematology, Mayo Clinic, Rochester, MN,[2]Mayo Clinic, Rochester, MN,[3]T Cell Engineering, Mayo Clinic, Rochester, MN,[4]Department of Oncology, Mayo Clinic, Rochester, MN,[5]Molecular Biology and Genetics, Izmir Institute of Technology, Izmir, Turkey

In chimeric antigen receptor T (CART) cell failure, the role of exhaustion has been studied extensively; however, the role of T cell senescence, another dysfunctional T cell fate, has remained unexplored. We developed an *in vitro* model for repeated CART cell activation and rest cycles, mimicking cancer relapse, to study the development of CART senescence. The resting period is essential to differentiate reversible exhaustion and irreversible senescence. Using CD19-targeted CART cells with costimulation via 4-1BB (BBζ) or CD28 (28ζ), we examined CART immunophenotype, cell cycle regulators, transcriptomic profile, and effector functions on days 0 (T cell), 8 (baseline CART), 15 and 22 (CART after one and two activation/rest cycles, respectively). Following an activation/rest cycle, T cell exhaustion markers CTLA4, LAG3, and PD1 returned to basal levels after an initial increase due to activation, while only TIM-3 levels remained elevated (BBζ; p=0.0090, 28ζ; p=0.0394). Despite the lack of an overt exhaustion immunophenotype, recurrent activation/rest cycles induce a state of dysfunction in BBζ and 28ζ *in vitro* and *in vivo*. Increased TIM-3 expression is associated with T cell senescence, which prompted us to further investigate the development of a senescence phenotype. We found elevated T cell senescence markers including p16 (BBζ; p=0.0103), p53 (BBζ; p=0.0248, 28ζ; p=0.0128), CD27 (BBζ; p=0.0080), and CD28 (BBζ; p=0.0327) in recurrently activated-rested CART cells. Overall, senescence-like phenotypes were observed more prominently in recurrently activated-rested BBζ than in 28ζ after one or two activation/rest cycles. Next, we evaluated the transcriptomic signature of recurrently activated-rested CART cells. Gene set enrichment analysis (GSEA) indicated enrichment of gene sets related to senescent T cells as well as senMayo, a novel gene set which is reported to be selectively enriched in senescing cells or tissues, in BBζ D15, but not in 28ζ D15, compared to T cell D0 or in BBζ D15 compared to 28ζ D15 (p=0.00014 and p=0.089, respectively). MYC-related pathways were found to be significantly enriched in recurrently activated/rested BBζ and depleted in 28ζ (p<0.1). Given these results, we induced senescence in CART cells using two alternative strategies: MYC activation (oncogene-induced senescence) and irradiation. Senescence induced by both methods impaired BBζ activity but improved 28ζ activity. Analysis with two independent patient data sets correlated the emergence of senescence with contradicting clinical outcomes to BBζ and 28ζ treatments. The presence of senescent 28ζ cells was associated with complete response in the clinic and longer disease-free survival (p=0.0029), while senescent BBζ cells were associated with non-response in the clinic (0.014). In summary, our study indicates that (i) CART cells are susceptible to a state of senescence, as demonstrated using three independent approaches (repeated activation/rest, irradiation, and MYC modulation), (ii) CART senescence is more prominent in BBζ-stimulated CART cells, and (iii) senescence is associated with worse outcomes following BBζ but not 28ζ treatment.

1337. Precision Biomaterials Enrich and Stimulate Long-Lived CD8+ CAR-T Cells for Supernumerary Expansion *Ex Vivo*

Xiao Huang[1], Yuanzhou Chen[2], Wendell Lim[2], Qizhi Tang[2], Tejal Desai[3]

[1]School of Biomedical Engineering, Drexel University, Philadelphia, PA,[2]University of California, San Francisco, San Francisco, CA,[3]School of Engineering, Brown University, Providence, RI

Chimeric Antigen Receptor (CAR) T cells have reshaped B-cell cancer treatment. However, tumor relapse remains a significant issue, mainly due to the limited post-infusion persistence. Persistence is an intrinsic feature of T cell differentiation, which can be modulated by biomaterials functionalized with precision immunomodulatory signals. Our previous research endeavors resulted in the development of a

DNA-scaffold platform capable of precision surface functionalization. Using microspheres made of biodegradable polymer PLGA (poly-(lactic-co-glycolic aid)) and decorated with controlled ratios of CAR-antigen (EGFR) to CD28 agonist antibody (a-CD28), we stimulated T cells engineered with 2nd-generation anti-EGFR CAR constructs. We identified an optimal ratio of antigen to a-CD28 antibody on microspheres that stimulated the best T cell proliferation for 4-1BB costimulatory (costim.) CAR T cells among 3 human donors (**Fig. 1A**). Importantly, repeated stimulations (every 8-10 days) with this optimal biomaterial formulation sustained continuous T cell proliferation *in vitro* for over 100 days, reaching an overall of ~10^12 folds of expansion (**Fig. 1B**). In comparison, CD28-costim. CAR-T cells did not show a difference of proliferation from various ratios of the two signals on microspheres during the first stimulation. However, T cells made from 2 out of 4 donors did require the optimally ratio-ed formulation for sustainable proliferation with repeated stimulations. Ultimately, we achieved a >100-day-continuous proliferation for both constructs *ex vivo* among >3 donors using precisely engineered biomaterials, whereas cancer cell (U87) stimulations led to cell apoptosis or dysfunction within 5 rounds of stimulation (**Fig. 1B**). Single-cell RNA sequencing shows a clear trend of clonal enrichment along the 100-day culture (**Fig. 1C**) for all tested donors with hallmarks of memory progenitor effector cells (MPEC). Although with extensive proliferation, cells did not show dramatically increased expression of the exhaustion markers (for both mRNA and protein data). Impressively, the massively expanded cells (~10^12 folds) throughout 8 rounds of stimulations retained their effector function from the *in vitro* tumor killing and cytokine secretion assays. Indeed, cells that underwent 3-5 rounds of stimulation showed enhanced effector function and mitochondria fitness compared to unstimulated cells (**Fig. 1D**). Whole-genome transcriptomic analysis 24 hours after the stimulation shows that biomaterials induce signaling that uncouple cell proliferation from excessive immune activation, compared to the cancer cell-mediated stimulation. The up-regulated signaling include DNA repair, mitochondria fitness, G1/S transition, and telomere maintenance. This data perfectly agrees with the phenotypes of massive proliferation, enhanced respiration capacity (by Seahorse assay), and temporally augmented telomerase activity (by TRAP assay). Our ongoing effort is to replicate these trends in CARs targeting different antigens and to test the in vivo anti-tumor efficacy of the expanded cells. This work can have a dramatic impact on our understanding of T cell signaling and fate decision, as well as shed new light on how to engineer cells for improved longevity.

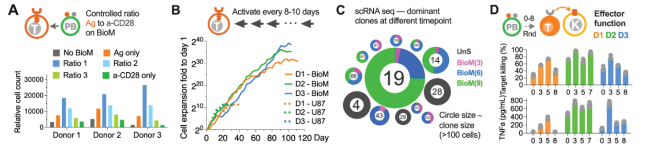


Figure 1. A) CAR-T cell proliferation 5 days after 1-round BioM stimulation. **B)** Expansion fold of T cells after repeated stimulations using BioM (Ratio 1) vs U87 cells. **C)** Distribution of dominant clones after 0 (UnS), 3, 6, 9 rounds of BioM-stimulation through single-cell RNA-seq based TCR repertoire analysis. Circle size of each clone is in scale with the clone size. **D)** Target killing and TNFa secretion of expanded CAR T cells when exposed to K562 cells for 2 days *in vitro* among 3 donors (D1-3).

Thursday Posters: Other Cellular and Regenerative Therapies

1338. Innovative Automation for iPSC Process in Cell Therapy Development: A Closed System Approach

Ranganatha R. Somasagara[1], Olga Cohen[1], Young Kim[1], Pushpalatha Cheluvappa[1], Carl Dargitz[2], Gregory B. Bonello[1], Namritha Ravinder[1]

[1]ThermoFisher Scientific, Carlsbad, CA,[2]Thermo Fisher Scientific, Carlsbad, CA

Induced pluripotent stem cells (iPSCs) provide a flexible platform for diverse clinical development that has incredible potential to address scalability issues and improve patient outcomes. iPSCs can be differentiated into various cell types, such as neurons, heart cells, or pancreatic cells, that can be transplanted into patients to replace damaged or diseased tissues. This approach has shown promise in preclinical and early clinical trials for conditions like spinal cord injury, heart disease, and diabetes. Allogenic cell therapy for cancer treatment has constantly been a developing field in the very recent years as iPSCs have drawn unique interest for the development of clinical manufacturing of CAR-NK and CAR-T cells. iPSC culture and differentiation involve multiple steps and require human intervention. To circumvent the drawbacks of human handling necessary during cell and gene therapy workflows, automation needs to be improved and there are currently limited automated, closed solutions available. he CTS™ Rotea cell processing system can minimize the human handling steps in multiple stages of iPSC process. It is a automated closed cell processing system that uses counterflow centrifugation technology that enables cell separation, washing, and concentration of different cell types. Utilization of instrumentation can eradicate manual effort, related mistakes, and time. In our current workflow, we cultured and expanded iPSC up to 4 billion cells in a 10-layer cell factory system similarly to clinical manufacturing scale for master bank cell preparation. iPSC manual process and harvest in 10-layer cell factory system is very labor intensive and prone to contamination. Utilizing of counterflow centrifugation system can minimize human intervention at multiple stages, including removal of media, washing of cells, addition of cell detachment media, collecting, concentrating iPSCs and delivery to collecting bags. Using this closed system, we can process and harvest large number of iPSCs in a single batch to create a master bank for cell therapy development. The iPSCs prepared using this method retains the pluripotency characteristics with good viability and expansion capability. In summary, using the closed counterflow centrifugation Rotea system reduces human error and minimizes the risk of contamination in large scale iPSC expansion and master banking. This system will be the best method to implement for iPSC workflow in cGMP-compliant clean room manufacturing environment.

1339. Evaluation of Flow Cytometry Utility in Hematopoietic Progenitor Cell Products for Patients with Multiple Myeloma Undergoing Stem Cell Collection

Sarah K. Grewal[1], Ayda Javanbakht[1], Marianne Yee[2], Jeanne Hendrickson[1], Edmund K. Waller[3], H. Cliff Sullivan[1]

[1]Department of Pathology and Laboratory Medicine, Emory University, Atlanta, GA,[2]Department of Pediatrics, Children's Healthcare of Atlanta, Atlanta, GA,[3]Department of Hematology and Medical Oncology, Emory University, Atlanta, GA

Introduction: Peripheral blood stem cell (PBSC) collection plays a pivotal role in the therapeutic paradigm for multiple myeloma (MM). While current regulatory requirements address essential components such as sterility, viability, and cell count, collection and processing practices among different institutions vary. For example, the evaluation of persistent disease is not mandated but some laboratories perform flow cytometry on human progenitor cell apheresis (HPCA) products. Herein, we aim to ascertain the clinical utility of this additional testing in ensuring the quality of PBSC collections for patients with MM. Methods: A retrospective review was undertaken of patients with MM who had autologous stem cell collections from October 2022 through January 2024. Flow cytometry results for HPCA products were unavailable for 4 patients and were excluded. Data extracted via chart review included flow cytometry results for HPCA products, as well as for pre-transplant and post-transplant bone marrow biopsies, and date of stem cell transplant, if applicable. Flow cytometry results were reported as a percentage of monotypic/clonal plasma cells identified in the specimen based on signed hematopathology reports of the leukemia/lymphoma panel using markers for CD19, CD27, CD38, CD45, CD56, CD81, CD117, CD138, KAPPA (cytoplasmic), and LAMBDA (cytoplasmic). Values were categorized as 0%, <1%, or >1%. The Fisher's Exact test executed using SAS v9.4 (Cary, NC) was used to compare results from the HPCA product and the pre-transplant flow result. Results: Of 354 patients with HPCA products, six (1.7%) had positive flow cytometry results, with all instances at <1%. Within the cohort, 109 patients (31%) had positive pre-transplant bone marrow biopsy results, with monotypic/clonal cell values ranging from <1% to 63%. On average, pre-transplant bone marrow biopsies were done 25 days before the date of collection (median 15), and 47 days before transplant (median 31). Out of 183 patients who had post-transplant data available, only 1 out of 6 positive HPCA cases had a result available, which showed no residual disease in the post-transplant bone marrow flow. Additionally, 21 cases (11.5%) displayed low levels (<1%) of monotypic/clonal cells in the post-transplant bone marrow biopsy, despite a negative HPCA product flow result. All positive HPCA products had positive pre-transplant flow results, while 69% of negative HPCA products had negative pre-transplant flow results (Table 1). Of note, none of the negative pre-transplant flow results were associated with a positive result for the HPCA product. Conclusions: These results highlight the infrequent occurrence of positive flow cytometry results for monotypic/clonal cells in HPCA products collected from MM patients undergoing stem cell collection. With less than 2% of patients demonstrating minimal residual disease in the HPCA product, flow cytometric evaluation would appear to represent test overutilization. Although the current study did not look at transplant outcomes, our findings prompt reconsideration of the clinical utility of routine flow cytometry during HPCA processing.

Table 1: Comparison of Pre-transplant and HPCA Product Flow Cytometry Results

	Pre-Transplant Flow Result		
HPCA Flow Result	**0%**	**<1%**	**≥1%**
0% (n=333)	230 (69%)	74 (22%)	29 (9%)
<1% (n=6)	0 (0%)	2 (33%)	4 (67%)

Fisher's Exact test: p<0.0001

1340. Off-the-Shelf *In Vitro* Expanded Peripheral Blood Stem Cells as a Source for Hematopoietic Stem/Progenitor Cell Transplant

Galaxy Gutierrez

Gift of Life Center for Cell and Gene Therapy, Boca Raton, FL

Timely transplant procurement is crucial for patients battling blood cancer since receiving a bone marrow stem cell transplant in the early stages of their disease significantly improves chances of survival. However, this timing remains a significant hurdle for patients in need, especially those of minority ethnic communities, as low availability of compatible donors and overwhelmed collection centers contribute to lengthy acquisition times. Optimistically, there is evidence that the *in vitro* expansion of hematopoietic stem cells (HSC) can be used to increase the restricted number of therapeutic cells in a transplant product. A single donation could potentially be multiplied to treat multiple patients. Having transplants readily available could reduce detrimental waiting time and directly improve patient outcomes. While there has been some documented success for expanding HSC sourced from umbilical cord blood, the greater challenge is achieving a sufficient dose of transplantable *in vitro* expanded HSC therapy from mobilized peripheral blood stem cells (mPBSC). To date, this has not been demonstrated. To contribute to this gap in knowledge we are investigating culturing mPBSC with small molecules from a library that targets key regulatory pathways involved in HSC self-renewal and differentiation. The aim of our project is to develop a cocktail of compounds that promote the *in vitro* expansion of mPBSC while maintaining stemness and therapeutic potential. This therapeutic potential is measured by assessing cell surface markers with flow cytometry for the presence of long term-HSC (LT-HSC), a subpopulation characterized by prolonged self-renewal and identified as the key to the success of bone marrow transplants. So far, we have preliminary results identifying two small molecules that expand LT-HSCs *in vitro* by affecting pathways that either arrest the cell cycle (Glycogen Synthase Kinase-3 inhibitor) or inhibit differentiation (Notch inhibitor). The milestones in our project will contribute to advancing the understanding of the mechanisms governing stem cell proliferation and the maintenance of stemness, ultimately paving the way for more refined and effective stem cell therapies.

1341. Optimizing Melanocyte Transplantation for Enhanced Vitiligo Treatment Outcomes

Sabrina Mai-Yi Fan[1,2], Wen-Jun Chen[1], Po-Hua Chen[3], Tzu-Ying Chen[1], Ming Yao[1,4], Sung-Jan Lin[1,2,3]

[1]Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan,[2]Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei, Taiwan,[3]Department of Dermatology, National Taiwan University Hospital, Taipei, Taiwan,[4]Division of Hematology, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan

Vitiligo, a prevalent disorder characterized by pigment loss, often undergoes various non-invasive treatments, including local immunosuppression and ultraviolet light irradiation. Recently, the transplantation of cultured autologous melanocytes as a cell suspension has emerged as a promising therapeutic option. This study focused on obtaining residual skin specimens of approximately 1 to 2 cm^2 post-surgery, which were subsequently dissociated into single cells and cultured in different Melanocyte Culture Mediums to selectively expand melanocytes. The research revealed significant differences in melanocyte growth across various media, including variations in morphology, growth rate, melanocyte markers, and melanin production. Additionally, the study investigated the expression differences of TYR, TRP1, c-kit, and other markers in different passages of melanocytes under each culture medium. Moreover, this study sheds light on the potential for enhancing melanocyte engraftment effects through careful consideration of culture conditions. The observed variations in melanocyte behavior and repigmentation outcomes emphasize the critical importance of selecting an optimal culture medium for the expansion of melanocytes before transplantation. These findings provide valuable insights into refining protocols for clinical melanocyte transplantation, offering a promising avenue for improving the efficacy of pigmentation treatments. In conclusion, our study establishes a foundation for future research aimed at advancing the field of melanocyte transplantation and optimizing treatment outcomes for patients with vitiligo. By unraveling the complexities of melanocyte behavior in different culture conditions, this research paves the way for more targeted and effective vitiligo treatments, ultimately enhancing the quality of life for affected individuals.

1342. Generation of Universal Stem Cells

Masakazu Hamada[1], Wenhan Yu[1], Crystal Bottenfield[1], Gargi Roy[1], Su Mi Choi[1], Lijuan Du[1], Christina Schindler[1], Christine M. Kiefer[1], Susan D. Wilson[1], Stephen Ireland[1], Ryan Hicks[2], Natalie J. Tigue[3], Michael R. Doran[1], Yasuhiro Ikeda[1]

[1]AstraZeneca, Gaithersburg, MD,[2]AstraZeneca, Gaithersburg, Sweden,[3]Astrazeneca, Cambridge, United Kingdom

Preventing allograft rejection is a major design objective when developing off-the-shelf cell therapy products. Universal stem cells (USCs), or pluripotent stem cells with gene edits that render them hypoimmunogenic, are ideal precursors from which to manufacture allogeneic off-the-shelf cell therapies. Hypoimmunogenic off-the-shelf products will enable therapies to reach more patients faster, by contributing to process standardization that will improve product consistency and reduce manufacturing costs. Herein we describe the engineering of human USC clonal line with an integrated kill switch, and its characterization. We employed two rounds of simultaneous gene knock-out/knock-in to interrupt B2M and RFXAP expression and overexpress HLA-E, CD47 and a simeprevir-inducible Caspase 9-based kill switch. The USC maintained a normal karyotype, stably expressed pluripotency markers, HLA-E, CD47 and the kill switch, and differentiated into three germ layers as well as beating cardiomyocytes or hepatocytes. The USC was resistant to both allo-reactive T and NK cell killing *in vitro*, and kill switch function was validated in undifferentiated USCs, cardiomyocytes and *in vivo*. Our protocol provides a template for the engineering of USCs suitable for the manufacture of off-the-shelf cell therapy products, including an integrated kill switch.

1343. Primary Lung Exosomes Specifically Interact with Hematopoietic Stem Cells and Enhance Long Term *In Vivo* Engraftment Potential

Sanam Rezaei Benam[1], Samaneh Maleknia[1], Wang Xuepeng[2], Reuben Kapur[2,3], Reza Shahbazi[1,3,4]

[1]Hematology/Oncology Division, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN,[2]Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN,[3]Indiana University Melvin and Bren Simon Comprehensive Cancer Center, Indianapolis, IN,[4]Brown Center for Immunotherapy, Indiana University School of Medicine, Indianapolis, IN

Hematopoietic stem and progenitor cells (HSPCs) are a critical population of cells responsible for maintaining the hematopoietic system throughout life. Despite their importance, the regulatory mechanisms controlling HSPC fate decisions, including self-renewal, proliferation, and differentiation, are not fully understood. Recently, exosomes, small membrane-bound vesicles secreted by cells, have emerged as important mediators of intercellular communication in various physiological and pathological processes [1]. Exosomes have been shown to regulate target cell function through the transfer of proteins, microRNAs, and other molecules. Our investigation into the communication between exosomes and HSPCs has yielded promising results, particularly in the context of LT-HSCs. LT-HSCs are a rare subpopulation of HSPCs that play a crucial role in maintaining the blood system over an individual's lifetime due to their unique capacity for self-renewal and differentiation, which is essential for the continuous production of all blood cells. The complex array of molecular mechanisms that govern LT-HSC maintenance, proliferation, and differentiation includes several molecular pathways such as the Wnt, Notch, JAK/STAT, PI3K/Akt, and BMP signaling pathways. Our study demonstrates that exosomes derived from healthy human primary lung (P. Lung) cells can directly interact with LT-HSCs, leading to an enhanced colony-forming potential and increased numbers of LT-HSCs. Flow cytometry analysis has confirmed that up to 80% of the exosome-positive populations exist within the LT-HSC population, compared to a lower interaction rate (5-10%) with MPP or CMP/GMP/MEP populations. Proteomics and miRNA sequencing were performed to identify potential molecules within the exosomes that play a key role in enhancing the stemness potential of HSPCs. Our in vivo data on NSG mice demonstrates that ex vivo treatment

of CD34+ HSPCs with P. Lung exosomes significantly enhances engraftment potential over a 28-week period. Analysis of different lineages reveals improved T cell, B cell, and myeloid cell development in mice engrafted with P. Lung exosome-treated HSPCs compared to untreated mock samples. To further validate these results, secondary transplantation was performed by retrieving cells from the mice bone marrow samples. The secondary transplantation results also confirm the high engraftment potential of P. Lung exosome-treated HSPCs, both in peripheral blood and bone marrow. In summary, our data reveal, for the first time in the literature, that P. Lung cells engage in cross-talk with HSPCs, demonstrating the significant impact of P. Lung exosomes on the modulation of stem cell behavior and engraftment potential.
References1. Butler, J.T., S. Abdelhamed, and P. Kurre, Extracellular vesicles in the hematopoietic microenvironment. Haematologica, 2018. 103(3): p. 382-394.

1344. Scalable Human IPSC-to-3D Bioprinting Pipeline: Successful Large-Scale Production Using Automated Bioreactor Systems

Rukmini Ladi[1], Debbie Ho[2], Stacey Lee[2], Jianyi Du[2], Jonathan Weiss[2], Tony Tam[2], Soham SInha[2], Danielle Klinger[3], Sean Devine[1], Art Hamfeldt[1], Hope Leng[2], Jessica Herrmann[2], Mengdi He[2], Lee Fradkin[2], Tze Kai Tan[2], David Standish[1], Peter Tomasello[1], Donald Traul[1], Noushin Dianat[4], Quentin Vicard[4], Kishore Katikireddy[1], Mark Skylar-Scott[2]

[1]Sartorius Stedim North America Inc, Bohemia, NY,[2]Stanford University, Stanford, CA, USA, Stanford, CA,[3]Sartorius Stedim North America Inc, Stanford, CA,[4]Sartorius Stedim France S.A.S, Aubagne, France

Human induced pluripotent stem cells (iPSCs) are pivotal in advancing tissue engineering, particularly for transplantation therapy and disease modeling. A sustainable approach to organ-scale tissue engineering demands the production of billions of human cells for bioprinting, especially wholly cellular bioinks. However, scalability challenges in conventional 2D cell culture methods, such as cost, space, and handling constraints, present significant hurdles. To address these issues, we optimized and developed a robust, scalable pipeline to produce iPSC aggregates (hAs) using an automated stirred-tank bioreactor system for bioprinting applications. Through a combination of Design of Experiments approach (MODDE® software) and multivariate data analysis, we identified an optimal culture condition from a dataset of 15 conditions with varied impeller speeds, seeding density, and use of polyvinyl alcohol. At the 250mL scale, we achieved a 23-fold cell expansion in 5 days, yielding 1 billion hiPSC hAs with optimal sizes and diameters. In terms of aggregate morphology, over 72% of hAs derived from SCVI-15, a versatile human iPSC line, and 90% of those from WTC-11, known for its genomic stability, had diameters ≤ 300 μm with an overall circularity of ≥ 0.73. Furthermore, pluripotency marker expression was maintained above 90% across three serial passages in SCVI-15 and WTC-11 cell lines. When scaled to a 1L stirred-tank bioreactor, the optimized parameters from the smaller scale remained effective. This successful scale-up to a 1L stirred-tank bioreactor resulted in ~20.4-fold cell expansion by day 4, yielding about 4 billion hiPSC hAs per vessel with >94% expression of pluripotency markers, while maintaining similar high cell density, viability, and circularity for both SCVI-15 and WTC-11 hiPSC cell lines. These pluripotent aggregates were subsequently differentiated into derivatives of the three germ layers, including cardiac, vascular, cortical, and intestinal organoids. The aggregates were successfully compacted into wholly cellular bioinks for rheological analysis and 3D bioprinting. The 3D bioprinted tissues exhibited high post-printing viability and the potential for vascular and neuronal differentiation, highlighting a promising pathway for billion cell-scale organ engineering. Our next objective is to further expand this process by implementing a 10L scale bioreactor system to enhance cell yield and process efficiency.

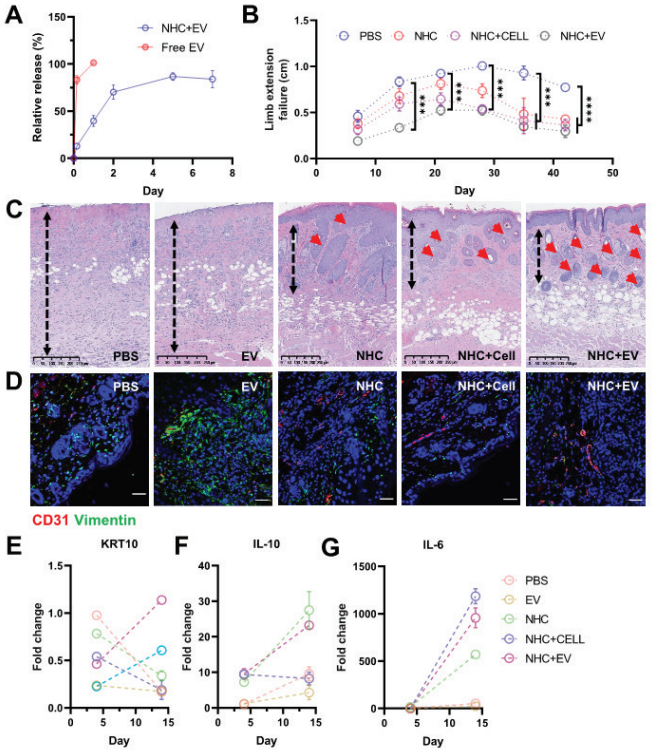
1345. Extracellular Vesicle-Incorporated Biostimulatory Matrix Prevents Radiation Dermatitis

Jiayuan Kong, Hexiang Feng, Kailei Ding, Kedar Krishnan, Sashank Reddy, Hai-Quan Mao

Johns Hopkins University, Baltimore, MD

State of Purpose: Radiotherapies have been a successful oncological treatment and widely conducted in clinical settings. However, around 95% of patients develop side effects associated with soft tissue damage after radiotherapy. These reactions often advance to chronic radiation dermatitis (RD).[1] Currently, there are no effective therapies to prevent or treat RD. There is an urgent need for standardized therapy. Here, we propose a nanofiber-hydrogel composite (NHC) matrix carrying adipose-derived stem cells (ADSCs) or ADSC-derived extracellular vesicles (EVs) to prevent the detrimental, chronic radiation-induced RD. We assessed the degrees of limb extension, hair loss, skin histology, fibroblast deposition, and inflammation-related gene expressions to evaluate the preventive outcomes. **Method**: The NHC matrix was generated according to our previous reports.[3] To assess the efficiency of preventing radiation fibrosis, B6 mice were treated with 30 Gy radiation to the left limb, and injected with NHC, EVs, NHC loaded with ADSCs, or NHC loaded with EVs, subcutaneously right after the radiation to mimic the prevention model of radiation fibrosis. Free EVs and PBS injection were used as controls. For NHC + EV group, the release of EVs was measured *in vitro* in PBS at 37°C. To evaluate the effect of fibrosis on tissue functions, limb extension was measured up to day 42. Skin histology was characterized by comparing the degree of inflammation and integrity of the skin. Fibroblast deposition was also investigated by immunostaining. Lastly, the expressions of three selected genes (KRT10, IL-10 and IL-6) were examined using qPCR. **Results**: Encapsulating EVs into NHC matrix extended EV retention from a few hours to around 5 days (**Fig. 1A**). For the *in vivo* study, a significant prevention of limb extension failure was observed in NHC groups starting on day 14. Moreover, NHC + EV group further enhanced preventive outcome (**Fig. 1B**). The H&E staining analysis suggests that compared to control groups, the NHC treatment with or ADSCs and EVs yielded lower dermal thickness, indicating reduced dermatitis responses. Moreover, NHC + EV showed robust hair follicle formation and preservation of native skin structure (**Fig. 1C**). The immunostaining for vimentin has similar trend as NHC groups showed lower numbers of fibroblasts from NHC containing groups (**Fig. 1D**). The qPCR assay showed that NHC + EV group had an increase of KRT10 and IL-10, indicating the regenerative responses at gene expression level (**Fig. 1E**, **F**). However, the IL-6 level was dramatically elevated in all NHC containing groups, especially in NHC + EV group

(~1000 fold; **Fig. 1G**). **Conclusion:** The ADSC- or EV-loaded NHC matrix allows for off-the-shelf therapy for radiation dermatitis. The stimulatory NHC matrix alone can reverse the detrimental effects of fibrosis, while the addition of ADSCs and EVs enhances the therapeutic effects. **References** 1. Berry, Charlotte E., et al. **Adv. Wound Care**, (2022). 2. Yao, Zhi-Cheng, et al. **Small**, 18.36 (2022): 2202309.



1346. FAST-STEM A Human Pluripotent Stem Cell Landing Pad System for Rapid Design-Build-Test-Learn Development of Human Cell Based Therapeutic Devices

Michael Garton

University of Toronto, Toronto, ON, Canada

Very recent clinical advances in stem cell derived tissue replacement have placed the possibility of human cell-based therapeutic devices firmly within reach. However, development of such devices has proven highly challenging, due to the need for generating stable cell lines for each design-build-test-learn engineering cycle. Current approaches to generating stable hiPSC lines are highly time-consuming and suffer from lack of control, poor integration efficiency, and limited functionality. Validation in clinically relevant stem cell derived tissues is also broadly lacking. We have developed an hiPSC landing pad platform called FAST-STEM (***F**acile **A**ccelerated **S**tem-cell **T**ransgene integration with **S**ynBio **T**unable **E**ngineering **M**odes*) that reduces the engineering time to 'differentiation ready' stem cell lines from several weeks to 6 days. Additional FAST-STEM innovations include: (i) rapid and highly efficient transgene integration; (ii) copy number control; (iii) simultaneous or consecutive integration of multiple gene cassettes; (iv) automatic landing pad reconstitution allowing unlimited consecutive transgene loading and (v) combinatorial library screen capability. In addition to this unique functional versatility, platform transportability and broad use case capability was confirmed by differentiation into eight different tissue types across nine different laboratories. This platform dramatically lowers the bar for integration of synthetic biology with regenerative medicine and paves the way for sophisticated human cell device development.

1347. Molecular Optimization of Transcription Factors and Vectors for High-Efficiency Generation of *In Vivo* Engraftable Motor Neurons

Nathan Wang, Katie Galloway

Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA

Replacement of damaged or diseased tissues and organs represents a major goal of cellular reprogramming. Potentially, autologous cell grafts generated from accessible cell donor populations may be used to regenerate and renew tissues and their function. However, low rates of direct conversion have limited the potential of reprogrammed cells for applications within the central nervous system (CNS) applications. While insight into the reprogramming process may guide new tools for reprogramming, the sparse and stochastic nature of reprogramming has obscured our understanding of how transcription factors drive cells to new identities. To address this challenge, we capitalized on our recently developed chemo-genetic cocktail that increases direct conversion to diverse neurons. Importantly, we first sought to reduce sources of extrinsic variation to resolve how individual transcription factors influence reprogramming outcomes. To this end, we tailored the transcription factor cocktail and minimized the number of viruses. In striking contrast to the prevalent assumption that optimal neuronal cocktails should drive early cell-cycle exit, our cocktail increases conversion to a post-mitotic identity and neuronal maturity by increasing the population of cells that undergo a period of rapid proliferation. By increasing the number of reprogramming events and titrating expression, we were able to identify transcription factor-specific correlations between expression and reprogramming events. Using these insights, we designed improved reprogramming cocktails for direct conversion of adult human fibroblasts to motor neurons and developed minimal vector systems that generate motor neuron grafts that can integrate with the murine central nervous system. With our two-vector system, we can generate induced motor neurons at scale (tens of millions of iMNs). Additionally, we achieve neurotrophic factor-free motor neuron reprogramming and show that our reprogrammed motor neurons survive in vivo grafting. As the in vivo environment may provide varying or limited neurotrophic support, our work highlights that cells can reprogram in the absence of neurotrophic support. Moreover, reprogrammed cells can be grafted following focal injury as needed for the replacement of damaged tissue, opening future studies for in vivo reprogramming and cellular therapies.

1348. Efficient and Reliable Measles Reprogramming Platform for the Generation of iPSC

Sarrianna Hoffer[1], Saksham Vashistha[1], Jessica Dade[1], Carter Caya[1], Brenna Sharp[1], Spencer Majerus[1], Qi Wang[1], Patricia Devaux[1,2]

[1]Department of Molecular Medicine, Mayo Clinic, Rochester, MN,[2]Mayo Graduate School, Rochester, MN

Previous studies from our lab have established the Measles virus (MeV) vector as an efficient and safe method for reprogramming somatic cells into induced pluripotent stem cells (iPSC). However, the efficiency was limited to 0.2%. Here, we present the next generation of the MeV reprogramming vector. The MV(O)(SK)(M) previously established by Rallabandi et al. was modified by substituting the P gene of the wild strain, MVPwt(O)(SK)(M). After validation of the four reprogramming factors OCT4, SOX2, KLF4, and c-MYC expression, and the propagation of the MVPwt(O)(SK)(M) vector, the reprogramming ability was analyzed in 6 adult human fibroblasts (AHF). Reprogramming was achieved at low multiplicity of transduction in 5 out of the 6 AHFs with average efficiency between 1.25-2.25%. Only one AHF was reprogrammed with a low efficiency (0.23%). At day 25 post-transduction, we isolated iPSC clones from each reprogramming. Following iPSC amplification, immunostaining was performed to confirm the induction of endogenous pluripotency-associated markers, SSEA-4, TRA-1-81, TRA-1-60, and NANOG. The iPSC pluripotency propensity was further confirmed by spontaneous and guided differentiation into three germ layers using immunostaining and confocal analysis. The rapid elimination of the vector from the iPSC at or before passage 5 was confirmed using qRT-PCR. Finally, we show the ability of the MeV-derived iPSC to differentiate into hematopoietic stem cells, pancreatic progenitor cells, neuronal progenitor cells, and terminal neurons. In conclusion, our study shows that the latest generation of MeV reprogramming vector is highly efficient and reproducibly reprograms adult human fibroblasts into iPSCs. Compared to other non-integrating reprogramming technologies, such as the Sendai system, MeV has a comparable to higher efficiency using a single vector, a lower multiplicity of transduction, and exhibits a faster vector elimination from iPSCs.

1349. High Throughput Multiomic Single Cell RNA Sequencing Revealing the Exosomal Communication with Long Term Hematopoietic Stem Cells

Samaneh Maleknia[1], Sanam Rezaei Benam[2], Xuepeng Wang[3], Reuben Kapur[3,4], Reza Shahbazi[1,4,5]

[1]Hematology/Oncology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN,[2]Hematology/Ocology, Department of Medicine, Indiana University School of Medicine, Indianapolis, IN,[3]Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN,[4]Indiana University Melvin and Bren Simon Comprehensive Cancer Center, Indianapolis, IN,[5]Brown Center for Immunotherapy, Indiana University School of Medicine, Indianapolis, IN

Hematopoietic Stem and Progenitor Cells (HSPCs) form a critical population in the human body responsible for the continuous replenishment of blood and immune cells. These multipotent cells reside in the bone marrow and have the remarkable ability to undergo self-renewal and differentiation, giving rise to a diverse array of blood cell lineages, including red blood cells, white blood cells, and platelets. HSPCs play a pivotal role in maintaining hematopoietic homeostasis throughout an individual's lifetime, ensuring the body's capacity to respond to various physiological demands and immune challenges. The intricate regulation of HSPC fate decisions, such as proliferation, self-renewal, and differentiation, involves a complex interplay of molecular pathways and signaling networks. Within the broader spectrum of HSPCs, Long-Term Hematopoietic Stem Cells (LT-HSCs) represent a rare subpopulation distinguished by their exceptional capacity for both self-renewal and differentiation [1]. LT-HSCs play a pivotal role in maintaining hematopoietic homeostasis over an individual's lifetime. The intricate regulation of LT-HSC behavior involves a complex network of molecular pathways, including Wnt, Notch, JAK/STAT, PI3K/Akt, and BMP signaling. Recently, we discovered distinctive populations of extracellular vesicles from the human primary lung (P. Lung) cells that significantly contribute to the homeostasis of LT-HSCs. Exosomes are small, membrane-bound extracellular vesicles secreted by cells, playing a pivotal role in intercellular communication. These nanosized structures carry a cargo of proteins, lipids, and nucleic acids, including microRNAs, reflecting the composition of their parent cells. We performed high throughput multiomics single-cell RNA sequencing to understand the potential effects of exosomal communication of P.Lung exosomes with both bone marrow-derived, and G-CSF mobilized peripheral blood HSPCs. We incubated P.Lung exosomes with CD34+ HSPCs for 48 h and performed CITEseq analysis using a full panel of antibodies defining the HSPC populations (CD34, CD38, CD90, CD45RA, CD49f). Our data show a significantly increased cell number and more defined LT-HSC cell type in P. Lung exosome-treated cells in comparison to untreated mock cells. We identified the upregulation of Krueppel-like factor 9 (KLF9) in LT-HSCs. KLF9 plays a key role in the TNF-alpha signaling pathway via NF-kB. This pathway is important in LT-HSC self-renewal, regulation of hematopoiesis, and maintenance of LT-HSCs. It is also important in keeping the balance between self-renewal and differentiation. Likewise, we identified significant upregulation of Nucleoporin 50 (NUP50) compared to the untreated mock sample. NUP50 is responsible for the regulation of the G2-M Checkpoint pathway which is critical in preventing uncontrolled proliferation or premature differentiation. At the same time, it contributes to the overall maintenance of the LT-HSC pool by ensuring the fidelity of the cell division process. In conclusion, we have identified multiple regulatory genes through our single-cell analysis of the exosomal communication with LT-HSCs. This is the first report revealing the intricate molecular pathways regulated by exosomes from human lung cells contributing to LT-HSC self-renewal and maintenance. **References** 1. Notta, F., et al. Science, 2016.

1350. Atelocollagen Supports Three Dimensional Culture of Human Induced Pluripotent Stem Cells

Yoshiki Nakashima, Masayoshi Tsukahara

Research and Development Center, CiRA Foundation, Kyoto, Japan

Currently, the standard approach for production of human induced pluripotent stem cells (hiPSCs) for clinical purposes is through use

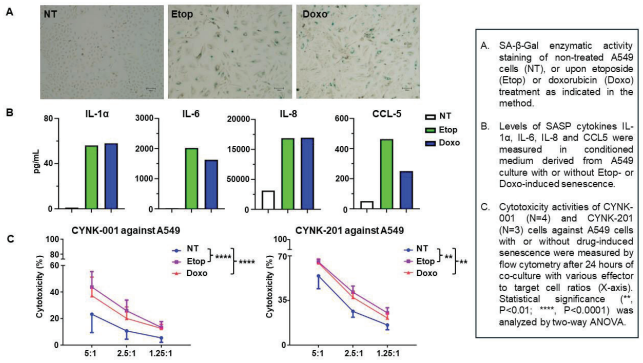
of two-dimensional (2D) cultures, i.e. cultures in which the cells are attached to a substrate coated with scaffold material. Although this approach provides viable hiPSCs, the method does have some limitations as it does not lend itself to upscaling for mechanized cell production using culture dishes or for automated culture processes for industrialized mass production of clinical iPSCs. As autologous iPSC therapy requires a custom-made small-lot cell production line, the cell production method differs significantly from the existing processes for producing allogeneic iPSC stocks for clinical use. Specifically, mass culture to produce stock is no longer necessary; instead, a series of operations from iPSC production to induction of differentiation of therapeutic cells must be performed continuously. A 3D culture method using small, closed-cell manufacturing devices is suitable for autologous iPSC therapy. Use of such devices avoids the need to handle many patient-derived specimens in a single clean room; handling of cell cultures in an open system in a cell processing facility increases the risk of infection. We have screened a number of candidate materials for 3D culture of hiPSCs. One of the most promising of these is atelocollagen, a low-immunogenic derivative of type I collagen obtained by removing the N- and C-terminal telopeptide components. Atelocollagen has an atomic mass of 300 kDa and has a rod-like structure with a length of 300 nm and a diameter of 1.5 nm. It has been clinically applied in various settings, such as wound healing, as a bone cartilage substitute, and as a hemostatic agent. In the present report, we describe the characteristics and quality of hiPSCs cultured on atelocollagen. We also investigated the mechanism underlying their adhesion ability. In order to reduce the risk of rejection during cell therapy transplants, the ultimate goal is to generate iPSCs from the patient's own cells as this will enable the production of self-compatible differentiated cells that can be transplanted into the patient. Here, we sought to establish a cell manufacturing process using autologous iPSCs and focused on 3D cultures as these have the desirable characteristic of supporting automated culture. This information has enabled us to propose a potential cell manufacturing process using atelocollagen. In this study, atelocollagen beads were evaluated as a three-dimensional biomaterial to assist 3D culture in the establishment, expansion culture, and induced differentiation of iPSCs. It was found that iPSCs can be handled in a closed-cell device with the same ease as use of 2D culture when Laminin-511 is added to the medium. In conclusion, atelocollagen beads enable 3D culture of iPSCs, and the quality of the obtained cells is at the same level as those derived from 2D culture.

1351. Human Placental Hematopoietic Stem Cell Derived Natural Killer Cells Preferentially Eliminate Senescent Cells Derived from a Tumor Model

Li Li[1], Irene Raitman[2], Valentina Rousseva[2], Lin Kang[2], Adrian Kilcoyne[2], Robert Hariri[2], Yuechao Zhao[2]

[1]Celularity Inc, Florham Park, NJ,[2]Celularity Inc., Florham Park, NJ

Although natural killer (NK) cells play central roles in controlling senescent cells, whether they can be effectively eliminated by NK cell therapies remains unexplored. Celularity is developing human placental CD34+-derived, cryopreserved, off-the-shelf, allogeneic NK cell products non-modified (CYNK-001), or genetically modified to optimize persistence and efficacy (CYNK-201) for the treatment of tumors and age-related diseases. Here we report the cytotoxicity results of both CYNK-001 and CYNK-201 against senescent cells derived from a tumor model. CYNK-001 cells were generated from human placental CD34+ cells in the presence of cytokines. CYNK-201 cells were generated similarly but transduced with a lentivirus vector expressing soluble IL-15 and a high IgG binding affinity and protease cleavage resistant CD16 variant. For both NK cell products, the phenotype and transgene expression were validated. Senescence was induced in the non-small-cell lung carcinoma tumor cell line A549 with chemotherapy drugs etoposide or doxorubicin followed by a 6-day recovery period. Senescence was confirmed by β-galactosidase (SA-β-Gal) activity or protein expression, monitoring the secretion of senescence associated secretory phenotype (SASP) cytokines, and analyzing the expression of cell surface stress ligands in the treated and non-treated A549 tumor cells by flow cytometry. Cytotoxicity activity and inflammatory cytokine secretion of CYNK-001 and CYNK-201 against A549 cells with or without senescence induction was assessed by flow cytometry and multiplex Luminex analysis. CYNK-001 and CYNK-201 cells were generated from multiple placental CD34+ donors (n=4) with high CD56+/CD3- NK purity. CYNK-201 displayed IL-15 secretion and high ectopic expression of CD16. Both CYNK-001 and CYNK-201 showed robust expression of activation markers such as CD11a, CD226, NKp30 and NKG2D. By etoposide or doxorubicin treatment, A549 tumor cells not only presented the phenotypical β-Galactosidase enzymatic activity (Figure A) or protein expression, but also showed increased expression of stress ligands including CD58, MIC-A/B and ULBP-2/5/6, which are known to be recognized by NKG2D expressed on the NK cells. Chemo-treated A549 cells also had increased secretion of SASP cytokines IL-1α, IL-6, IL-8 and CCL-5 compared to non-treated A549 (Figure B). Functionally, both CYNK-001 and CYNK-201 cells demonstrated significantly enhanced cytotoxicity at 24h against A549 cells with chemo induced-senescence (Figure C). With CYNK-001, there was significantly increased cytolysis of etoposide or doxorubicin treated cells vs. cytolysis of control non-treated A549 cells. Similarly for CYNK-201, there was significantly increased killing of chemo-treated cells vs. non treated A549 cells. Consistently, significantly increased secretion of inflammatory cytokines GM-CSF, G-CSF, IFN-γ, and TNF-α was also observed following CYNK co-culture with chemo-induced senescent A549 cells compared to non-treated cells, further confirming the preferential senolysis. Our preclinical data indicates that CYNK cells could serve as potent and selective senolytic agent for age-related diseases and also following conventional cancer treatment, CYNK may improve overall therapeutic outcomes and delay disease recurrence. Further investigation of CYNK cells in this regard, including in vivo tests using animal models and clinical studies, is ongoing.



1352. Using Microtissues to Study Bone Marrow Stromal Cell Chondrogenesis

Michael Robert Doran[1], Kathryn Futrega[2]

[1]AstraZeneca, Gaithersburg, MD,[2]National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD

Arthritis is a serious health crisis, with approximately 20% of the US adult population suffering from some form of the disease. Strategies to extend cartilage function, and/or regenerate damaged cartilage, are needed to increase productivity and extend healthy lifespans. Bone marrow stromal cells (BMSC) have been touted as an ideal cell source from which to regenerate cartilage. However, despite BMSC chondrogenesis being first described over 25 years ago, generating functional chondrocytes from BMSC is not yet possible, and no BMSC-mediated cartilage repair therapies have been approved. BMSC chondrogenesis is typically studied in large diameter pellet or tissue models; such models suffer steep diffusion gradients, yielding radially heterogeneous cell and tissue output. We reason that this heterogeneous tissue readout obfuscates the biological intricacies of BMSC chondrogenesis, and has contributed, in part, to delayed progress. By contrast, small diameter microtissues have reduced diffusion gradients, yielding a relatively homogeneous cell and tissue readout. Herein, we review our exploration of BMSC biology using microtissues, summarizing critical insights not visible in commonly used tissue models. In 2010, we described the adaptation of a microwell cell culture platform to generate hundreds of uniformly sized small diameter microtissues, in place of single large diameter pellets. Using microtissue cultures we identified that a 2% oxygen atmosphere yielded radially homogeneous high-quality cartilage-like tissue, while 20% oxygen inhibited chondrogenesis, and that large diameter pellets yielded radially heterogenous tissue regardless of culture conditions. As we incrementally improved BMSC chondrogenesis, displacement or loss of sticky cartilage microtissues from microwells became problematic over 14 to 21-day cultures. In response, we invented the Microwell-mesh; the Microwell-mesh has a nylon mesh bonded over an array of microwells. The pores in the mesh are large enough to allow single cells to be centrifuged through the mesh and pelleted in microwells, but small enough to retain cells once they self-assemble into microtissues. The Microwell-mesh enables extended culture with no loss or displacement of microtissues. Since 1998 it has been presumed that *in vitro* BMSC chondrogenesis required 2-3 weeks of TGF-β1 stimulation; in 2021 we reported that, in fact, BMSC chondrogenesis is triggered by a single day of TGF-β1 stimulation, and that subsequent differentiation is driven by intrinsic signaling cascades. Many presume that chondrogenesis and hypertrophy are sequential processes, but our data revealed that these differentiation processes are intertwined, and both are initiated in BMSC following a single day of TGF-β1 stimulation. *BMP2* and *SP7* signaling is upregulated within hours of TGF-β1 stimulation, likely contributing to hypertrophic differentiation. We attempted to obstruct BMP signaling with a small molecule, and used gene engineering to silence *SP7* signaling. Both strategies dampened hypertrophy, but also compromised chondrogenesis, again suggesting that these processes are intertwined. We subsequently compared chondrogenesis in response to TGF-β1, GDF-5 or BMP-2 stimulation. TGF-β1 is the most potent, and the only growth factor for which brief stimulation triggers intrinsic differentiation cascades that progress independent of further growth factor stimulation. In totality, this body of work provides insights into BMSC chondrogenesis not discernable in the common large dimension tissue models embraced by the field. The microtissue provides a high resolution, homogenous readout that aids in the optimization of complex bioprocesses, such as BMSC chondrogenesis.

1353. Engraftment of Engineered Plasma Cells into Humanized Mice Shows for the Feasibility of Engineered Plasma Cell Therapies

Tyler Hill[1], Rene Yu-Hong Cheng[2], Anna Helmers[2], Andee Ott[2], Leah Homad[3], Greg Asher[2], Parnal Narvekar[2], Andrew Mcguire[3], David J. Rawlings[4], Richard G. James[2]

[1]Seattle Children's Research Institute - Seattle, Washington, Seattle, WA,[2]Seattle Children's Research Institute, Seattle, WA,[3]Fred Hutch, Seattle, WA,[4]University of Washington, Seattle, WA

Advances in genome-engineering have enabled the generation of engineered plasma cells (ePCs) that secrete large quantities of therapeutic proteins. However, *in vivo* modeling of human ePCs has largely been limited to short-term studies following adoptive transfer of ePCs into immunodeficient mouse models including immuno-deficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice. In this study, we tested the hypothesis that immunodeficient mice humanized by engraftment of syngeneic human CD34^{+} hematopoietic peripheral stem cells would better mimic the pharmacodynamics of human ePCs. ePCs engrafted more efficiently and in additional lymphoid compartments in mice engrafted with syngeneic CD34 cells (NSG-huCD34) than in control mice without. ePCs migrated to and localized long-term in the bone marrow and spleen while maintaining a mature plasma cell phenotype. Plasma cells engineered to express an anti-EBV antibody produced robust anti-EBV titers that lasted for at least 3 months. We used surface display to identify highly expressed antibody sequences, and found that SARS-CoV2-RBD297 was more highly expressed in B cells than AMMO1. Finally, we showed that humanized mice engrafted with ePCs expressing SARS-CoV2-RBD297 were capable of neutralizing SARS-CoV2 pseudovirus. Taken together, these data demonstrate that the presence of autologous human hematopoietic cells in NSG-huCD34 mice permit the establishment of a robust model for studying the in vivo biology of long-lived human ePCs.

1354. Identifying Metabolite Markers for Detection of Flaviviruses in Human Induced Pluripotent Stem Cells (hiPSCs)

Tahira Fatima[1], Aaron Scholl[2], Sandip De[2]

[1]FDA, Tumor Vaccine & Biotechnology Branch, Division of Cellular Therapy 2, Office of Cell Therapy and Human Tissues, Office of Therapeutic Products, Silver Spring, MD,[2]Tumor Vaccine & Biotechnology Branch, Division of Cellular Therapy 2, Office of Cell Therapy and Human Tissues, Office of Therapeutic Products, Silver Spring, MD

Introduction: Human cells, tissues and cellular and tissue-based products (HCT/Ps) are being evaluated for use as therapeutic agents. HCT/Ps are required to comply with the donor eligibility requirements as per 21CFR 1271. HCT/P donor testing and screening includes screening for Zika and testing and screening for West Nile viruses. Moreover, rising global temperatures and expanding insect vector populations have led to a surge in vector-borne diseases, including flaviviruses. So, a rapid, sensitive, and precise pan-flaviviral detection tool is imperative for testing donor cells and tissues. Objectives: 1. Compare the sensitivity, speed and specificity of metabolomics to the NATs and immunogenic detection assays. 2. Develop metabolite markers for detecting flaviviruses in hiPSCs. Methods: hiPSCs were infected with Dengue virus (DENV) - DENV3 and Zika virus (ZIKV)-MR766 strains. We assessed impact of viral infections on hiPSCs by examining cell viability, cytopathic effect (CPE), and viral load using immunofluorescence assay (IFA) and qRT-PCR. For metabolite extraction standardization, we analyzed 0.2, 0.8, 2, and 8 million control and infected cells using untargeted FIA-MS. Ions were annotated by matching their inferred mass with compounds in the Human Metabolome Database. We performed LC-MS of hiPSCs infected with both viruses at 0, 8, 24, 48 and 96 hpi to identify virus specific shift in metabolome and to confirm FIA-MS data. Raw data was normalized to the caffeine standard, and "differential metabolites" analysis was done using ANOVA. Results: We did not observe significant differences in cell viability and CPE in hiPSCs infected with DENV3 & ZIKV vs control. However, IFA and qRT-PCR results indicated higher virulence of ZIKV compared to DENV3. FIA-MS detected same number of annotated compounds in all tested samples concluding that 0.2 million cells are sufficient for extracting maximum number of metabolites. Downstream analysis of the FIA-MS data identified 5 differentially expressed compounds in infected samples at 24 hpi. LC-MS resulted in identification of 271 annotated metabolites including several unique metabolites in the mock, DENV and ZIKV infected samples. In addition, 15 metabolites were differentially expressed (fold change ≥2) upon either DENV or ZIKV infection. Further data analysis is under progress. Conclusions: We demonstrated that 'Metabolomics' is a reproducible, and sensitive method for detecting flaviviruses in infected cells. Our subsequent objective is to validate the 'specificity' of the identified metabolites for flaviviruses detection.

Thursday Posters: Vector Product Engineering, Development, and Manufacturing

1355. Revolutionizing mRNA Production: the Paradigm-Breaking Nfinty™ Continuous RNA system

Patrick Thiaville[1], José Castillo[2], Emilie Gateau[1]

[1]Exothera, Jumet, Belgium,[2]Univercells, Brussels, Belgium

The field of RNA manufacturing has witnessed significant progress in recent years, fueled by advancements in synthetic biology, enzymology, and process engineering. Our thorough technology and process development led to the deployment of the first available high-performance continuous RNA manufacturing system. Our predefined and standardized process does not require scale-up or process development and combined with our robotic equipment produces high quality RNA drug-substance (DS) compatible with therapeutics and vaccines. We performed > 250 IVT reactions using more than 45 different DNA constructs varying in length from 900 bases to > 4kb. The data demonstrates the process efficiency scales going from single milligram to multi-gram batches of mRNA. The process produced mRNA with > 85% integrity, > 90% capping efficiency, > 90% poly-A tail length. Also, the process produces very low impurities with < 130 ng/mg of dsRNA and < 1.2 ng/mg residual DNA while maintaining > 80 % product recovery. Finally, we will show how our production process enabled the implementation of a decentralized manufacturing sites to quickly produce mRNA for cell and gene therapies or vaccines at or near point of use. As a conclusion, by using this groundbreaking technology, pre-clinical or clinical development is already at the final scale, which accelerates process and product development and significantly speeds up the access of RNA therapies to patients.

1356. Rapid Optimisations for Cell and Gene Therapy Applications

Thomas M. Evans, Charles Moore-Kelly, Anurag G. Kulkarni, John O'Driscoll, Kyriacos A. Mitrophanous, Nicholas G. Clarkson, Andre Raposo

Oxford Biomedica (UK) Limited, Oxford, United Kingdom

The process of lentiviral vector production is complex and presents unique challenges. Optimal production is heavily dependent on the concentration and ratios of the plasmid DNA components contributing to the transfection process. Improving titres can determine whether a new gene therapy product is commercially viable. A considerable amount of research is aimed at establishing the best titre and one of the ways to do this is by ensuring optimal transfection ratios that can improve the productivity or recovery of upstream/downstream platform processes, respectively. This means that a multifactorial design approach is often required to address the biological complexity of the system and achieve optimised vector particle production. We have developed and optimised a cutting-edge digital and physical platform coupled with automated liquid handlers enabling: 1) Automated Design of Experiments (DoE) and multivariate systems optimisation; 2) High-throughput screening, including small molecules, siRNA, shRNA; 3) Machine Learning and Deep Learning Data Analysis; 4)

Robustness and Ruggedness Testing to Support Analytical Quality by Design (AQbD) Methods. Our platform routinely screens and models 5 to10 factors. We have proven capacity for modelling 100+ factors with execution of thousands of experimental runs in a single iteration. The new platform, High-throughput Optimisation Services (HtOS), has significantly reduced manual operator time, increased throughput while meeting high demand and with improvements in precision and robustness. We will describe the capabilities and present a Case Study where the new platform was used to successfully optimise the transient transfection plasmid ratios in upstream production of lentiviral vector.

1357. PKC Agonists as Small Molecule Inducing Agents for Enhancing Lentiviral Vector Production

Charles Moore-Kelly, Thomas M. Evans, Jordan Wright, Daniel C. Farley, Kyriacos A. Mitrophanous, Nicholas G. Clarkson, Andre Raposo

Oxford Biomedica (UK) Limited, Oxford, United Kingdom

Achieving high upstream lentiviral vector (LV) titres is fundamental to the development and manufacture of a commercially viable gene therapy product. Product titres can vary considerably with different therapeutic transgene sequences despite being produced in the same mammalian cell system. Implementing new technologies able to recover low titres or improve high titres further is invaluable. Induction is a key stage of the upstream LV production process that typically involves increasing the expression of LV genes in production cells with the histone deacetylase inhibitor, sodium butyrate. A series of recent screening experiments revealed that titres of LV products can be further increased by using an additional class of molecules known as PKC agonists alongside the induction step. Here, we describe how, through optimisation of dosing concentration and timing, LV titres can be enhanced 2- to 9-fold with PKC agonists prostratin and ingenol 3-angelate in a product-specific manner. Importantly, LV produced using PKC agonists have comparable or superior particle-to-infectivity ratios, and these PKC agonists are removed from the vector product following downstream processing. Furthermore, we demonstrate that PKC agonists act synergistically with an in-house technology based on LV RNA-targeted U1 snRNA to recover log-fold-increases in titre without detriment to product quality attributes particularly for large, multiple or complicated expression cassettes. Having established the benefits of introducing PKC agonists to our platform process and in our packaging/producer cell lines, we provide insight into the mechanisms by which these agonists are acting on production cells and their transfer to GMP production.

1358. Leveraging Automation in Analytical Analysis of Viral Vectors

Gareth McCathie, Gareth Price, Samuel Stockdale, Thomas M. Evans, Benni Juckes, John O'Driscoll, Kyriacos A. Mitrophanous, Nicholas G. Clarkson, Andre Raposo

Oxford Biomedica (UK) Limited, Oxford, United Kingdom

The commercial scale manufacture of lentiviral vectors (LV) presents unique problems in QC release testing. Many of the methods employed require days or weeks to complete often with multistep cell culture combined with quantitative analytical readouts. Lengthy protocols, operator-to-operator variability, and a heavy dependence on manual setup are common shortfalls. To successfully meet these challenges, further gains in throughput by enhancing processes and automating analytics are needed. Oxford Biomedica are at the forefront of developing technologies to address these issues. The Computer Aided Biology (CAB) Group design, implement and optimise lab-based robotics and digital tools to enable bench-scientists to work more efficiently, generate more robust data and reduce time-to-insight. We have leveraged automation to increase assay throughput, robustness, repeatability, reduce operator hands-on-time, and perform Design of Experiments (DoE) to identify optima at a speed that would be impossible using manual methods. Our automated systems were first introduced in Research laboratories and later in GMP-regulated environments, further increasing the return on investment. We will describe the evolution of our automated qPCR workflows as a case study - from initial concept, validation, continuous improvements, and lessons learnt. At the first stage, automated liquid handler methods were developed for the most heavily used qPCR standard operating procedures (SOPs) to allow batching of large numbers of samples into multiple assay plates in one run. At the second stage, we developed a single Universal qPCR method with expanded functionality to allow extensive adaptations to assay setup and combine multiple, completely independent assays in one run. This was achieved via the use of the familiar Excel front-end to translate user input into machine-readable instructions. These dynamic Worklists enable a single validated method to adapt to any qPCR assay parameters, thus expediting validation and minimising the burden of SOP updates and new method qualifications. Continuous improvements in assay robustness and error-handling (e.g. automated, traceable recovery of aborted assays) have further reduced QC paperwork and repeated experiments. At a time when lab automation devices and digital tools are rapidly becoming more varied and accessible than ever before we hope that presenting some of the issues that we have faced and the solutions that we have implemented will aid other teams looking to introduce automation in their own labs in the future.

1359. Tuned Silencing of HLA-I Expression in Allogeneic CAR T-Cells Strikes a Balance Between Immune Rejection by CD8 T-Cells and NK Cells

Marco Alessandrini, Antonija Šakić, Bénédicte Ponsard, Marine Mansuy, Vincent Blancheteau, Inès Lanz, Antoine Marteyn, Yasmine Genolet, Bryan Bourrat, Amelie Turc, Ophelie Cherpin, Sten Ilmjärv, Audrey Roussel-Gervais

Antion Biosciences, Geneva, Switzerland

Allogeneic chimeric antigen receptor (CAR) T-cell therapy offers a promising solution to overcome key challenges of autologous CAR T-cells, including high manufacturing costs and limited widespread adoption. Allogeneic CAR T-cells have the absolute need for removal of T-cell receptor (TCR) expression to mitigate the risk of graft vs host disease (GvHD). To enhance cellular persistence, knockout of HLA-I in CAR T-cells very efficiently allows bypassing of rejection by host CD8 T-cells; yet it provokes rejection by natural killer (NK) cells. To

overcome this, NK cell inhibitory molecules are also required for co-expression with the CAR. We have developed a proprietary microRNA (miRNA) gene silencing technology, which not only enables silencing of one or more target genes, but also "tunable" silencing of targets. When co-expressed with a CAR, our "miCAR" platform allows for highly efficient, multiplex engineering of next generation CAR T-cells. We sought to develop allogeneic anti-CD19 CAR (CAR19) T-cells with complete silencing of the TCR and tuned silencing of HLA-I with the intention of striking a balance between immune rejection by host CD8 T-cells and NK cells - without the need for co-expression of an NK cell inhibitory receptor. Primary T-cells were modified via lentiviral vector transduction of optimized miCAR19 gene constructs for tuned silencing of HLA-I, expanded in G-Rex cell culture plates, and purified by depletion of TCR-expressing cells. Produced miCAR T-cells were characterized by flow cytometric immunophenotyping, tumor cell cytotoxicity, loss of alloreactivity and mixed lymphocyte reactions (MLRs) with allogeneic CD8 T-cells and NK cells.Our data demonstrates engineering of distinctly pure miCAR19 T-cell products, all of which were completely silenced for TCR and with tuned silencing of HLA-I over a range of 70-95%. While CAR functionality was maintained in recursive cytotoxic assays against tumor cells, our novel allogeneic miCAR19 T-cells were non-alloreactive and remained largely protected from both CD8 T-cell and NK cell mediated cytotoxicity when HLA-I was silenced within a range of 80-90%. We therefore demonstrate key benefits of our technology platform, which not only facilitates multiplex engineering of fully functional allogeneic CAR T-cells, but also the ability to optimally tune down expression of molecules for optimal therapeutic benefit.

1360. Automating a Lentivirus Infectious Titer Assay (ITA)

Kristen Kellar, Sadik Canca, Meiling Miranda, Matt Troutman, James Richardson, Tim D. Culp, Philip R. Johnson

Interius BioTherapeutics, Philadelphia, PA

Lentiviral vector (LVV) potency, as measured by transduction efficiency, is a Critical Quality Attribute (CQA) that must be monitored for every LVV sample. At Interius, the *in vitro* potency of LVV samples is assessed in an infectious titer assay via the transduction of SupT1, a suspension T cell line, that serve as model CD7+ target cells. Transduction of target cells by the LVV, encoding a CAR20 protein, is detected through antibody staining of SupT1 cells using an anti-idiotype monoclonal antibody targeted to the scFv domain of the CAR20 protein using flow cytometry. LVV containing samples are serially diluted and added to a fixed amount of target cells in a 96 well plate format. The transduced cells are incubated for 7 days, with intermittent medium refresh, followed by live cell staining for CD7+ and CAR+. The transducing units (TU) per mL are calculated by assessing the CAR+ cells amongst total live SupT1 cells assuming a Poisson distribution of single cell infections in the range of 2 - 20%. The manual execution of the ITA method is a labor-intensive, multi-day process consisting of many discrete steps, followed by a long day of live cell staining and signal acquisition by flow cytometry. Standard 96 well plate reading flow cytometers are not well suited for high-throughput flow cytometry. To support the sample numbers required to develop multiple process development efforts simultaneously, a high throughput solution was required. To this end, we have developed a fully automated, multi-step method covering sample dilution, SupT1 transduction, medium refresh, moving from 96-well to 384-well plates, 384-well live cell staining and HT flow cytometry on a Sartorius iQue, followed by automated data reduction. Each of these discrete steps is designed to reduce hands-on time and eliminate operator to operator variability while providing the added benefits of increased throughput and speed. Importantly, the same assay framework can be utilized for different products and target cells with minor modifications.

1361. Evaluation of Residual Host Cell DNA Clearance and Sizing During Production of a Lentiviral Vector

Elaine M. Youngman, Meiling Miranda, Shanice Edwards, Thomas M. O'Malley, James C. Richardson, Tim C. Culp, Philip R. Johnson

Interius BioTherapeutics, Philadelphia, PA

Viral vector-based gene therapy drug products can contain residual DNA derived from the production cells. Production typically occurs in continuous cell lines with tumorigenic potential and residual host cell DNA carries a theoretical risk of oncogene transmission to recipient cells. This risk decreases as a function of reduced concentration and size of residual DNA fragments. It is therefore important to monitor residual host cell DNA concentration and size in gene therapy products. We have evaluated methods for quantitation of residual host cell DNA concentration and size during production of an engineered lentiviral vector manufactured in HEK293T cells and used those methods to compare the performance of two commercially available nucleases in downstream processing steps. We evaluated qPCR-based methods for quantitation of residual host cell DNA from multiple vendors, and multiple options were found to be accurate using the newly available ATCC/USP quantitative HEK293 genomic DNA reference standard. We find a substantial difference in performance between the two evaluated nucleases, DENARASE® and M-SAN HQ, across the downstream processing steps in our lentiviral production process, with material produced using M-SAN HQ having at least 10-fold lower residual DNA concentrations than the comparable material made with DENARASE®. To assess the size of residual DNA fragments, we developed a method based on universal linker ligation followed by PCR amplification (LiLi-PCR) and size analysis by capillary electrophoresis. The method accurately reports on the size distribution of mixed synthetic DNA fragments in the range of 50-1000 base pairs in concentrations ranging from 10 ng/uL to 0.5 ng/uL, with some bias against larger fragments at lower concentrations. Further, the method faithfully recapitulates the size distribution of a smear of genomic DNA fragments produced by enzymatic digestion. We will report on progress in evaluating whether available nucleases result in differing distributions of residual DNA fragment size after digestion and downstream processing.

1362. Efficient and Reliable Cell Separation Using Microbubbles for CAR-T Cell Manufacturing

Jason S. Ellis, Dominique N. Badea, Yifan Huang, Tiffany Snow, Jesse Chen, Jon Roussey, Chia Ku, Casey Wegner

Akadeum Life Sciences, Ann Arbor, MI

The chimeric antigen receptor-modified T cell (CAR-T) therapy has recently expanded. Despite several CAR-T products being approved and commercialized since 2017, CAR-T cell therapy still has several manufacturing challenges. Conventional CAR-T therapies are manufactured by collecting apheresis material (leukopaks) from a patient's blood, then modifying the isolated T cells from the apheresis material to express a cancer cell-specific CAR, and eventually infusing the CAR-T cells into a patient. Unfortunately, there are several challenges during the development of these therapies, including early exhaustion of the therapeutic cells, manual and laborious processes, and complex workflows. Akadeum's innovative microbubble technology can help shorten multiple CAR-T production steps to streamline the workflow. At the heart of the technology is the silica microbubble with an air-filled core. Utilizing the buoyant nature of these microbubbles has allowed Akadeum to develop a variety of kits for isolating desired cell populations. The microbubble's outer layer is composed of streptavidin. Cells are labeled with biotinylated-antibody cocktails, and the streptavidin-biotin bond allows the microbubbles to capture the labeled cell populations and lift them up to the top of the culture. This technology not only provides specificity in cell selection and isolation, but also significantly reduced processing time and a gentler process than magnetic selection methods. Akadeum's **Human T Cell Leukopak Isolation Kit - GMP** utilizes negative selection to remove unwanted cell populations from leukopak material. Isolated T cells are left untouched and can be used for cryopreservation for future research or immediate processing. Next, Akadeum's **Human T Cell Selection, Activation, and Expansion Kit - GMP (SAE)** uses a positive selection method to isolate T cells from PBMCs for immediate activation and expansion. The SAE kit not only provides over 95% purity but also allows 80-fold expansion of total cell counts, reduced PD-1 exhaustion marker, and increased CD69, CD25, and HLA-DR T-cell activation markers. Finally, Akadeum's newly launched **Human T Cell Depletion Kit - GMP** uses positive selection for the removal of CD3-expressing cells from genetically engineered CAR-T cell products.

1363. Lentiviral Production at the 50l Scale Using the Gibco CTS™ LV-Max™ Lentiviral Production System

Arjen van Den Berg[1], Paula Decario[2], Nils Williston[1], Jonathan Zmuda[1]

[1]Thermo Fisher Scientific, Frederick, MD,[2]Thermo Fisher Scientific, Logan, UT

As viral vector gene therapy processes reach the clinical and manufacturing stages, the ability to scale up these processes becomes crucial. Traditionally, Lentivirus (LV) has been produced using adherent 293T cell systems in the presence of fetal bovine serum (FBS). These systems have proven the be difficult and costly to scale up and the presence of the T-antigen as well as the use of animal serum are of regulatory concern. The Gibco™ CTS™ LV-Max Lentiviral Production System addresses these shortcomings by combining a 293F-derived suspension cell line, Viral Production Cells (VPC), adapted to growth in a chemically-defined media, a cationic lipid based transfection reagent and chemically defined LV production enhancers into a high titer transient production platform. In the present study we demonstrate scalability of the Gibco CTS LV-Max system at the 50 L scale producing an anti-CD19CAR-vector at a titer ≥1x10^8 TU per mL. We describe optimal conditions for growth and transfection of VPCs in Thermo Scientific™ Hyperforma™ 5:1 Single-Use Bioreactors as well as a scalable depth filtration train suitable for clarifying LVs produced in the LV-MAX system.

1364. Establishing Modified Adenovirus Serotype 35 Manufacturing Process Development

Toshihiko Okazaki

OSAKA University Hospital, Suita City, Japan

The development of gene therapy is progressing rapidly worldwide. In order to accelerate practical application, it is necessary to establish a system for providing high-quality, multi-item investigational drugs in small scale for clinical trials, which is different from commercial production. We are developing a novel adenovirus serotype-35 (Ad-35), to see if it could be useful as a vaccine (non-replicating vector) or oncolytic virus. So far GMP compliant manufacturing process of Ad-35 has not been established. This time, we challenged to establish Ad-35 vector manufacturing process development for the usage in the investigational clinical trial. Based on the pre-conditioning analysis of optimizing culture condition using the suspension HEK293 cell line with serum-free culture medium, initial seed virus prepared in flask culture was transferred to the shaking back-type of closed mass culture system. The virus suspension was prepared from the supernatant of the upstream 5 liter volume of Ad-35 vector-infected whole culture cells and further subjected to subsequent downstream virus purification and concentration steps. Subsequently, clarified harvest was subjected to fraction separation using ion exchange membrane chromatography to capture the target Ad-35 vector, and finally 50 mL of viral-contained fractions were collected. The virus fractions collected were subjected to the final steps of polishing and sterilization. Additional quality tests were confirmed, we finally could establish Ad-35 manufacturing process development.We were able to identify critical upstream and downstream process parameters. These findings will provide useful and important information for subsequent technology transfer aimed at establishing a scaled-up GMP-compliant virus production process.

1365. Comparison of Two Process Protocols for Allogeneic CART Production with Reduced Cost

Chunxia Lu

OBIO TECH, Shanghai, China

Introduction Autologous CD19 CAR-T cells have demonstrated outstanding efficacy in B-cell malignancy. CAR-T therapy mainly focuses on autologous T cells at present due to the restriction of MHC. However, autologous preparation of CAR-T cells has drawbacks in terms of manufacturing time, cost, feasibility and scalability that can

affect clinical outcome. In order to prepare off-the-shelf CAR-T cells, CRISPR/CAS9 technology was used to knock out TRAC and CD52 genes in donor T cells to avoid the potential GVHD and acquire resistance to anti-CD52 mAb based lymphodepletion after allogeneic T cell infusion. In this study, we report the allogeneic CAR-T cells production process using selected T cells and PBMC. **Methods and results** 2.1 Cell Banking, selection and stimulation T cells and PBMC were selected directly from apheresis for cell banking. One bag of T cells was thawed and then stimulated with TransAct or Dynabeads CD3/CD28 CTS. One bag of PBMC was thawed to isolate T cells and then stimulated with Dynabeads CD3/CD28 CTS. 2.2 Transduction and Electroporation Lentiviral transduction was performed in a serum-free activation and transduction system. The positive rate of CAR+ is more than 80%. After transduction, the T cells were gene edited by electroporation of CRISPR/Cas9 RNP complex to knock out TRAC and CD52. The maximum volume of electroporation is 20 ml with 2E+09 Cells. The knockout efficiency of double genes of TRAC and CD52 is more than 80%. 2.3 Cell expansion Perfusion culture process with Wave Cell Expansion System was used to expand T cells. The perfusion volume is up to 25 L with cell density ≥1E+7cells/mL and more than 90% cell viability, which generated more than 100 fold of T cell amplification. **Conclusion** Allogeneic CAR-T production process was developed by knockout TRAC and CD52 genes. Using two starting cell sources from T cell bank or PBMC bank, we demonstrated higher activated T cells with more than 60% recovery rate. Alternately, a low cost method was developed to use PBMC banks, but the activated T cells could be affected by the positive selection efficiency of PBMC.

1366. Personalized High-Throughput cGMP Manufacturing of Neoantigen-Based Plasmid DNA Cancer Vaccines

Ernst Boehm

Biomay, Vienna, Austria

Personalized medicine is an emerging therapeutic approach in cancer therapy, based on the unique genetic makeup of an individual tumor patient. One of the most rapidly advancing areas of application is an individualized cancer therapy where the mutational alterations of the patient's tumor cells - the individual profile of neoantigens - are evaluated. Based on the respective patient-specific neoantigen profile, a customized therapeutic cancer vaccine specific for that individual tumor patient is developed, manufactured and administered. Here we present our manufacturing platform, including a dedicated and specialized manufacturing facility for patient-specific plasmid DNA vaccines, which is operated under the conditions of current Good Manufacturing Practice (cGMP). More than 100 individual patient-specific DNA batches have been GMP-produced so far, to support a clinical trial for the treatment of different types of cancer. Manufacturing of individualized vaccines requires rapid turnaround times and robust processes across the complex supply chain, including tumor biopsy, identification of tumor-specific neoantigen biomarkers, vaccine design, generation of the patient-specific plasmid DNA starting material and manufacturing and quality control of the individualized drug substance and drug product. During the parallelized small-scale manufacturing process, a patient-specific *E. coli* cell bank is generated, which is then used in a fed-batch fermentation to propagate and produce the plasmid. After harvesting and alkaline lysis of the bacterial cell mass, the DNA plasmid is purified in a two-step chromatography process, formulated by ultra/diafiltration and finally aseptically filled. Key parameters of product quality, identity, purity and safety were characterized with a comprehensive set of analytical quality control assays. We will present our approaches to facility design, process optimization (e.g. DoE), quality control and quality parameter trending over a high number of batches. Considerations particularly crucial for personalized manufacturing will be made, e.g. a small footprint, rapid turnaround time and cost effectiveness. It could be shown that efficient GMP manufacturing of high-quality individualized tumor vaccines can be achieved within a short period of time, which is in the category of days to weeks rather than months.

1367. Design and Characterization of Next-Generation SMAR Nanovectors for Stable Cell Modification: Towards Safer Allogeneic and Autologous Adoptive CAR-T Cell Therapy

Toros Tasgin, Julia D. Peterson, Alice De Roia, Anna Hartley, Luisa Burger, Patrick Derigs, Richard P. Harbottle

German Cancer Research Centre (DKFZ), Heidelberg, Germany

Adoptive cell therapy (ACT) is one of the most prominent approaches to cancer immunotherapy and is attracting attention with recent promising clinical outcomes obtained in the treatment of melanoma and small cell lung cancer patients. The generation of CAR and TCR engineered T-cells allowed further advancement of the field by enabling off-the-shelf target specificities to peripheral T-cells, eliminating the need for isolation of tumor infiltrating lymphocytes (TILs). Moreover, the incorporation of induced Pluripotent Stem Cells (iPSCs) in cell engineering, coupled with subsequent differentiation into mature T-cells, has the potential to revolutionize ACT. This innovative approach offers an alternative means to generate a clinically significant quantity of CAR or TCR engineered T-cells, eliminating the necessity for stringent ex vivo T-cell expansion protocols that frequently result in T-cell exhaustion and diminished T-cell activity. Despite these advancements in ACT, conventional cell engineering strategies poses certain risks in clinical settings. Typically used integrating vectors can present inherent risks of genomic instability and genotoxicity and has recently led to the FDA warning of the risk of secondary malignancies. On the other hand, non-integrating systems, using viral replication-driving sequences such as Epstein-Barr Nuclear Antigen 1 (EBNA-1) or the SV40 large T antigen, are known to have transforming potential in the host cell. In this study, we address this limitation in current ACT protocols by refining and implementing a safer pipeline for engineering cells by utilizing an episomal DNA nanovector platform. Virus- -free S/MAR nanovectors are designed to adhere to the nuclear matrix, forming episomes that sustain stable transgene expression without genomic integration. Our findings demonstrate the efficiency of S/MAR vectors in introducing stable, and high level of CAR expression in transfected iPSCs and Jurkats, under both in selection-free and positive cell enrichment regimes. Additionally, our study aims to enhance episomal vector functionality, specifically focusing on matrix attachment stability and prolonged vector persistence within the transfected cell population. We have achieved this functional enhancement by designing and implementing a next-generation of S/MAR vectors with improved composition compared to our previously

described vectors. Results indicate that these vectors markedly improve vector persistence and transgene-positive cell population stability during long-term iPSC cultures, where transgene-positive cells are periodically enriched through Fluorescence-Activated Cell Sorting (FACS) Overall, the results of this study demonstrate the effectiveness and stability of S/MAR nanovectors for modifying iPSCs, providing a safer alternative to protocols that carry vector-mediated risks. Furthermore, we highlighted the potential for improving S/MAR vectors through a deeper understanding of the underlying mechanisms of S/MAR function. By gaining more insights into these mechanisms, it becomes possible to develop more clinically adapted and safer protocols for CAR-iPSC-T cell ACT by utilizing a more efficient S/MAR cell modification platform.

1368. Primary CD4 T-Cell Biofactory for Antigen-Inducible *In Situ* Synthesis of Engineered Proteins

Harikrishnan Radhakrishnan, Sherri Newmyer, Harold S. Javitz, Parijat Bhatnagar

Biosciences Division, SRI International, Menlo Park, CA

T-cell Biofactory is an engineered cell-based platform that assesses the disease burden and synthesizes calibrated amounts of complex protein-based biologics with desired properties at the disease site. This offers the potential to exert a broad range of functions without any dose misalignment, which otherwise causes side effects. Clinical translation of this technology has however been impeded by insufficient expression of the biologics from the T cell and manufacturing bottlenecks specific to the T-cell products. Compared to the pan T cells, the CD4 T cells offer to overcome these challenges due to their propensity for higher transduction, faster expansion, and a more productive transcription machinery. The results presented herein show that capitalizing on the CD4 T-cell biology, the CD4 T-cell Biofactory, compared to its CD8 counterpart, when configured with an appropriate chimeric antigen receptor (CAR), they achieve ~3X transduction efficiency of CD8 T cells, ~2X expansion rates, generating ~5X more biologic, and exhibit minimal cytolytic activity. Cumulatively, this address two main hurdles in the translation of cell-based drug delivery: scaling the production of engineered T cell *ex vivo* and generating sufficient biologics *in vivo*. When programmed to induce IFNβ upon engaging the target antigen, the CD4 T cells outperforms CD8 T cells, effectively suppressing cancer cell growth *in vitro* and *in vivo*. In summary, this platform enables precise targeting of disease sites with engineered protein-based therapeutics while minimizing healthy tissue exposure. Leveraging CD4 T cells extended persistence could enhance disease management by reducing drug administration frequency, addressing critical challenges in cell-based therapy.

1369. Lentiviral Vector Capture Using Anion-Exchange Chromatography: Removing an Industry Bottleneck through Rational Design

George Pamenter[1,2], Lee Davies[1], Carol Knevelman[1], James E. Miskin[1], Kyriacos Mitrophanous[1], Duygu Dikicioglu[2], Daniel G. Bracewell[2]

[1]Oxford Biomedica, Oxford, United Kingdom,[2]Department of Biochemical Engineering, University College London, London, United Kingdom

Lentiviral Vectors (LVs) have emerged as the preferred vector of choice for *ex vivo* modification of patient cells, particularly in Chimeric Antigen Receptor T-cell (CAR-T) therapies, with several products approved for commercial use. LV therapies hold immense potential for further application and future commercial success. However, challenges in manufacturing these therapeutics have resulted in a restricted global supply and limited early line use. As CAR-T developers endeavour to enhance therapy accessibility, efficacy, and safety, the demand for high-quality LV raw material is anticipated to rise. The manufacture of LV drug substance poses specific challenges arising from the large size and structural complexity of LVs. Downstream purification is particularly problematic and product recoveries of 15-25% are typical. Product capture primarily relies on charge-based separation using convective anion-exchange chromatography (AIEX) due to low cost and wide applicability across LV constructs. However, despite its near ubiquitous use across the industry, this purification step alone results in product losses of 60-85%, making AIEX a major bottleneck in the design of economical LV manufacturing processes. Despite its critical role in LV AIEX manufacture, widely variable AIEX performance is observed with little to no consensus on the optimal chromatographic configuration. This variability stems from a lack of focused research and a poor understanding of the complex LV-specific adsorption behaviours that result from the large size and structural complexity of LVs. The poor understanding of product loss mechanisms that underpin charge-based LV separation has led to a largely heuristic approach to process development. This highlights the need to further elucidate LV adsorption phenomena to pave the way for a more rational process development approach. In this work, we first identify the major product loss mechanisms driving poor recovery of LV on current commercial AIEX membrane adsorbents. Loss mechanisms such as entrapment and shear related damage are ruled out, with the importance of strong avidity effects that cause irreversible binding following prolonged adsorption periods highlighted. We further demonstrate the heterogeneous nature of LV binding and identify at least two binding subpopulations. The role of LV envelope structure in determining LV binding behaviour within these populations was explored, with the major LV envelope species responsible for binding identified. The role of thesespecies in driving time-dependent product loss mechanisms was theninvestigated, demonstrating that practicable improvements cannot be achievedusing current commercial adsorbents, thus advocating for an adsorbent redesign.The use of AIEX membrane prototypes developed specifically for LV purificationby Sartorius demonstrates high process recoveries are achieved in materialsdesigned for purpose. Finally, we highlight how we aim to enable *in silico* process development by applying the core understanding gathered to develop a mechanistic model of the AIEX process using the CADET Python package. LV-specific challenges are discussed such as the requirement for higher order binding isotherms, high-throughput analytical data and characterisation of non-conventional chromatography geometries.

1370. Improvements in Baseline Lentivirus Production Using Design of Experiment (DOE) Tool and Scale-Up in Bioreactors

Sonal Patel[1], John Mumira[1], Gregory Piscitello[1], Hiral Gami[1], Eva Fong[2], Joe S. Orlando[1], Scott Bahr[1]

[1]MilliporeSigma, Bedford, MA,[2]MilliporeSigma, Carlsbad, CA

Lentiviral vectors (LV) have gained popularity as modified gene therapy and gene delivery vehicles for treating a variety of acquired and inherited diseases. There is a growing demand for highly purified and concentrated viral vector products. To meet the market demands as research studies progress into pre-clinical and clinical phases, scalable manufacturing poses a big challenge. One of the areas that can help alleviate this challenge is optimization of transfection conditions to achieve higher viral titers. During lentivirus production processes using suspension-adapted HEK293T cells, 4 plasmids are complexed with a PEI-based cationic polymer and added to the cells at a specific cell density. Key factors for optimal transfection include transfection cell density, DNA to cationic polymer ratio, plasmid DNA molar ratio, transfection complex volume and transfection complex formation time. The work in this poster reflects the successful exploration of these factors using statistical software - JMP® and DOE approach, along with a new GFP transfer plasmid, to achieve higher lentivirus harvest titer. After obtaining optimal level for each factor, findings were confirmed in shaker flasks and scale-up was demonstrated in Mobius® 3 L and 50 L Single-Use Bioreactors.

Thursday Posters: Cell Therapy Product Engineering, Development, and Manufacturing

1371. Impact of Temperature Conditions on Cell Stability in Leukopaks During Shipping

Diana C. Chiluiza[1], Courtney Noah[2]

[1]Immune Cell Isolation, BioIVT, Watertown, MA,[2]Scientific Affairs, BioIVT, Westbury, NY

Human Peripheral Blood Mononuclear Cells (PBMCs) are critical components for cell and gene therapies (CGT) and regenerative medicine[1]. PBMCs are defined as a highly diverse heterogenous population of immune cells, which include lymphocytes such as CD4+ and CD8+ T cells, B cells, natural killer cells, monocytes which differentiate into macrophages and dendritic cells, and a small percentage of stem cells[2,3]. Leukopaks, sourced from leukapheresis, offer reduced variability as a single-donor origin for PBMCs. Proper storage and shipping conditions are important to ensure immune cell activation[4] as it plays a crucial role in the success of novel therapies. In this study, we investigated the impact of ambient and cold shipping conditions over time on white blood cells in leukopak products, focusing on cell concentrations, viability, and differential leukocyte counts (DLC). Study Design: Leukapheresis was performed on three healthy donors, and leukopaks were divided into aliquots stored at room temperature or 4°C to mimic shipping conditions. Viability, complete blood counts (CBC), and DLC were assessed daily for five days. Results: Viability of PBMCs remained stable for 72 hours in cold conditions but rapidly declined in ambient conditions (Fig. 1A). Lymphocyte to monocyte ratios (Lym/Mon) exhibited significant changes, with a notable increase in ambient conditions at 72 hours ($p<0.0001$, Fig. 1B). Total white blood cell counts were unaffected by time or temperature. Conclusions: Ambient shipping led to a significant drop in PBMC viability after 48 hours ($p=0.018$), suggesting it may not be ideal for prolonged transport. Lym/Mon ratios revealed distinct changes, indicating potential cellular effects. Cold shipping maintained PBMC viability and Lym/Mon ratios up to 72 hours, making it the recommended choice for extended shipping. Implications: The study supports the guarantee of cell product quality up to 72 hours when shipped in cold conditions, emphasizing the importance of optimal shipping conditions to prevent unexpected changes in leukopak cell populations and ensure successful research and therapy outcomes in the field of cell and gene therapy. Beyond 72 hours, the study suggests the need for further analysis of time and temperature impact on cellular functions. References: 1. Kobayashi, D. T. et al. Evaluation of Peripheral Blood Mononuclear Cell Processing and Analysis for Survival Motor Neuron Protein. PLOS One 7, e50763 (2012). 2. Abbas AK, L. A. Cellular and molecular immunology. vol. 5 (Saunders, Philadelphia, 2005). 3. Jerram, A. et al. Effects of storage time and temperature on highly multiparametric flow analysis of peripheral blood samples; implications for clinical trial samples. Biosci Rep 41, (2021). 4. Johnson, R. K., Overlee, B. L., Sagen, J. A. & Howe, C. L. Peripheral blood mononuclear cell phenotype and function are maintained after overnight shipping of whole blood. Sci Rep 12, (2022).

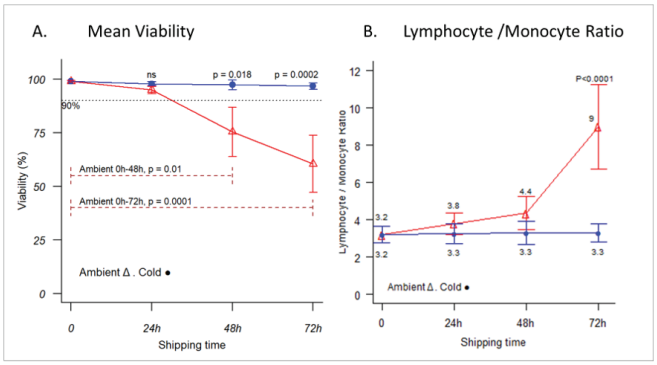


Figure 1. A. Mean viabilities per temperature and shipping time. B. mean lymphocyte to monocyte ratios per temperature and shipping time. Error bars represent one standard deviation from the mean. Statistical analysis performed using ANOVA with post-hoc Tukey's Honest Significant Difference Test.

1372. Autologous CD19 CART Manufacturing from Whole Blood Collection for the Treatment of Autoimmune Disease

Vicki Stratton, Ashley Martynchuk, Yan Li, Jinmin Lee, Rebecca Dryer-Minnerly, Gwen Binder, Samik Basu, Chris Schmitt

Cabaletta Bio, Philadelphia, PA

Chimeric antigen receptor T (CART) cells targeting B-cells have been approved for treating various cancers and have now also demonstrated efficacy in refractory autoimmune diseases, including, but not limited to, systemic lupus erythematosus, myositis, systemic sclerosis, and myasthenia gravis. Despite the efficacy of CART cells in autoimmune disease and cancer, significant logistical barriers exist, limiting access

to patients. Access to apheresis centers is one of these barriers, as patients need to undergo leukapheresis to isolate T-cells for CART cell manufacture. To address this barrier and to improve the patient experience, we have developed a novel approach to isolate, transduce, and expand T-cells from whole blood collections. Studies were conducted to evaluate the isolation efficiency, determine the optimal blood volume(s), and characterize the immune phenotype of whole blood derived T-cells as compared to apheresis derived T-cells. T-cells isolated from whole blood collections were used to generate CD19 CART cells and compared against apheresis derived CD19 CART cells. These whole blood derived CD19 CART cells demonstrated comparable transduction efficiency, phenotype, cytolytic activity, and proliferative capacity as compared to apheresis derived CD19 CART cells. Taken together, our data support the ability of whole blood derived T cells for manufacturing CD19 CART cells which may alleviate some of the logistical burden to patients and enable broader access to CART cell therapies.

1373. Rethinking Transduction in Cell Manufacturing: Applying Functional Viral Titer (TU/mL) in Place of Multiplicity of Infection Improves Process Robustness

Gabriela Brumatti[1], Krystal Bridgeman[1], Seungyoul Oh[1], Jo Leen Lim[1], Ellen Otte[1], Andreas Gille[2]

[1]CSL, Melbourne, Australia,[2]CSL, Pasadena, CA

Introduction: Autologous CD34 positive hematopoietic stem and progenitor cells (HSPCs) can be genetically modified using lentivirus, making them an attractive target for gene therapy in the treatment of various genetic disorders and blood-borne diseases. One challenge in the development of such therapies is creating robust processes for a range of cellular product sizes based on variations in yield from mobilization and isolation (based on patient age, for example). The concept of multiplicity of infection (MOI) has been used to address this, where vector quantity scales with cell concentration based on protocols developed in the 1930s for T4 infection of *E. coli*. This concept (MOI) presumes that absorption of infectious particles is a dominant factor in transduction so that a constant ratio of particles to cells will achieve consistent transduction. However, this has never been systematically examined in the context of cell manufacturing for gene-therapies that use lentiviral vector (LVV) infection of HSPCs. These cells are substantially restricted and require high LVV concentrations for successful transduction. We hypothesized that considering diffusive limits in such a system and the number of LVV transducing units (TU) required for transduction, controlling LVV concentration (TU/mL) versus MOI (TU/cell) would improve the robustness of transduction processes independent of cell density. **Methods:** Human purified CD34 positive HSPCs isolated from GCS-F mobilized leukopaks, were transduced with two different lentiviral vectors encoding green fluorescence protein (GFP). Cells from 3 donors were plated at cell densities of 0.25, 0.5, 1, 2 and 4 x 10^6 cells per mL, followed by 24 h cytokine stimulation. LVV was added at different concentrations (0, 0.01, 0.1, 1 and 10 x 10^6 TU/mL) in the presence of transduction enhancers for 24 h. Cell viability and proliferation were determined by acridine orange/ propidium iodide or 7AAD staining, using the automated cell counter Cellaca MX and flow cytometry respectively, on days 3, 6, 9 and 13 post LVV addition. Transduction efficiency was determined at the same time-points through the detection of GFP expression by flow cytometry and quantification of vector copy number by ddPCR. **Results and Conclusion:** Transduction efficiency in HSPCs was LVV concentration dependent. Surprisingly, LVV potency on HSPCs strongly varied when expressed as MOI (TU/cell) but much less with LVV concentration as (TU/mL) (Figure 1). This finding is consistent with insignificant LVV adsorption to cells during the incubation. Moreover, high LVV concentrations did not impact on the viability or proliferation of HSPCs. Contrary to common practice, our data show that the use of MOI is an inadequate measure of LVV concentration for the transduction of HSPCs when varying cell densities. LVV concentration applied as TU/mL achieves robust HSPC transduction over the typical range of cell densities used in gene therapy cell manufacturing. Predictable transduction efficiency across a wide range of cell densities enables the use of automation when processing partial or pediatric cell products and allow significant savings of HSPCs in large scale manufacturing product development.

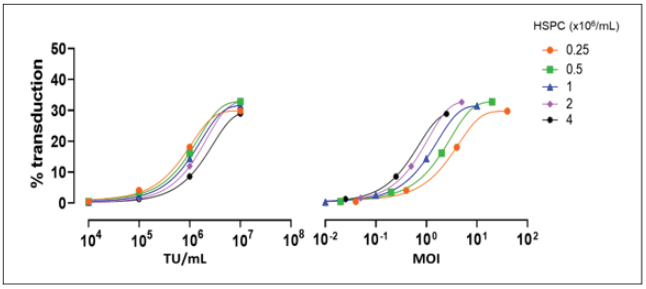


Figure 1. *LVV dosing by functional titer (TU/mL) versus MOI (TU/cell) improves robustness of HSPC transduction across cell densities.* Transduction was assessed as GFP-expressing cells by flow cytometry. Shown are the means of 2 donors. Error bars omitted for clarity.

1374. Establishing a Novel Reimbursement Strategy for Drugs Developed for "Angel" Ultra-Rare Disease Patients

Richard Horgan, Eric Faulkner

Cure Rare Disease, Woodbridge, CT

Our exponential advancement in genomics, systems biology, and cell and gene therapies have ushered in unprecedented opportunities to address devastating diseases at their core. One key group that has not had the opportunity to benefit from these novel therapy options are the majority of ultra-rare disease patients for which limited and often no therapeutic options exist. Around 70% of these diseases disproportionately impact pediatric patients. These patients and their caregivers have historically fallen through cracks in a US healthcare system never built for uncommon diseases. But viewed in the aggregate, patients with ultra-rare diseases constitute one of our largest US healthcare unmet needs - broader than focal, high-impact diseases like diabetes and many of the top cancer indications combined. Further, because our treatment development process in the US operates with a lens on individual diseases, many of these ultra-rare diseases do not rise to the level of commercial interest and thus will not be a target for potentially life-changing advanced therapy development. This sizable subset of rare disease patients with poor-to-no options have been dubbed "Angel" patients for purposes of this report. This research report intends to examine and layout a potential pathway for

offering these patients access to transformative cell and gene therapies. This report - available initially in interim form -methodologically involved targeted literature research, qualitative stakeholder research with providers, regulators, payers, patients and patient advocates and treatment manufacturers, as well as a quantitative multistakeholder survey en route to finalization. The concepts described herein are intended to serve as a foundation for multi-stakeholder dialogue and action needed to advance solutions for this often forgotten patient population with ultra-rare diseases. There are several key findings of this report, including the following: • Based on the numbers, the unmet need for rare and ultra-rare disease is cumulatively one of our most profound health challenges, with numbers of aggregate patients that rival or exceed many diseases considered the most high impact diseases of our time. • Across all dimensions of the Angel Treatment Pathway outlined in this report (i.e., **definition of "Angel" patients, evidence requirements, regulatory pathway, reimbursement pathway, and patient follow-up**), there *is* a foundation of precedent upon which to rapidly build and execute this pathway in the US - we would not need to start from scratch and solutions could be rapidly and broadly enacted. Despite this potential, it will take collective will and multi-stakeholder collaboration to action a replicable Angel Treatment Pathway in the US. This effort offers the most comprehensive, end-to-end view on addressing non-commercially applicable rare disease to date in the US, while acknowledging multiple areas where progress towards the goal of treating an aggregate rare disease population with profound and unaddressed unmet need. Our goal is offering up a fully fungible strawman and advancing dialogue and action towards pilots that can address the burden of rare disease in a scientifically sound, responsible, and life-changing manner, while taking into account the changes required beyond a model never built and infrequently adapted for niche populations.

1375. Developing a Novel Large-Scale Process for B Cell Therapy Manufacturing

Eileen Elliott, David Bengford, Kerri Somebang, Inosencio Casanova, Grant Li, Johnny Arenas, Robert Chang, Andy Rakestraw, Nallakkan Arvindan

Process Development, Walking Fish Therapeutics, South San Francisco, CA

Cell therapies have garnered much success as potent, versatile, and long-lived treatments for mostly oncological indications. Owing to advances in gene-editing, viral delivery, and large-scale culturing, B cells have emerged as new therapeutic modalities in the oncological, auto-immune, and rare disease spaces. B cells are particularly attractive cell-therapy because they are engraftable, long-lived, and have the potential to secrete high levels of biologic therapeutics. Walking Fish has developed a versatile, robust process to create multiple clinical doses of engraftable B cells engineered to secrete therapeutic levels of recombinant protein. In this report, Walking Fish describes a scalable, closed, GMP-compatible process to create its first B cell therapy WFX-001. This process uses the MaxCyte GtX™ together with CRISPR genome editing and AAV-based gene delivery to engineer B cells to secrete alpha-galactosidase (α-GAL) for the treatment of Fabry disease: a condition caused by poor α-GAL function. This autologous process edits Fabry-patient cells with functional α-GAL and then expands them to levels that are suitable for dosing. Walking Fish has adapted traditional and novel analytical tools to identify critical process parameters (CPPs) and critical quality attributes (CQAs) that contribute to engineering and manufacturing proficiency. Additionally, in vivo murine models illustrate the effectiveness of the WFX-001 therapy in secreting therapeutic levels of α-GAL and clearing the Gb3 intermediate that characterizes Fabry disease. As such, engineering B cells as live "factories" to deliver therapeutic cargo has become a reality.

1376. Importance of Clinical Trial Design on Therapy Commercial Access

Katie Moses, Jen Klarer

The Dedham Group, New York, NY

Background: In this abstract, we aim to share historical trends and expert analysis on how clinical trial design impacts market access and associated commercial dynamics manufacturers need to consider before pivotal clinical trials start. **Discussion:** Payers reference inclusion and exclusion clinical trial criteria to formulate coverage policies for cell and gene therapies. Once a payer has developed a coverage policy the criteria can be difficult to remove. To ensure appropriate access, manufacturers may wish to develop clinical trial inclusion and exclusion criteria with commercial management in mind. The goal of clinical trial criteria is to ensure controlled efficacy results for products, but this can inadvertently narrow patient populations and access for those products post-approval. For example, policy criteria of "no prior BCMA-targeted therapy" is relatively prevalent for multiple myeloma CAR-Ts, despite data supporting patient response greater than the control following BCMA exposure[1]. Now reaching 48% of commercial medical lives, this criteria can limit access particularly with the entrance of 5L+ BCMA-targeted bispecifics. On the gene therapy side, Zynteglo[2], clinical trial criteria included ineligibility for patients that have a known and available HLA-matched family donor[3]. This criterion, which has been widely used in commercial coverage policies covering 61% medical lives, indicates that if a donor is available, the patient must be evaluated for transplant prior to Zynteglo treatment. Although transplant is viewed as a functional cure, patients who undergo transplants can experience serious clinical complications, including morbidity and mortality (e.g., GvHD). The recently launched sickle cell disease gene therapies had similar clinical trial criteria, which will be important to watch in how payers manage the therapies[4]. Additionally, primarily in the gene therapy space, manufacturers have developed an innovative payment model to contract with payers. Such models need to be proactively planned during the clinical trial design phase to ensure the right endpoints are studied and available for outcomes-based agreements. Outcomes-based rebate models are one of the more commonly leveraged innovative payment models used to increase product access but can be challenging as outcomes are sometimes operationally difficult to measure. Manufacturers should consider clinical trial criteria that may be seen in claims processing or have ease of measurement to enhance the likelihood that payers will adopt these payment models and execute on them. **Methods:** Payer coverage for BCMA-targeted CAR-T therapies and Zynteglo was evaluated across ~93% Commercial medical lives to identify prevalence of respective clinical trial criteria in policies. **Conclusion:** Clinical trial design shapes the landscape of therapy access. Understanding how clinical trials criteria influence payer policies is crucial for ensuring appropriate patient access to innovative therapies. Strategically selecting control criteria, or proactively planning to mitigate potential

access risks associated with necessary control criteria, in pivotal clinical trials can avoid clinically inappropriate implications on commercial access. Therefore, as the field continues to evolve, proactive and thoughtful clinical trial design is key for manufacturers to overcome access challenges and advance patient care. **References:**[1]https://pubmed.ncbi.nlm.nih.gov/37558706/ [2:] https://www.fda.gov/media/161472/download [3]https://www.molinahealthcare.com/~/media/Molina/PublicWebsite/PDF/Common/Molina%20Clinical%20Policy/Zynteglo_betibeglogene%20autotemcel_R.pdf. [4] https://www.ncbi.nlm.nih.gov/books/NBK538515/

1377. Programmable Gene Silencing for Engineering Adaptable Cell Therapies

Vlad C. Seitan

Laverock Therapeutics Ltd, Stevenage, United Kingdom

Cell therapies are still facing considerable challenges relating to efficacy, safety, and cost. A significant proportion of these challenges can be tackled by genetically engineering therapeutic cells to become adaptable, so that they can respond to distinct cell state transitions and/or disease environments - such as the tumour microenvironment (TME). We have developed a new technology called Gene Editing-induced Gene Silencing (GEiGS) that enables programmable gene silencing, and thus allows the engineering of programmable therapeutic cells. GEiGS is a new way of harnessing the RNAi pathway that works by introducing specific gene edits within endogenous redundant miRNA genes, to redirect their silencing activity towards new desired targets. We have previously shown that this technology is able to effectively silence targets in human iPSCs in a tunable manner and with no detectable off-target effects. We now show that GEiGS activity can be programmed to silence target genes in a developmental- and cell state-specific manner, in a range of human cells including primary T cells, iPSC-derived macrophages and iPSC-derived pancreatic cells. By editing miRNAs that are differentially expressed in response to developmental and environmental cues, we show these dynamic changes can be leveraged to silence desired target genes in a programmable manner. This feature of the GEiGS technology allows the generation of adaptable cell therapies that can deploy their therapeutic activity 'at the right time, and in the right place'. We exemplify this through the design of GEiGS-engineered immune cells that are programmed to respond to the TME, and iPSC-derived hypoimmunogenic pancreatic cells with an improved safety profile.

1378. High-Throughput Characterization of Subvisible Particle Contamination in Cell and Gene Therapies

Karessa White

Halo Labs, Burlingame, CA

A key measure of biologic stability in cell and gene therapies are detecting and characterizing subvisible aggregates. The Aura+ is a high-throughput, low-volume approach that allows the detection of these subvisible aggregates by using a method called Backgrounded Membrane Imaging coupled with Fluorescence Membrane Microscopy. Here we demonstrate the complete characterization of subvisible aggregates and external particle impurities in a variety of cell and gene therapy products.

1379. Large-Scale Quantitative Live-Cell Tracking Using Label-Free Imaging of Individual iPSCs for Monitoring Pluripotency

Anthony J. Asmar, Zackery Benson, Michael Halter, Anne L. Plant

NIST, Gaithersburg, MD

The ability to quantitatively image induced pluripotent stem cells (iPSC) to monitor their state of pluripotency and differentiation in a non-invasive manner is important for establishing better metrics for pluripotency, and to assure consistency and efficiency in iPSC manufacturing. To address this, we have developed a label-free imaging and image analysis pipeline that allows tens of thousands of individual hiPSCs and their progeny to be segmented and tracked over multiple cell divisions using phase contrast microscopy. The AI model for this analysis is trained using automated segmentation of fluorescent nuclei. This also obviates the need for manual annotation and provides access to the large diversity of cells within an isogenic population and also allows for the development of a model in a minimal amount of time. The rapid imaging (all cells are interrogated every 2 minutes) allows detection of mitosing cells and tracking of their daughters with a high rate of accuracy. It has been frequently observed that each individual cell expresses its phenotype (e.g., expression of transcription factors, level of pluripotency, morphology, etc.) slightly differently that its neighboring cells. This distribution of observed phenotypes across a population seems to be indicative of the intracellular processes that produce the phenotype, and therefore can provide information about mechanistic aspects of regulatory networks. To demonstrate the usefulness of this knowledge to the expansion of pluripotent stem cell cultures, we are adapting our imaging and image analysis pipeline in order to interrogate different reporter stem cell lines. The cell lines produce fluorophores that report on the transcription factors relating to stem cell pluripotency. Quantitative optical imaging and AI-assisted image analysis of large numbers of individual live cells will allow quantification of the relative expression of single or multiple transcription factors within each cell. The presence of the reporter probe provides information that can used to inform models for the label-free identification of cell state. These data can provide a new metric that allows better characterization of the state of an expanding pluripotent or differentiating population of cells. These findings will help to establish the validity of this approach for use in hiPSC culture optimization and development.

1380. Optimization of Automated Selection and Lentiviral Vector Mediated Transduction of MSCs

Alessandra Gatti[1], Federica Mingotto[2], Paola Terreni[2], Ralitsa Arnaudova[2], Federico Lo Presti[2], Claudia Gentile[2], Nunzia Mangano[2], Fabio Ferrazzi[2], Giuliana Vallanti[2]

[1]AGC Biologics, Bresso, Italy,[2]Development, AGC Biologics, Bresso, Italy

Mesenchymal stem cells (MSCs) are adult stem cells that can be isolated from several adult tissues and are of great interest for the development of new MSC-based products in advanced therapies or regenerative medicine. They have a wide range of therapeutic applications and are a valuable tool to treat different pathologies. MSCs have traditionally

been isolated by manual flask-based methods based on their property of adherence to plastic. However, this classic approach includes many open handling steps that can be error prone and labor intensive. Automation has the potential to address these problems and so, aiming to an industrial translation of cell and gene therapies, we investigated Quantum cell expanssion system (Terumo BCT) for isolation and first cultivation passages of MSCs from unprocessed bone marrow (BM). The system is a hollow fiber bioreactor with a 2.1-m^2 surface area for cell growth and continuous medium perfusion. In addition, in the context of product early stage development, we demonstrated the feasibility of lentiviral vector-genetically modified MSCs production to generate cell-therapy-based drugs or for downstream application as exosomes production. Lentiviral vectors (LVV) are a platform to deliver and permanently express therapeutic genes and have displayed great potentiality in clinical trials.Here we present proof of concept results concerning the setting up of optimal MSCs transduction with a highly-purified VSV-G pseudotyped LVV encoding for GFP protein in order to obtain optimal genetic modification and process yield. Briefly, BM donor-derived MSCs were cultured in different chemically defined MSC-specific serum free media and seeded at different concentrations in tissue colture 2D supports. Transduction was performed at different timing up to 24 hours from seeding at MOI ranging from 20 to 50. Different conditions were tested and cells were maintained in culture up to fourteen days, thus obtaining optimal transduction efficiency (up to 80% GFP positive cells).

1381. Designing Instructional Materials for Synthetic Notch Receptor Activation in a 3D Context

Joanne C. Lee, Jonathan M. Brunger
Biomedical Engineering, Vanderbilt University, Nashville, TN

Introduction: To overcome tissue degradation and combat inflammation caused by injury, metabolic disorders, autoimmune diseases, and aging, regenerative engineers often leverage therapeutic cells *in situ* to deliver biologic factors, such as pro-inflammatory cytokine antagonists, or to engineer tissue substitutes. As adverse events can result from unreliable dosages of biologic factors and a lack of control over transplanted cell behaviors, it is extremely important to be able to precisely generate engineered, defined cell responses. In previous work, we have presented a 2D system leveraging synthetic biology tools to create a customizable platform where cells engage materials to overcome such limitations, termed MATRIX for material activated to render inducible expression. Here, we extend our platform to a 3D context for applicability in *in vivo* environments through implantation of the co-engineered cell and material system. **Materials and Methods:** Our system leverages the synthetic Notch (synNotch) receptor platform to program cellular responses to cues in their microenvironment. Like the native Notch receptor, synNotch is a juxtacrine receptor that regulates ligand-dependent transcriptional programs in cells. By exchanging Notch's extracellular domain with alternative recognition motifs and intracellular domain with synthetic transcription factors, we create synNotch receptors that produce user-specified sense/response behaviors. Since we can customize synNotch to drive expression of any transgene, we can tune defined cellular responses to selected orthogonal inputs, reducing crosstalk from native pathways. SynNotch, like native Notch, requires force on the order of piconewtons for activation. Thus, soluble monomeric ligands do not activate synNotch. We leveraged this feature to create biomaterials capable of capturing synNotch ligands, resulting in a privileged material:cell channel of communication, requiring co-localization of the engineered cell, the designer biomaterial, and the selected synNotch ligand. Here, we implemented our 3D platform to enable engineered cells to respond to GFP, a bioinert cue, and activate cells. **Results and Discussion:** Polystyrene scaffolds with fiber diameters of 150 microns and pores of 200 microns were functionalized with a nanobody for capturing GFP, termed GFP-TRAP. Mouse mesenchymal stem cells (mMSCs) engineered to produce secreted alkaline phosphatase (SEAP) and mCherry when activated were seeded across the functionalized scaffolds and then exposed to GFP in cell culture media over a period of 19 days. An increase in mCherry fluorescence and SEAP production as measured by a chemiluminescence assay was observed at the end of the culture period, demonstrating successful synNotch cell activation in a 3D context (Fig. 1A and Fig. 1B). **Conclusions:** Our system has the potential to be highly versatile with custom inputs and outputs through the customizability of synNotch. Our platform represents an innovative approach to co-engineer cells and biomaterials for increased cellular and spatiotemporal control, as the requirement for co-localization of all components gates responses spatially. Further work will test the platform's capabilities for regenerative functions, such as delivery of pro-inflammatory cytokine antagonists and cellular differentiation.

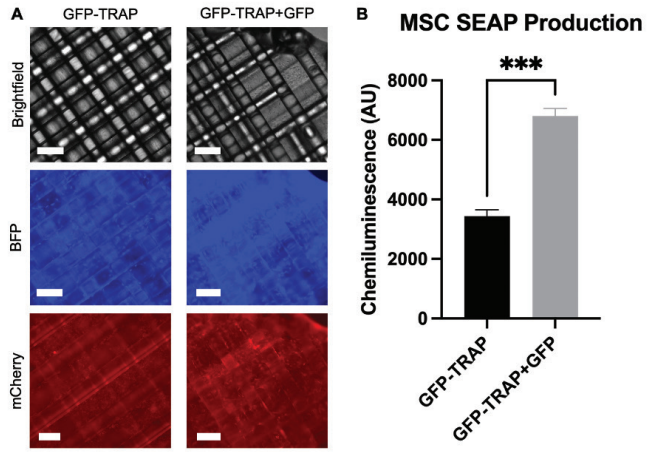


Fig. 1: A. SynNotch activation of mMSCs on functionalized GFP-TRAP scaffolds with and without GFP added as denoted by mCherry reporter fluorescence. Cells are constitutively BFP+, shown here for ease of visualization. Scale bar = 200 μm. B. SEAP production as measured by a chemiluminescence assay shows a significant increase in mMSCs exposed to GFP on functionalized GFP-TRAP scaffolds. (***p<0.001, Student's t-test).

1382. Novel Immortalized Human Bone Marrow Derived Mesenchymal Stem Cells Overcome Primary Cell Limitations

Nicholas Lee, Larisa Saari, Xiangshan Zhao, Fang Tian
ATCC, Gaithersburg, MD

Mesenchymal stem cells (MSCs) are adult stem cells with the ability to differentiate into other types of cells. Additionally, MSCs can

modulate the immune response and promote tissue repair. Due to their properties, primary MSCs have a significant application potential in the treatment of various diseases. However, primary MSCs also have limitations such as limited life span, donor variability, source limitation, and heterogenicity. These limitations hinder the utilization of primary MSCs in preclinical applications. These constrains could be overcome with cell immortalization. Cell immortalization technology enables the establishment of new cell models that retain normal physiology, minimize cellular heterogeneity, and gain an indefinite lifespan. In this study, we generated a clonal cell line immortalized by stably expressing human telomerase reverse transcriptase (hTERT) gene in normal human primary bone marrow-derived mesenchymal stem cells (BM-MSCs). This immortalized cell line has been cultured for over 200 days and has continued to proliferate over 110 population doublings. The immortalized BM-MSCs exhibited normal karyotype and had a similar cell growth profile and cell doubling time as the parental primary cells. The immortalized BM-MSCs were positive for CD73, CD90, CD105, and negative for CD14, CD34, CD45. In this study, we also investigated the adipogenic, osteogenic, and chondrogenic differentiation abilities of these immortalized BM-MSCs. In summary, the immortalized BM-MSCs present a novel cell model that avoids the limitations of primary cells and can be used as a valuable tool for gene and cell therapy.

1383. Clinical Scale Genetic Engineering of MSCs with Complex Genetic Cargos

AJ Walters[1], Caleb Bashor[1], Scott D. Olson[2]

[1]Bioengineering, Rice University, Houston, TX,[2]University of Texas Health Science Center, Houston, TX

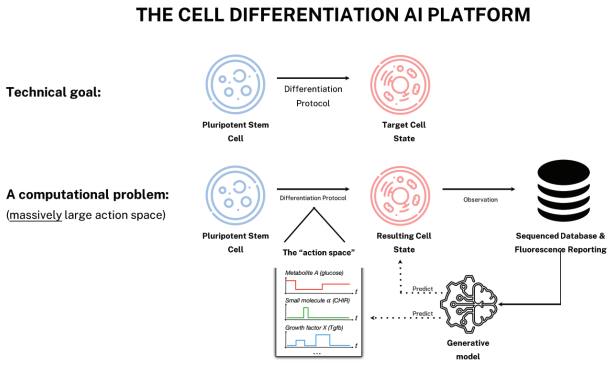
Mesenchymal stem/stromal cells (MSCs) are a widely studied cell type with potential therapeutic value. However, these cell therapies generally fail to show efficacy in large scale clinical trials. Genetic engineering represents an opportunity to augment cell therapies with novel mechanisms of action. Unfortunately, efforts to deploy genetic engineering in MSCs have been stymied by technical and logistical challenges that limits the manufacturing scale and size of DNA cargo. Through systematic optimization, we identified improved transfection and manufacturing conditions then produced genetically engineered MSCs (eMSCs) that meet clinical criteria. These eMSCs expand to a clinical scale product, maintain the growth kinetics, phenotype, and immunomodulatory properties of unmodified MSCs. This approach demonstrates comparable transfection outcomes to mRNA transfection with RNAiMAX, and resulted in a similar cell product to lentiviral transduction. We then installed DNA cargo greater than 15 kb, outside the typical capacity of lentivirus. To demonstrate the modularity and tunability of this approach, we installed an IL-10 transgene under multiple promoters. The multi-gene eMSCs expressing variable amounts of IL-10 proportionally decreased inflammation in myeloid cells. This demonstrated proof of concept that clinically-compatible eMSCs can be augmented with a user-defined transgene that introduces a novel and quantifiable mechanism of action. Importantly, this work enables the development of a new generation of MSC-based therapeutics.

1384. Somite-Derived Cells from Human Pluripotent Stem Cells for Cell Replacement Therapy

Kristy J. Brown, Lu Yan, Carl Morris, Cliff Tabin, Jonathan Rosenfeld, Allon Klein, Olivier Pourquie

Somite Therapeutics, Boston, MA

The delivery of cell replacement therapies requires producing cells of the appropriate identity and maturity, and to do so at scale while minimizing cellular byproducts, batch variability and cost during production. The search over protocol variations to optimally achieve these goals represents a considerable task that can be navigated efficiently using statistical learning approaches (artificial intelligence, or AI). A principled framework for AI-guided protocol optimization has been established. The initial focus of the framework is on optimizing somite-derived lineages for treating musculoskeletal and metabolic diseases. This will be applied towards large-scale production of differentiated muscular, dermal, tendon and skeletal (bones and cartilage) and brown fat tissues from human pluripotent stem cells (PSCs). The first proposed clinical indication is Duchenne muscular dystrophy (DMD), a rare X-linked genetic progressive muscle loss disease. Production of iPSC-derived muscle satellite stem cells (MSSCs) was optimized and their efficacy demonstrated through muscle force recovery in mice subjected to muscle injury. Additionally, iPSC-derived brown adipocytes capable of normalizing glycemia in a Type 1 diabetes mouse model has also been developed. The regenerative capacity of these somite derived cells, characterized by self-renewal and differentiation into mature functional tissues, positions them as promising therapies for long-term patient benefits and establishes a systematic framework for protocol optimization.



Thursday Posters: Pharmacology/ Toxicology Studies and Analytics/Assay Development

1385. Transduction Efficiency of rAAV8 Comparing Mouse and Non-Human Primate (NHP) Tissues Following Intravenous Administration

Nakyo Heo, Kentaro Konishi, Joshua Chang, Takeshi Sato, Megumi Adachi, Whitney Blankenberger, Marie Stark, Sam Sutton, Bala Medicherla, Damir Simic, Carlos Fonck

Astellas Gene Therapies, South San Francisco, CA

Introduction: Data from mouse and cynomolgus macaque studies were analyzed to investigate transduction efficiency following rAAV8 gene transfer. In the nonclinical development of AAV-based gene therapies, animal models of disease such as transgenic mice are typically used to investigate doses and vector exposures that are associated with efficacy. In contrast, species such as cynomolgus macaques are often utilized to investigate potential toxicity and biodistribution of the AAV vector. To the extent that non-clinical studies are used to select first-in-human doses, we were interested in any generalities or trends in terms of rAAV8 transduction patterns. We compared species, mouse versus cynomolgus macaque, and tissues, including skeletal muscle, heart and liver. The potential impact stemming from differences in DNA construct content and design was also assessed. The dependent variable in this analysis was vector copy number (VCN) measured in tissues. ***Materials and Methods:*** Four mouse and three non-human primate (NHP) studies across three gene therapy programs that utilized rAAV8 were selected to analyze VCN data from liver, heart, and quadriceps. These studies utilized the same methods to evaluate rAAV8 vector titers. rAAV8-treated animals received doses ranging between 1E12 and 8E14 vg/kg and included 202 mice and 39 NHPs. There were 241 liver samples, 193 quadriceps samples and 249 heart samples that included atria, ventricles and intraventricular septa. Body weights, animal age at the time of dosing or necropsy, and mouse genotype varied across studies. The VCN was measured in tissues by quantitative polymerase chain reaction (qPCR) or droplet digital PCR (ddPCR). The log-log linear regression between dose and VCN were analyzed on R and RStudio, assuming no differences between the bioanalytical methods utilized across the studies. ***Results and Discussion:*** The highest VCN levels were measured in the liver across all experiments which is consistent with several publications that investigated the ability of multiple capsid serotypes to transduce mammalian tissues. Heart showed slightly higher transduction efficiency compared to quadricep, while quadricep displayed the highest variability in terms of VCN measurements. Using tissue as an independent variable, dose-dependent increases in VCN were observed across species in all tissues, with 95%, 93% and 87% of the VCN values within 5-fold of the regression values in the heart, liver, and quadricep, respectively; indicating that the most consistent results were measured in the heart, with similar proportional increases across the two species in each tissue. There were no apparent differences due to construct design. The slope estimates were 0.96, 1.18, and 1.17, intercept of -11.2, -16.0, and -16.4, and r^2 of 0.77, 0.89, and 0.75, in liver, heart, and quadricep, respectively. When species was set as the independent variable, similar slope and intercept estimates in mouse heart (slope=1.23, intercept=-16.6) and NHP heart (slope=1.20, intercept=-16.4) were observed, suggesting that heart transduction is relatively more predictive when translating across species, compared to liver and quadricep. NHP showed slightly lower transduction in liver, and slightly higher transduction in quadricep compared to mice. There were no measurable differences in transduction efficiency across tissues or species when construct sequences were considered.

1386. Spatial Transcriptomics Decodes AAV2 and AAV9 Specific Signatures in the Mouse Liver

Bettina Amberg[1,2], Fabian Köchl[1], Nadine Kumpesa[1], Megana Prasad[1], Filip Bochner[3], Mar Hernández-Obiols[1], Michael B. Otteneder[1], Lucas Stalder[1], Björn Jacobsen[1], Sven Rottenberg[2], Alberto Valdeolivas[1], Petra C. Schwalie[1], Kerstin Hahn[1]

[1]Roche Pharma Research and Early Development, Pharmaceutical Sciences, Roche Innovation Center Basel, Basel, Switzerland,[2]Institute of Animal Pathology, University of Bern, Bern, Switzerland,[3]Department of Biosystems Science and Engineering, ETH Zurich, Basel, Switzerland

Introduction: Assessing the biodistribution of adeno-associated virus (AAV) gene therapy (GT) vectors and corresponding transcriptomic changes is invaluable to estimate the efficacy and safety of GT compounds. In this regard, spatial transcriptomics (ST) based on unbiased in situ capturing of the mRNA provides the unique opportunity to spatially map transgene expression and investigate vector or transgene-mediated responses. Moreover, the integration of ST and single-nucleus RNA sequencing (snRNA-seq) can reveal the distribution of numerous markers at nearly single-cell resolution, alongside mapping putative interactions and identification of cell communities. To validate this approach for AAV-based GT, we applied ST and snRNA-seq, to evaluate the serotype-inherent spatial biodistribution of AAV2 and AAV9 in the mouse liver. Methods: C57BL/6 mice (n=4 male/female per group) were intravenously injected with $5x10^{11}$ vg/kg of AAV2-CMV-EGFP, AAV9-CMV-EGFP or PBS. Frozen liver tissue was processed using the ST 10X Genomics Visium spatial gene expression and Chromium Single Cell 3' Reagent kits (v3.1 Chemistry Dual Index) for snRNA-seq. Spatial data were compared to EGFP in situ hybridization (ISH; RNAscope™, ACD) and immunohistochemistry (IHC) to assess the capturing of the AAV vectors. Results: Visium ST revealed spatial sex- and serotype-specific patterns, with periportal or pericentral accentuated transgene expression for AAV2 and AAV9, respectively. ST findings were in line with ISH, IHC and snRNA-seq results. The transcriptomic signatures in transduced liver zones included panlobular changes in the androgen and EGFR pathways, providing additional evidence for the relevance of both pathways for AAV2 and AAV9. We further used the spatial localization of the transgene to investigate its co-expression with receptor-encoding genes. This analysis identified known AAV entry factors such as the laminin receptor and syndecans as well as potential novel receptors. Conclusions: We demonstrate for the first time that AAV transgene levels and associated transcriptomic signatures can be robustly assessed using the commercially available 10X Genomics

Visium platform. This method can be extrapolated to other preclinical species and tissues and proves to be a valuable tool for GT development including efficacy and safety assessments.

1387. Transcriptomic Analysis of Vasculature Grown in 3D and 2D Platforms Helps Enable Complex *In Vitro* Model Selection in Pharma

Maryam Tarazkar, Monica Romero-Lopez, Chidubem Onyeagoro, Fjodor Melnikov, Kimberly Homan, Catrin Hasselgren

Genentech, South San Francisco, CA

Cell culture conditions can affect phenotypic and genotypic signatures of endothelial cells. 2D and transwell endothelial models have been widely used to study endothelial cell behavior and interactions. Vascular in vitro models are advancing in physiological complexity, yet few studies directly analyzed transcriptomic differences between endothelial cells cultured in 2D and 3D systems. Here, we compared the expression of key endothelial cell markers across various culturing platforms: 2D cultures (traditional cell culture plates and transwells), 3-lane Mimetas Organo-plate chips, and an in-house bioprinted 3D-Chip, aiming to provide foundational data to help drug development users select an endothelial model whose cellular function matches their need (e.g. effective drug transport or immune system regulation, etc). Bulk RNA-Seq data demonstrate the differences in gene expression profiles between 2D and 3D cultures. Specifically, the 3D-Chip generates a gene expression profile that is distinct from those observed in 2D cultures and/or Mimetas. It is known that endothelial cells exposed to various extracellular matrix (ECM), geometries, and flow patterns exhibit different gene expression pathways related to mechano-sensing, inflammatory, and endothelial cell damage markers. Here, we observed that the inflammation and endothelial activation pathways were downregulated in the 3D-Chip compared to other systems; suggesting that no-flow or bidirectional-flow contributes to the establishment of a more pro-inflammatory environment relative to the 3D-Chip culture. Our findings identified the value of 3D complex models compared to 2D cultures and highlighted the importance of considering specific culture conditions and their influence in cell behavior. This research provides an insight into the utilization of models by drug developers for internal decision-making and highlights the essential characteristics required for successful integration of new models into existing preclinical drug development pipelines. Specifically, it demonstrates how model-omics data generated across diverse vascular platforms assists users in selecting appropriate models for particular contexts of use.

1388. Qualification of a Preclinical to Phase I ELISpot Assay for AAV Immune Monitoring

Emma Kraus, Lauren Huffman, Hakan Cam, Sapna Varia, Victoria M. Velazquez

Analytical Sciences, AmplifyBio, West Jefferson, OH

The ability to identify cellular immune responses to advanced therapies, such as AAV gene therapy, is key in documenting their overall safety. The interferon-gamma (IFN-γ)-based Enzyme-Linked ImmunoSpot (ELISpot) assay is a robust and highly sensitive tool for this purpose. The goal of this study was to develop and qualify an IFN-γ ELISpot method suitable for identifying and enumerating AAV9-specific T cells in both non-human primates (NHPs) and in humans. The IFN-γ ELISpot was first evaluated by measuring human T cell responses to CMV in cryopreserved PBMCs, for: linearity, limit of detection (LOD), precision, specificity, and accuracy. When testing a PBMC input range of 0 to 1×10^6 PBMCs/well, the assay was determined to be linear ($R^2 \geq 0.95$), and the countable frequency of spot forming units (SFUs) were between 0 to 700 per well. To establish the LOD, human CMV "responder" PBMCs were supplemented with an increasing concentration of CD3-depleted cells from the same donor (non-responder cells) to artificially create a range of measurable CMV-specific T cell frequencies. The LOD was determined to be 11.2 SFU/million PBMCs, or 2.24 SFUs/well. To test precision, three CMV-responsive PBMC donors were tested over three different days by two independent operators. Intra-assay precision was determined to have a coefficient of variation percent (CV%) of 8.9-18.4% and inter-assay precision was 27.2-29.8% CV. These responses were deemed specific, as when human and NHP PBMCs were tested within the same assay for responses against human (hCMV, pp65) and cynomolgus CMV peptide sequences (CyCMV, UL55/UL83), each showed only a species-specific response. To address accuracy, the frequency of HLA-A2 CMV tetramer-positive cells, as identified using flow cytometry, was directly compared to the frequency of SFUs, as measured by the IFN-γ ELISpot assay. The two different methods were interchangeable, resulting in a Pearson's correlation coefficient of r=0.9. Finally, to assess suitability for use in preclinical safety (NHPs) and in phase I trials (humans), the assay was used to identify AAV9 specific T cell responses. A total of 20 (each) NHP and human PBMCs were tested. Responses were determined to be positive using an empirical method (positive = 2X background and ≥ 50 SFU/ 10^6 PBMCs). Positive stimulation controls included a CEFX peptide pool and mitogen PHA-L. The AAV9 peptides were a single pool of 182 peptides (15mers overlapping by 10, >95% purity). Responses were reproducibly detected in 2 of the 20 commercially purchased human PBMCs. Interestingly, while the NHP PBMCs did show responsivity to CyCMV peptides, confirming functional PBMCs, none of the animals responded to the AAV9 peptide pool. In summary, the qualification of this IFN-γ ELISpot method demonstrates its potential and suitability for use in both late-stage preclinical testing as well as in Phase I immune monitoring. Future directions will include further evaluation of PBMC stability and storage requirements, and continued monitoring of assay performance and suitability over time. We believe the IFN-γ ELISpot method, due to its sensitivity and reduced requirement for cells compared to other methods, will continue to be a useful immunological tool across discovery, preclinical and clinical applications.

1389. Safety of AMP-101 (AAV.Dok7) in Non-Human Primates

Patricio Sepulveda

Amplo Biotechnology, La Jolla, CA

Amplo is a late pre-clinical, gene therapy company, focused on diseases that affect the neuromuscular junction. AMP-101 (AAV-Dok7) was designed and optimised to express Dok-7 protein. AMP-101 has been successfully manufactured in suspension with projected COGS of ~$40,000 / patient. The product is efficacious in several disease mouse

models and safe in mice. We have performed a non GLP non-human primate study to assess the safety of AMP-101 that supports clinical translation.

1390. Inline Variable Pathlength Chromatography Application for High Concentration Oligonucleotide Solutions

Teva Smith, Ramsey Shanbaky
Analytics, Repligen, Waltham, MA

With the increased focus on oligonucleotides within biopharmaceutical projects real-time measurements of product concentration can lead to increased efficiency within bioprocessing and manufacturing. A collaboration between C-tech/Repligen and Ionis Pharmaceuticals assessed the viability of inline measurement of oligonucleotide concentration during reverse phase chromatography. This incorporated a variable path length to measure highly concentrated anti-sense oligonucleotide (ASO) eluate. ASO's have become a group of interest in the field of oligonucleotide therapeutics due to successful implementation for diseases such as spinal muscular atrophy and hereditary transthyretin amyloidosis. Now many pharmaceuticals aim to assess the viability and capabilities of ASO's to combat diseases such as Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases. The inline variable path length system ran at wavelength 260nm for a total of two elutions. The first run used a quick kinetic with auto pathlength adjustments to determine the optimal pathlength based on concentration of the eluate during elution. The second run used the optimal fixed pathlength range determined from the first run to maximize measurement frequency. The results were compared to traditional 1mm fixed path UV monitoring. With the 1mm fixed path, the absorbance of the eluted ASO solution is consistently out of the linear range of detection when using either the standard lambda max 260nm wavelength or the 295nm wavelength at which the operation was previously optimized. The test system successfully collected real-time concentration data, within the linear range of detection, at wavelength 260nm during both runs, despite the product solution reaching a peak concentration of 83.6mg/ml during elution. Real-time integration of the UV data from the first run with the auto pathlength setting estimated a collected product mass of 562g, while the results from the fixed pathlength range second run estimated a collected product mass of 526g. These values were compared to the determination of the product mass via an analytical HPLC method, which reported product masses of 506g and 511g, respectively. This represents a 11% (first run) and 2.9% (second run) difference. The project findings support the reliability of inline variable pathlength chromatography application to provide reliable data at target wavelength when compared to traditional fixed path chromatography for high concentration oligonucleotide solutions. The capabilities of inline variable pathlength UV monitoring found in this project provide a reliable alternative for biopharmaceutical laboratories looking to streamline manufacturing process eliminating the need to wait for in-process concentration result to proceed to downstream processing.

1391. Systemic Delivery of SGT-003 Microdystrophin Gene Therapy Using the Novel Capsid AAV-SLB101 Ameliorates Muscle Pathology and Rescues Muscle Function in the *mdx* Mouse Model of Duchenne Muscular Dystrophy

Jamie L. Marshall[1], Jessica Boehler[1], Prushti Bhavsar[1], Jennifer C. Gifford[1], Margaret Beatka[2], Hui Meng[2], Mariah Prom[2], Michael Lawlor[2], Jenny Marlowe[3]
[1]Solid Biosciences, Charlestown, MA,[2]Diverge Translational Science Laboratory, Milwaukee, WI,[3]Solid Biosciences, Cambridge, MA

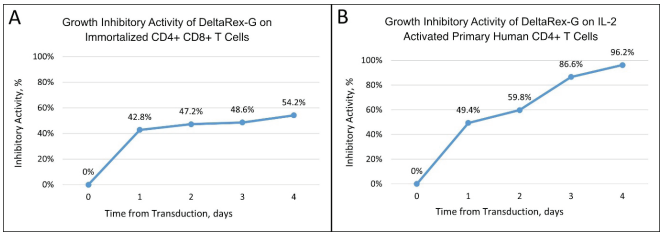
Duchenne muscular dystrophy (DMD) is a rare, X-linked neuromuscular disease caused by mutations in the *DMD* gene that prevent the production of functional dystrophin protein. The lack of functional dystrophin protein results in severe, progressive muscle wasting and premature death. Solid Biosciences' next generation therapeutic gene therapy candidate SGT-003 utilizes a novel adeno-associated virus vector (AAV-SLB101) and contains a codon-optimized human microdystrophin transgene driven by the muscle-specific CK8 promoter. This unique microdystrophin transgene contains the R16/R17 nNOS binding domain that has been shown to be required for proper nNOS membrane localization and function, which is essential for increasing blood flow to the muscle during exercise to prevent damage that can occur due to functional ischemia. To test the efficacy of SGT-003, systemic injections were evaluated in *mdx* mice at escalating doses. Three months post-SGT-003 treatment, functional efficacy was observed at doses ≥ 6.0E12 vg/kg as measured by forelimb grip strength, EDL specific force production, and treadmill exhaustion. Microdystrophin and beta-sarcoglycan proteins, as well as nNOS enzymatic activity, were detected at the sarcolemma. Improvements in muscle pathology were also observed in response to microdystrophin expression. Overall, these data provided proof of concept for the use of SGT-003 in the treatment of DMD and supported initiation of a Phase I/II clinical study in boys with DMD.

1392. DeltaRex-G Inhibits Growth of Primary Human CD4+ and Immortalized Human CD4+CD8+ T Cells: A Potential Treatment Option for Parkinson's Disease

Erlinda M. Gordon[1], Nadezhda Omelchenko[2], Sant P. Chawla[3], Don A. Brigham[4], Rebecca A. Reed[4], Frederick L. Hall[4], Paul Chang[5], Paul Song[6], Bo Han[7]
[1]Neurodegenerative Disorders, Cancer Center of Southern California/Sarcoma Oncology Research Center and Aveni Foundation, Santa Monica, CA,[2]Cancer Center of Southern California, Santa Monica, CA,[3]Neurodegenerative Disorders, Sarcoma Oncology Center, Santa Monica, CA,[4]Neurodegenerative Disorders, Aveni Foundation, Santa Monica, CA,[5]Neurodegenerative Disorders, NKGen Biotech Inc., San Diego, CA,[6]Neurodegenerative Disorders, NKGen Biotech, Inc., Santa Ana, CA,[7]Tissue Engineering Lab, USC Keck School of Medicine, Los Angeles, CA

Background and Rationale: a-Synuclein, a key pathological component of Parkinson's disease, has been implicated in the activation of the innate and adaptive immune system. This immune activation includes

microgliosis, increased inflammatory cytokines, and the infiltration of T cells into the CNS. More recently, peripherally circulating CD4+ and CD8+ T cells derived from individuals with Parkinson's disease have been shown to produce Th1/Th2 cytokines in response to a-synuclein, suggesting there may be a chronic memory T cell response present in Parkinson's disease. In an animal model of Parkinson's disease, the authors found that T cell responses associated with a-synuclein pathology may be damaging to key areas of the CNS in Parkinson's disease and that targeting these T cell responses could be an avenue for disease modifying treatments (Williams et al., Brain, 2021). DeltaRex-G, an amphotropic retrovector displaying a pathology-targeted decapeptide and encoding a cytocidal *CCNG1* inhibitor gene, has shown clinical benefit and improved dose dependent overall survival in Phase 1 and Phase 2 clinical trials. DeltaRex-G is currently being studied in an FDA authorized expanded access program for an intermediate size population of pancreatic cancer, sarcoma and breast cancer. DeltaRex-G has also gained FDA emergency use authorization to treat severe COVID-19 by killing the activated immune cells that cause ARDS. Methods: To determine if DeltaRex-G, an amphotropic retrovector displaying a pathology-targeted decapeptide and encoding a cytocidal anti-cyclin G1 gene, would inhibit growth of immortalized (Jurkat) human CD4+ CD8+ T cells and IL-2 activated primary human CD4+ T cells in vitro, these cells were transduced with DeltaRex-G as previously described (Zhang et al., Plos Pathogens, 2010). Briefly, $5x10^3$ T cells were transduced with 300 ml DeltaRex-G vector/R10+polybrene or Control R10 + polybrene in 24-well plates, spun for 90 min at 1000 x g at 32°C, followed by incubation at 37°C for 4 hours. Fresh R10 was then added to transduced T cell cultures and further incubated at 37°C overnight. DeltaRex-G -induced growth inhibitory activity was then measured over 4 days post transduction. Results: Figure shows that DeltaRex-G progressively induced 54% inhibition of immortalized CD4+ CD8+ T cell growth (A) and 96% inhibition of IL-2 activated primary CD4+ T cell growth (B) over 4 days post transduction. Conclusion: DeltaRex-G inhibits growth of both immortalized CD4+ CD8+ expressing human T cells and IL-2 activated primary human CD4+ T cells *in vitro*, suggesting that DeltaRex-G has the potential of reducing T cell induced inflammation and neuronal damage in patients with Parkinson's disease.



1393. Assessment and Elimination of Pluripotent Cell Contamination in Stem Cells Derived Cell Therapy Products

Louise Delsing[1], Joana Ferreira[1], Isabelle Matthiesen[1], Märta Häll[2], Marcella Sini[1], Magnus Söderberg[1], Mick Fellows[1]

[1]Clinical Pharamcology and Safety Sciences, AstraZeneca, Mölndal, Sweden,[2]EarlyCVRM, AstraZeneca, Mölndal, Sweden

Stem cells have near limitless potential for use in cell therapy as they can be generated in large amounts and, in theory, differentiate to any cell type. However, in vitro differentiation is rarely 100% efficient and any remaining pluripotent cells could generate a teratoma, creating a significant safety concern and a major hurdle for clinical application. As such, contaminating pluripotent cells need to be identified and eliminated prior to clinical delivery. We describe a combination approach using marker evaluation (flow cytometry and qPCR), the high efficiency culture assay *in vitro* and *in vivo* tumorigenicity studies, to detect undifferentiated cells with high sensitivity. By comparing batches of stem cell-derived therapy product with different levels of pluripotent contamination, we translate how marker levels correlate with contamination of viable stem cells. In addition, we provide data to support the value of a bead based pluripotent cell contamination elimination method targeting a pluripotency marker protein, which ultimately produces a cell therapy product with a pluripotent contamination of less than 1 cell in a 1 million cell dose. We demonstrate the translatability and relevance of these methods to qualify release criteria setting for a stem cell-derived cell therapy product. Furthermore, we demonstrate that *in vitro* methods have superior sensitivity to standard *in vivo* tumorigenicity studies to detect pluripotent contamination, which should be considered in future safety assessment of cell therapy products to deliver more accurate predictions of clinical safety and reduced animal use in research.

1394. The Somatic Cell Genome Editing Consortium Toolkit

Melinda R. Dwinell[1], Jennifer Smith[1], Jeff De Pons[1], Jyothi Thota[1], Angela Lemke[2], Michael Grzybowski[1], Marek Tutaj[1], Monika Tutaj[1], Anne E. Kwitek[1], Aron M. Geurts[1]

[1]Physiology, Medical College of Wisconsin, Milwaukee, WI,[2]Physiology, Medical College of Wiscsonsin, Milwaukee, WI

The Somatic Cell Genome Editing (SCGE) Consortium Toolkit is a publicly available portal to provide access to data and resources generated by members of the SCGE Consortium. The focus of the first phase of the SCGE Consortium includes novel approaches for delivery of genome editing technologies with cell and tissue specificity, independent validation, and approaches to detect adverse effects of genome editing. The SCGE Toolkit has been developed to showcase the results from the SCGE projects and provide access to experimental details including tools, reagents, and protocols with the overarching goal of promoting collaborations and accelerating the use of the validated technologies to the broader scientific community. Data generated by the SCGE Consortium members have common elements, including genome editors, delivery technologies, and models in which these are tested, including large and small animals, and in vitro human biological systems. Metadata fields were identified and defined to standardize the data submission pipeline. The majority of the submissions to the SCGE Toolkit were from the delivery technology initiative and the small animal validation studies designed to provide second site validation of the primary results generated by the delivery projects. Where applicable, the validation studies are highlighted and linked to their respective delivery studies to allow users to discern similarities and differences between the studies and interpret the extent of independent validation. Public access to the submitted data

is through the SCGE Toolkit (https://scge.mcw.edu/toolkit/). Data is organized by SCGE initiative, experiments, and data types. Users can explore and visualize data using common interface components, including filtering functions, standardized structure, and sorting and download options. As submitted data is published, Toolkit data is linked to publications, providing access to the overarching goals of the studies and broader implications of the findings.

1395. Regional Limb Perfusion of AAV1 Gene Therapy Vectors Results in Robust Muscle Transgene Expression in Cynomolgus Macaque

Debora Pires Ferreira[1], Qiushi Tang[1], Darcy Reil[1], April Destefano[1], Janet Benson[2], Jane Lindborg[2], Alisha M. Gruntman[1], Terence R. Flotte[1]

[1]Gene Therapy, University of Massachusetts Chan Medical School, Worcester, MA,[2]Lovelace Biomedical, Albuquerque, NM

Alpha-1 antitrypsin (AAT) is the primary protease inhibitor in human serum, required to mainly protect the lungs from excessive proteolytic damage caused by neutrophil elastase during inflammation. Alpha-1 antitrypsin deficiency (AATD) is a genetic disorder caused by mutations in the *SERPINA1* gene, with the prevalent deficient variant (E342K mutant allele) leading to the PiZ phenotype. This phenotype is characterized by misfolded and polymerized AAT protein, causing hepatic accumulation and impaired plasma secretion, potentially contributing to liver (e.g., chronic hepatitis, cirrhosis) and lung diseases (e.g., COPD, bronchiectasis). As AATD is a monogenic condition with no cure or effective treatment, gene therapy has emerged as a definitive therapy for the disorder. We have previously shown that delivery to skeletal muscle using a venous limb perfusion approach resulted in similar, or higher, levels of serum AAT compared to intra-muscular (IM) injection. In order to bridge these results from the IM injection route used in our last clinical trial to a clinical trial using limb perfusion delivery, we performed a study with doses bracketing our previous clinical trial and comparing the manufacturing method used for the last trial (HSV co-transfection) to the planned method for future trials (triple transfection). We used two vectors packaged in adeno-associated virus serotype 1 (rAAV1), one carrying human AAT (rAAV1-CB-hAAT) that was manufactured using triple transfection and the other rhesus AAT (rAAV1-CB-rhAATmyc) that was used in our last study and manufacturing using HSV. These constructs were administered into the right pelvic limb of a non-human primate (cynomolgus macaque) using a venous regional limb perfusion method. The animals were divided into four groups, with two to three animals in each group that included both male and female members: Group 1 received vehicle (CTRL); Group 2 received rAAV1-CB-hAAT at a dose of 3E12 vg/kg; Group 3 was received rAAV1-CB-hAAT at a dose of 1.2E13 vg/kg; and Group 4 received with rAAV1-CB-rhAATmyc at a dose of 6E12 vg/kg. Our findings demonstrated that the rAAV1-CB-hAAT vector successfully transduces the skeletal muscle and produces relatively high M-AAT serum levels (300-400 μg/ml) (analyzed by ELISA and Western blot for hAAT or c-mycAAT) in a safe manner at both lower (3E12vg/kg) and higher (1.2E13 vg/kg) dosages. When comparing the transduction efficiency and safety profile of the rAAV1-CB-hAAT and rAAV1-CB-rhAATmyc vectors, there was no significant difference. The toxicity profile and potential for organ toxicity were examined through complete blood counts, platelet counts and serum chemistry, there was no evidence of toxicity. The rAAV1-CB-hAAT and rAAV1-CB-rhAATmyc vectors show potential as a viable option for skeletal muscle gene replacement therapy at high doses, particularly in cases of pre-existing liver conditions, offering a "liver-sparing" approach.

1396. Navigating Viral Safety: A Patient-Centric Approach to Biotherapeutic Development

Alysia Birkholz

Texcell - North America, Inc., Frederick, MD

Traditionally, safety assessments for biotherapeutics or drug products are conducted prior to human clinical trials, serving as a vital prerequisite for regulatory approval. Notably, the recently revised ICH Q5A, now R2/revision 2, stands out as a globally harmonized and influential Viral Safety document. This update brings attention to patients with less common or 'orphan diseases,' expanding its scope to encompass a more diverse patient population. The document also provides insights into addressing the imperative of viral testing at earlier stages of development. Alysia Birkholz, a patient and leader in the biotech industry, will share her unique perspective in a deeply personal narrative. She will delve into the significance of such safety testing and emphasize the criticality of early consideration in the development of manufacturing processes. Her story serves as a compelling call to action within the scientific community.

Thursday Posters: Molecular and Cellular Methods

1397. A Versatile Assay for Same-Section Spatial Visualization of Small RNA, mRNA, Exon Junction, Protein and Protein-Protein Interactions

Sonali A. Deshpande, Ge-Ah Kim, Manvir Sambhi, Julia Yu, Li-Chong Wang, Maithreyan Srinivasan

ACD-A Bio-Techne Brand, Newark, CA

Biologics and small molecule therapeutics have been the mainstay of treatment options despite only 0.05% of the human genome targeted by currently approved biologics and 85% of protein targets lack appropriate structural features for efficient small molecule binding and modulation (PMID: 19740696). RNA molecules such as antisense oligos (ASOs) and small interfering RNAs (siRNAs) expand the range of targets available for therapeutic intervention by directly binding to target RNA transcripts to modulate gene function. In addition, biological vector platforms are increasingly used to deliver payloads to either correct a gene function or engineer cell therapies *ex-vivo* or *in vivo*. All these studies require technologies to understand the biodistribution of the potential therapy in pre-clinical models and patient samples to ensure there are no off-target effects. To understand the biodistribution of cell and gene therapies (CGTs) and evaluate their safety and efficacy, we have developed a novel TSA based, protease-free, highly flexible

approach that integrates the ability to visualize miRNAs/ASOs/siRNAs, exon regions, mRNAs, proteins, and protein-protein interactions. The same-slide, 6-plex assay uses newly improved RNAscope chemistry with robust sensitivity of detection for exon junctions and point mutations while retaining the sensitivity and specificity for mRNA and small RNA targets. Here we present a proof-of-concept study to assess the impact of BRAF V600E mutation on miRNA, mRNA and protein targets in the MAPK signaling pathway. These targets enabled visualization and identification of different cell types in the tumor immune microenvironment (TIME). Co-expression of targets resulted in detection of specific tumor infiltrating immune cell populations and analysis of their activation states. The assay offers a powerful technique for multiomic analysis and accurate interrogation of complex tissues to obtain insights into novel biomarkers and therapeutic targets. In addition, the method can contextualize the biodistribution of CGTs with potential protein markers for cell types and further interrogate cell-cell/protein-protein interactions.

1398. RNA/Protein Multiomics Imaging Using RNAscope Technologies to Understand Nonclinical Biodistribution (BD) and Persistence of Gene Therapy (GT) Products

Sonali A. Deshpande, Anushka Dikshit, Manvir Sambhi, Li-Chong Wang, Maithreyan Srinivasan
ACD-A Bio-Techne Brand, Newark, CA

With over ten approved gene therapies and over 1500 ongoing GT clinical trials, the FDA has issued recommendations to generate nonclinical biodistribution (BD) data for "the evaluation and interpretation of nonclinical pharmacology and toxicology findings prior to initiation of human clinical trials". While several technologies to assess BD exist, RNA *in situ* hybridization is increasingly used to spatially visualize the delivered therapeutic, target gene, transgene and/or cell markers and complement information gathered from molecular technologies such as quantitative polymerase chain reaction (qPCR). Recently, RNAscope technologies have been widely used to co-detect the presence of vector and transgene expression as well as ASO/siRNAs and their intended mRNA targets. To enhance the information gained from these technologies and to satisfy FDA recommendations on understanding BD in the context of on and off-target cell types and tissues we have developed a novel codetection method that enables the detection protein targets along with of miRNAs/ASOs/siRNA and mRNAs. Built on the flagship RNAscope technology, the RNAscope Plus assay can detect 1 small RNA and 3 mRNA targets using TSA-based fluorescent readouts. Fixed, fresh frozen samples and formalin fixed paraffin embedded (FFPE) tissues are supported by manual and automated workflows using the Leica Bond Rx system. We have leveraged this technology to investigate spatial expression profile of miRNA and associated RNA targets across different tissue types and applications. We can also combine protein detection using a target antibody to visualize cell-type specific markers. This assay was used to demonstrate expression of miRNAs and target genes implicated in tumor initiation, progression, and angiogenesis. Expression of miR-205, associated tumor target genes such as *PTEN* and tumor suppressor *TP53* was visualized in head and neck cancer tumors. Downregulation of tumor-suppressor, *TP53* resulted in upregulation of miR-205 which downregulates *PTEN* expression. PanCK (protein) stained the tumor region in the tissue. This novel platform will enable researchers to visualize regulatory RNA simultaneously with target RNAs, protein, cell type and morphology markers in intact cells/tissues with single cell resolution. This technology can provide meaningful insights into disease pathology driven by miRNAs as well as assess biodistribution and efficacy of oligonucleotide therapeutics.

1399. Efficient Delivery of Lentiviral, DNA, and mRNA Reporter Gene Vectors in Human Colonic Organoids

Yanghao Fu[1,2], Mathieu Derouet[3,4], Samuel Asfaha[3,4], John Ronald[5]
[1]Medical Biophysics, University of Western Ontario, London, ON, Canada,[2]Robarts Research Institute – Imaging, London, ON, Canada,[3]Medicine, University of Western Ontario, London, ON, Canada,[4]Schulich School of Medicine & Dentistry, London, ON, Canada,[5]Medical Biophysics, The University of Western Ontario, London, ON, Canada

Introduction: Colorectal cancer (CRC) is a leading cause of cancer-related mortality, ranking second for men and third for women (Favoriti et al., JGO, 2019). Colonic organoids, developed from single colonic epithelial stem cells, effectively replicate the gut lining and are instrumental in studying human colonic physiology and diseases like CRC. These organoids offer significant advantages over small animal models, particularly in high-throughput screening of therapies and modeling human colon cancer progression (Yoo et al., WJG, 2019). To date, few laboratories have successfully modified organoids using transgene delivery methods using plasmids (Sato et al., Nat Prot, 2015) or lentiviral vectors (Spence et al., 2022). Given the transient and safe nature of mRNAs, exploring novel mRNA delivery into colonic organoids as a method of delivering gene editing systems is of significant interest. Our research focuses on comparing the efficiency of plasmids, lentiviruses, and mRNA gene delivery vectors for carrying a green fluorescent reporter gene into colonic organoids. **Methods:** We generated healthy human colonic organoid lines from patient-derived crypts, isolated and dissociated into single cells, then cultured in Matrigel. Plasmids and mRNA encoding green fluorescent protein (GFP) were delivered using electroporation, with rock inhibitor to reduce cell death during dissociation. Lentiviruses were mixed with dissociated organoids before embedding in Matrigel-coated wells, followed by fluorescence imaging at various time points post-delivery. Flow cytometry was used to assess delivery efficiency, and organoid growth was measured on the 7th day. Organoid establishment rates were tracked from day 1 to day 7. **Results:** Delivery efficiencies were approximately 80% for lentiviruses, 55% for DNA, and 85% for mRNA. Notably, mRNA delivery was significantly more efficient than plasmid-based methods ($p < 0.01$). Organoid growth post-delivery showed no significant differences across the groups, indicating the viability of all these methods for gene delivery. **Discussion:** This study underscores the versatility of gene delivery methods in colonic organoids, with lentiviral vectors enabling permanent gene integration and mRNA providing a safe, transient alternative for gene editing. The development of an efficient mRNA delivery system, reminiscent of its application in COVID-19 vaccines, represents a significant stride in CRC research, offering new avenues for modeling the disease and testing therapeutic

interventions. The differential permanence of gene expression by these vectors enriches the toolkit for genetic studies in colonic organoids, facilitating in-depth investigations of CRC onset and treatment efficacy.

1400. Mapping AAV Integrations Using Long-Read Sequencing (MAILR) to Solve the Structure of Complex AAV Integrations Associated with Genotoxicity

Eli J. Kaufman[1], Randy J. Chandler[2], Charles P. Venditti[2], Paul N. Valdmanis[1]

[1]Medicine (Medical Genetics), University of Washington, Seattle, WA,[2]National Institutes of Health, Bethesda, MD

Background: Recombinant AAV integration after gene therapy in preclinical models and clinical trials remains a critical determinant of long-term safety in patients. While considered a non-pathogenic virus, rAAV exposure can cause hepatocellular carcinoma (HCC) formation in mice (PMID: 28293963) and is associated with clonal expansion of transduced hepatocytes in hemophiliac dogs (PMID: 33199875). Additionally, the recent observations that integrated AAV vectors not only contribute to long term transgene expression in nonhuman primates but display complex rearrangements after integration (PMID: 37932420) further highlight the need to comprehensively investigate rAAV integration. Previous studies have largely relied upon LAM PCR, with primers anchored into the ITR, to amplify integration junctions, and the recovery of both 5′ and 3′ ends of integrated vectors has remained elusive and recalcitrant to chromosomal walking. **Methods:** To systematically evaluate rAAV integrations in an unbiased manner, we developed a pipeline that relies upon whole genome Nanopore long-read sequencing in an approach we designate as Mapping AAV Integrations using Long-Read sequencing (MAILR). DNA was obtained from from 21 mouse HCCs, many of which were associated with AAV integrations at the mouse *Rian* locus on chr12qF1 (PMID: 25607839). Samples were barcoded and then multiplexed and sequenced on a PromethION machine, yielding 72 million reads. We split the library into an initial flow cell for whole genome sequencing (achieving an N50 of 13.9kb) and two subsequent flow cells with alignment to an adaptive sampling file corresponding to regions of integration and the delivered AAV vector to enrich for integration reads. We used minimap2 for genome alignment, sniffles2 (v2.2) for calling insertions and deletions, and the MinKNOW integrated basecaller to query read methylation. **Results:** We successfully retrieved long reads that spanned both AAV integration junctions and the complete integrated sequence for 11/21 HCCs (52%), including several samples that failed LAM PCR based characterization. Each integration was unique, ranging from 255 to 4,545 bp, and was specific to the *Rian* locus on chr12qF1. However, within each tumor, AAV reads were homogenous and approached 50% of reads (1 of 2 chromosomes) at integration sites, suggesting clonality of the tumor sequence. All integrations contained promoter or enhancer elements, though only two contained the complete transgene. Importantly, we recovered several classes of integrations that would not be detected using conventional techniques, including one completely devoid of ITR sequence, another with a complex inversion event within the locus, and an integration coupled with a chromosomal translocation event between *Rian* and mouse chromosome 8. As Nanopore sequencing also permits methylation analysis, we determined that all integration-containing reads (43 total) were associated with DNA hypomethylation *in cis*, while the corresponding reads on the non-integrated allele were universally hypermethylated (46 reads). RNAseq confirmed that integration-associated DNA hypomethylation was associated with microRNA and RNA over-expression at the *Rian* locus. **Conclusions:** We demonstrate that our long-read sequencing and analysis pipeline, MAILR, is a cost-effective and sensitive approach to recover complex rAAV integration events associated with genotoxicity. MAILR addresses a critical gap in identifying potentially oncogenic rAAV integration events longitudinally across various organs both in preclinical models and in human subjects after gene therapy.

1401. Automated Analysis of High-Resolution Whole Genome Maps for Biodosimetry

Christopher J. Tompkins[1], Matthew McGowan[1], Douglas Brugge[2], Susan Bailey[3], Erin Cross[1], Aaron Datesman[4]

[1]KromaTiD, Longmont, CO,[2]University of Conneticut, Farmington, CT,[3]Colorado State University, Fort Colins, CO,[4]University of Virginia, Washington, DC

Understanding the relationship between a past exposure to radiation or cytotoxic compounds requires a highly sensitive measure of long-lasting DNA damage markers typically through a single cell cytogenetic test for structural variation. Historically, however, these cytogenetic biodosimetry assays have measured DNA damage in only a fraction of the genome or focused on individual classes of structural events, such as dicentric chromosomes. The dGH SCREEN assay developed by KromaTiD Inc. is whole genome cytogenetic test that uses ten million individual oligonucleotide hybridization produces targeting all the unique sequence in the genome. dGH produces high-resolution, single-cell whole genome maps of genomic rearrangements as small as 5Kb. Because of the unique nature of the dGH platform, dGH SCREEN is an unbiased measure of *all classes* of structural variants, including those involving orientation changes, such as inversions. Previous work with the SCREEN assay has demonstrated that this whole genome approach provides a higher sensitivity measure of structural variation and thus a more robust correlation of rates of structural variation with received dose. Inversions, in particular have proven to be more sensitive biodosimeters as they are more common and more stable than translocations. However, a critical limitation to the throughput of any single-cell cytogenetic assay is the labor involved in assessing the images of many individual genomes, labor that increases exponentially with the genomic coverage. For instance, in a typical unautomated cytogenetic analysis, the requirements of human scorers limit the number of single cell genomes analyzed to tens or hundreds and our human poweredl biodosimetry SCREEN assay is usually performed on only 50 single cell genomes. To address this limitation, which is most significant for low dose exposures with their corresponding rarer DNA damage events as well as for modified cells with low rates of off-target editing we have developed dGH SCREEN into a high-throughput automated cytogenetic DNA damage test. Using an efficient data pipeline combined with a well-trained AI, we are now measuring the long-lasting DNA markers of radiation exposure in a cohort exposed during and after the accidental Three Mile Island radiation release, analyzing tens, hundreds or even thousands of genomes with essentially no human image analysis labor. For the TMI cohort in particular, the extent and types of radiation released was not enough to explain the

reported clusters of cancers and other health effects downwind from the accident. Using AI SCREEN we are today providing the only method with the required sensitivity to understand the biological mechanisms of DNA damage after low dose exposures while at the same time, measure the true received dose of the exposed population. Finally, the method developed for measurement of historical radiation exposures is today being used to detect off-target DNA damage in batches of edited cells which occur at low frequency but provide similar long term cancer risk to patients receiving gene therapies.

1402. Genomic Integrity G-Banding for Cell and Gene Therapy Drug Product

Christopher J. Tompkins, Greg Husar, Matt McGowan, Michael Vernich

KromaTiD, Longmont, CO

T-Cell gene therapy drug products show great promise for treating refractory hematological diseases. However, when moving from an autologous to allogeneic treatment strategy, additional editing steps are required to prevent graft versus host disease and the edited cell population must be stimulated and expanded to produce batches large enough to treat multiple patients. Both the editing and the expansion are robust cellular processing steps, but they do create additional opportunities unwanted editing by-products, sub-clonal outgrowth, and/or genomic instability. To advance these novel gene therapies from R&D to the market while minimizing new risks to the patients, requires measurements of structural variation, sub-populations and markers of instability.G-banded Karyotyping is a classic cytogenetic method for imaging the structure of metaphase chromosomes typically applied to measure the high frequency structural variation associated with genetic diseases. While the resolution of G-banding is poor when compared to DNA hybridization based methods such as metaphase FISH or dGH, as a stain of secondary chromosome structure, G-Banding is an orthogonal and complementary approach to any measurement of DNA structure or sequence. As a metaphase chromosome "mapping" method, Single-cell g-banding karyotyping offers a direct visualization of individual chromosomes and the method is sufficient to detect large structural variations such as translocations or aneuploidy along with markers of instability such as complex rearrangements. When producing cell and gene therapy drug product from donor cells or cell lines, maintaining the integrity of the genome throughout editing and expansion is critical. Here we describe an adaption of classical G-Banding: **Genomic Integrity G-Banding** optimized for Cell and Gene therapy products. Rather than focused on high-frequency events driving genetic diseases, Genomic Integrity G-Banding uses larger numbers of cells (typically 100 or 200 rather than 20) to measure the low frequency events that occur during editing and expansion. This measurement is performed using a matched sample pair: the pre-editing donor or wild-type cells and the final drug product after editing and any required expansion. Genomic Integrity G-Banding is then a "before" and "after" comparison measuring the differences between edit associated structural variants and karyotypic variation from the baseline levels in donor cells. Importantly, the risk associated with instability or a specific variant is only related to the identity of the variant, not the source. Genomic Integrity G-Banding is a true, unbiased method and an equivalent measure of structural variation regardless of causation, whether that be an edit, impaired DNA damage repair capacity, or a variation contributed to the drug product from the donor cells themselves.

1403. dGH In-Site: An Assay System for the Simultaneous Single Cell Measurement of Edit Associated Structural Variation and Transgene Localization

Christopher J. Tompkins, Erin Cross, Stephen Gross

KromaTiD, Longmont, CO

Cell and Gene therapy products such as CAR-T or CAR-NK often require complex editing systems combining nuclease-based editing with insertion of one or more transgenes into the target genome. This complexity leads to additional opportunities for on- and off-target double-strand breaks which can mis-repair to form structural variants, such as inversions and translocations. Even in systems with highly specific targeted transgene insertions, there is the potential for off-target insertions at random or off-target editing loci within the genome.These number (and sometimes location) of the transgenes in these complex edited products can be measured on average by NGS or dPCR methods. However this averaging divorces structural variant and transgene data from any cellular context, making it impossible to observe co-localization of events or distribution of transgenes in a batch of edit cells; with only dPCR data, it is not possible to differentiate one cell with ten transgenes from two cells with five transgenes.This type of quantitation is best performed by a comprehensive, single cell method such as directional Genomic Hybridization™ (dGH™). dGH is a unique cytogenetic technique for mapping the structure and structural variation of individual genomes in single cells. Based on images of fluorescently labeled DNA probes designed to target specific loci and hybridized to metaphase chromosomes, dGH in-Site assays provide direct detection of structural variation, including DNA inversions, a common editing by-product. Combining this data with the number and location of transgene insertions however, is challenging due to the relatively small size of therapeutic CARs, often smaller than 2Kb. By combining advanced DNA probe design strategies that avoid homology, repetitive sequences and other sources of fluorescent signal noise with the unique sample preparation inherent in dGH, we have designed and qualified a single cell assay system for the simultaneous measurement of edit associated structural variation and transgene localization *in the same single cell*. Using a model transgene system we demonstrate the potential of this system for understanding the true structural heterogeneity in batches of edited cell products.

1404. A High Throughput, Multi-Plex, ddPCR-Based Platform for Cellular Kinetics and Biomarker Detection in Biofluid and Tissue Samples

Neha Sangewar, Jean-Pierre Minembe, Jing Mo, Hiroshi Sugimoto

Global DMPK, Takeda Pharmaceuticals (TBOS), Cambridge, MA

Droplet digital PCR (ddPCR) is a widely used platform for the detection and quantification of cell/gene therapy drugs, genetic mutations and disease biomarkers. It allows absolute quantification of target with high

accuracy and precision, and is compatible with various sample types. A typical ddPCR assay used in discovery and clinical research involves using FAM and HEX probes for detecting a target chimeric antigen receptor (CAR) gene for monitoring the CAR cells and a reference gene for normalization in blood samples. However, due to shared homology, the detection of CAR cells in mouse models xenografted with human tumors is challenging. Also, the CAR-specific 2-plex assay isn't designed for detecting un-transduced (UTD) control cells in the xenografted mouse model. Therefore, we applied a higher order of multi-plex using the QXOne ddPCR system where we developed a 4-plex assay for concurrently tracking CAR and UTD cells in the xenograft model. Using the same 4-plex assay format, we also designed an RT-ddPCR assay for detecting multiple target mRNA biomarkers in support of quantitative biomarker studies. Lastly, we developed a high throughput, automated, multi-plex assay workflow that we apply to rapidly bulk-analyze the *in vivo* study samples for CAR and biomarker quantification (Fig. 1). Hence, we are reporting here two case studies outlining the application of this multi-plex platform to generate cellular kinetics and genomic biomarker data. In the first study, we analyzed the blood samples from tumor xenografted mice that were dosed with either CAR cells, UTD cells or PBS using our 4-plex ddPCR assay. We successfully measured the proprietary CAR (FAM) and armoring (Cy5.5) constructs in CAR cell dosed mice. In the UTD group, UTD cells were detected using a human-specific gene (Cy5) whereas in PBS group, only the reference mouse- and human-specific gene (HEX) used for genomic DNA input normalization was detected. Our results demonstrate that our 4-plex assay allows simultaneous detection of CAR cells, armoring payload and UTD cells in *in vivo* samples which is highly crucial for the efficacy, toxicity and long term persistence analysis of the cell therapy product. In the second case study, we used the 4-plex one-step RT-ddPCR assay for mRNA quantification in the control non-human primate (NHP) biofluid and tissue samples. We observed that compared to the MetAP2 (HEX) and NONO (CY5) mRNA levels, the PIKfyve (FAM) mRNA levels were extremely low in NHP biofluid samples where PPP2R5C (Cy5.5) mRNA was used as housekeeping reference for normalization. In contrast, all three biomarkers were detected consistently in the tissue samples. Additionally, the developed 4-plex RT-ddPCR assay successfully detected these biomarkers, that are potential therapeutic targets for treating cancer such as glioblastoma (NONO and MetAP2) and B-cell lymphoma (PIKfyve), in control human tissue samples. So, our data shows that the 4-plex RT-ddPCR can be a valuable tool for supporting biomarker studies for drug target engagement and treatment prediction. Overall, we have designed a multi-plex genomic assay platform which is high throughput, automated, fast and adaptable across non-clinical and clinical studies.

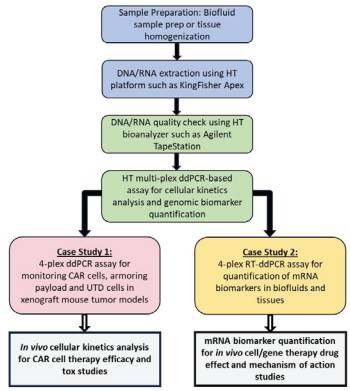


Figure 1. High throughput ddPCR-based multi-plex genomic assay workflow.

1405. Analyzing CAR T-Cell Genomes: Heterogeneity, Integration Patterns, and Safety Considerations

Maya Wright, Emily Caulfield, Dirk Kruit, Irina Sergeeva, Bergboer Judith, Georgios Georgiou, Nicole Hendriks, Ellen Stelloo

Cergentis, Utrecht, Netherlands

Chimeric Antigen Receptor (CAR) T-cell therapy has demonstrated substantial efficacy in hematological malignancies, yet concerns persist over the safety of viral and transposon-based vectors used for gene transduction due to the potential risk of insertional mutagenesis. This is not unprecedented, as the FDA has received reports of T-cell malignancies in patients who received treatment with CAR T-cell immunotherapies. Targeted Locus Amplification (TLA), an NGS technology based on proximity ligation, allows comprehensive genetic characterization of engineered CAR T-cells, focusing on the distribution of vector integrations and their proximity to cancer-related genes. Viable frozen retroviral transduced human T-cells were processed using Cergentis' TLA protocol. Genetic analysis, employing high-throughput sequencing, identified 1,078 different integration sites based on breakpoint sequences. The integration sites were distributed across all chromosomes in gene exons (5%), gene introns (48%) and within 1 kb upstream of genes (8%). Viral integration in gene exons occurred in 49 genes from which 11 were listed as cancer genes in the *NCG7.0 network of cancer genes*. While no integration site occurred more frequently than other sites, the sample exhibited significant heterogeneity; most of the integration sites were present in a limited number of reads with no overlap between the TLA data of 2 primer sets. Additional amplifications and/or sequencing could further illuminate integration patterns and sample heterogeneity. The sample's heterogeneity prohibited variant detection within the integrated provirus in individual integration sites but allowed analysis of abundant variants. In conclusion, TLA has proven valuable to identify integration sites, their distribution, and proximity to cancer-related genes. The occurrence of viral integrations within cancer-related genes emphasizes potential risks. A comprehensive understanding of CAR T-cell genetics is crucial for advancing these therapies safely and aligning them with stringent regulatory standards.

1406. Comparative Analysis of U937 and THP-1 Monocytic Cell Lines as Macrophage Models for Drug Discovery

Shasha Li, Cindy Merino, Miguel Calero-Garcia, Mihee Kim

CSL Behring, Pasadena, CA

In drug discovery, high-throughput screening plays a pivotal role, especially in gene therapies. Robust and scalable cellular models are crucial for these endeavors, but challenges with primary monocytes and macrophages, such as donor variability and limited cell numbers, lead researchers to monocytic cell lines. This study thoroughly characterizes the responses of U937, and THP-1 cell lines after priming with phorbol 12-myristate 13-acetate (PMA) and exposure to polarizing stimuli. Differentiation into macrophage-like cells is induced using lipopolysaccharide (LPS), interleukin-4 (IL-4), or interferon-stimulating DNA (ISD). Evaluation includes immunophenotyping with 242 markers via flow cytometry, cytokine secretion profiling (16 cytokines) using the meso scale delivery platform (MSD), and interferon-stimulated gene (ISG) transcriptomic signatures via quantitative polymerase chain reaction (qPCR). Additionally, U937 and THP-1 cells show distinct transduction availabilities when transduced with lentiviral vectors. Despite differences from primary macrophages, these unbiased findings provide critical data for future drug screening assays using U937 and THP-1 cells, contributing insights to optimize gene therapy and drug discovery strategies.

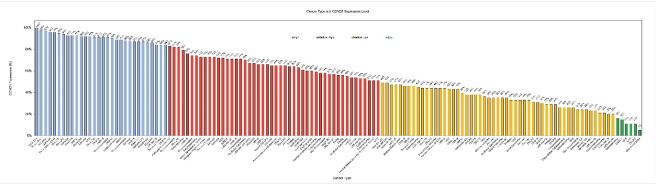
Thursday Posters: Gene Therapy Trials - In Vivo Modification

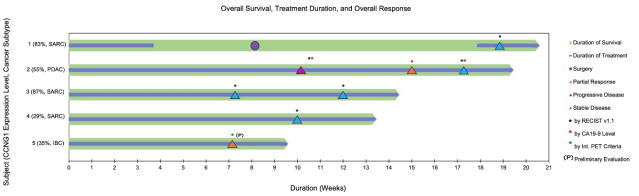
1407. Deltarex-G Plus (Deltarex-G + FDA Approved Drug) Demonstrates Clinical Benefit in Advanced Chemoresistant Pancreatic Cancer, Sarcoma, and Breast Cancer (NCT04091295)

Samantha Jeffrey[1], Nadezhda Omelchenko[2], Vaishali Kumar[3], Rheanna Carter[4], Anmol Dia Agarwal[3], Rebecca A. Reed[1], Neal S. Chawla[3], Don A. Brigham[5], Ishrat Bhuiyan[3], William H. Isacoff[2], Michael A. Morse[6], William Swaney[7], Sant P. Chawla[8], Erlinda M. Gordon[1], Frederick L. Hall[5]

[1]Medical Oncology, Cancer Center of Southern California/Sarcoma Oncology Research Center and Aveni Foundation, Santa Monica, CA,[2]Medical Oncology, Cancer Center of Southern California, Santa Monica, CA,[3]Medical Oncology, Cancer Center of Southern California/Sarcoma Oncology Research Center, Santa Monica, CA,[4]Medical Oncology, BostonGene, Morgan Hill, CA,[5]Medical Oncology, Aveni Foundation, Santa Monica, CA,[6]Medical Oncology, Duke University Medical Center, Durham, NC,[7]Medical Oncology, Expression Therapeutics Inc., West Chester, OH,[8]Medical Oncology, Cancer Center of Southern California/Sarcoma Oncology Center, Santa Monica, CA

Background: Expanded access for DeltaRex-G, a tumor targeted retrovector encoding a CCNG1 inhibitor, is being studied for advanced cancers. **Methods:** CCNG1 expression levels in 137 tumors were assessed by RNA analysis. Nine cancer patients with known CCNG1 levels received DeltaRex-G with an FDA-approved drug (DeltaRex-G Plus) or as monotherapy. Overall survival, duration of treatment, and responses were evaluated. **Results:** Enhanced CCNG1 expression levels are depicted in Figure 1 by cancer type. Thirty (22%) tumors showed High CCNG1 expression, 48 (35%) showed Medium-High CCNG1 expression, 53 (39%) displayed Medium-Low CCNG1 expression, and six (4%) had Low CCNG1 expression. One hundred (73%) patients had metastatic disease, and 37 (27%) had localized disease. There was no correlation between CCNG1 expression and disease stage. Four patients with advanced pancreatic cancer, HR+Her2+ invasive breast cancer, triple negative breast cancer, and squamous cell carcinoma received DeltaRex-G between 2008 and 2021 and are alive 15, 3, 2.5, and 2.5 years from treatment initiation, respectively. Their tumors showed CCNG1 expression levels of 24%, 23%, 74%, and 40% respectively. One patient with pancreatic cancer and 2 patients with breast cancer enjoy sustained remission. Five patients are currently being treated with DeltaRex-G Plus: one patient with advanced breast cancer had a partial response, one patient with advanced pancreatic cancer and three patients with sarcoma had stable disease. All 5 patients had previously progressed with standard doses of chemotherapy. All patients are alive from 9 to 20 weeks since treatment initiation. Figure 2 illustrates the cancer type/CCNG1 expression level on the vertical axis and time from treatment initiation, duration of survival, and duration of treatment on the horizontal axis. No serious treatment-related adverse events have been reported. **Conclusions:** Taken together, the data shows that (1) 100% of 137 tumors tested have enhanced CCNG1 expression, further reinforcing the 2021 FDA-CBER authorization for use of DeltaRex-G Plus, and (2) DeltaRex-G Plus is a promising therapy for advanced pancreatic cancer, sarcoma, and breast cancer. Randomized Phase 2/3 studies are warranted to confirm the efficacy and safety of DeltaRex-G Plus.





1408. Preliminary Safety Results from the Ph1/2 Study of NGN-401, a Novel Regulated Gene Therapy for Rett Syndrome

Bernhard Suter[1], David Lieberman[2], Sravan Jaggumantri[3], Cynthia Feng[3], Stuart Cobb[3], Julie Jordan[3]
[1]Departments of Pediatrics & Neurology, Texas Children's Hospital, Baylor College of Medicine, Houston, TX,[2]Department of Neurology, Boston Children's Hospital, Harvard Medical School, Boston, MA,[3]Neurogene Inc., New York, NY

Introduction: Rett syndrome (RTT) is an X-linked severe neurodevelopmental disorder of the nervous system caused by mutations in the *MECP2* gene that lead to deficiency of the methyl-CpG binding protein 2 (MeCP2). Conventional gene therapy approaches have not successfully advanced beyond preclinical development, as the MeCP2 levels required for efficacy are commonly associated with rapid onset toxicity, driven by the overexpression of MeCP2 in highly transduced cells. NGN-401, a recombinant AAV9 gene transfer therapy containing the full-length human *MECP2* gene, uses Neurogene's proprietary transgene regulation EXACT technology, designed to express a therapeutic level of MeCP2, while avoiding toxicity associated with overexpression in each transduced cell. Head-to-head biodistribution comparison of various routes of delivery for AAV9 demonstrated in non-human primates that intracerebroventricular (ICV) administration yields a 10-100x higher level of vector biodistribution to key areas of the brain that drive fundamental phenotypes in RTT relative to intrathecal lumbar administration. As a result, all nonclinical studies of NGN-401 were conducted using ICV to maximize its therapeutic potential in clinical studies. **Study Design:** NCT05898620 is an ongoing Phase 1/2 open-label study evaluating the safety, tolerability, and preliminary efficacy of NGN-401 in pediatric female subjects ages 4-10 years old with classic RTT, and Clinical Global Impression-Severity scores of 4-6. NGN-401 is delivered via ICV administration and prophylactic immunosuppression with corticosteroids is initiated at Day -1. Subjects in Cohort 1 receive a one-time total dose of 1E15 vector genomes. **Study Results:** The first three enrolled subjects were ages 7, 4 and 6 years at baseline, with a milder missense mutation in the first subject and mutations shown or predicted to be more severe in subjects two and three. Follow up data at the time of the abstract submission was approximately 6 months, 3 months, and 2 weeks for the three subjects. NGN-401 administration was generally well tolerated by all 3 subjects with no procedure-related adverse events. No clinical or laboratory signs or symptoms of MeCP2 overexpression (ie, no evidence of respiratory, gastrointestinal, or neurologic manifestations) have been observed in any subject post NGN-401 administration. Alltreatment-related adverse events have been mild (Grade 1). There has been oneGrade 1 ALT elevation and one Grade 1 AST elevation. All elevated liver enzyme levels have resolved or are resolving with corticosteroid administration. There have been no treatment-related serious adverse events. **Conclusions:** NGN-401 given via one-time ICV administration has been generally well tolerated, with an adverse event profile consistent with AAV9 administration. No clinical evidence of MeCP2 overexpression was observed in three pediatric female subjects with differing pathogenic mutations of classic RTT, including one predicted to result in residual MeCP2 function.

1409. Optogenetic Therapy UGX-201 for Patients Inflicted by Advanced Retinitis Pigmentosa: Interim Results of an Investigator Initiated Trial to Evaluate the Safety, Tolerability and Preliminary Efficacy at First Affiliated Hospital of Soochow University

Peirong Lu[1], Ziyue Song[1], Gaoqin Liu[1], Xiaohua Liu[2], Sijie Sun[2], Shanzhong Zhang[2,3], Li Li[2]
[1]The First Affiliated Hospital of Soochow University, Suzhou, China,[2]Ugenex Therapeutics Ltd.Co, Suzhou, China,[3]Otovia Therapeutics, Shanghai, China

Background: Retinitis pigmentosa (RP) is a genetic disorder characterized by progressive loss of vision due to degeneration of retinal photoreceptors. Optogenetic therapy represents a novel therapeutic approach, aiming to confer light sensitivity to retinal neurons which are normally light-insensitive. UGX-201 is an investigational optogenetic therapy designed to restore partial vision in advanced RP patients. It utilizes an adeno-associated virus (AAV) vector containing a gene of interest that expresses recombinant opsin upon transfection of retinal ganglion cells (RGCs), enabling them to perceive light and convert it to electrical signals. Methods: An open-label, single-arm investigator initiated trial (IIT) was conducted to evaluate the safety and efficacy of UGX-201 in advanced RP patients at the ophthalmology department of the First Affiliated Hospital of Soochow University, China. The trial was approved by the Science and Ethical Committee of the hospital. It planned to enroll two distinct cohorts: Cohort 1 included patients with extremely low visual acuity (Best-corrected visual acuity, BCVA<0.02) but with light perception, and Cohort 2 included patients without light perception, 6 patients each. Every patient signed an Informed Consent Form (ICF), underwent all necessary screening tests, and then received the investigational treatment. Following the treatment, the patients were followed for up to 52 weeks. The primary outcome measures of the study were focused on evaluating the safety of UGX-201, while the secondary outcome measures were aimed at assessing its efficacy. To date, enrollment has been completed for Cohort 1, with 6 patients, and has begun for Cohort 2, with 1 patient enrolled. Results: The interim results of the investigator-initiated trial evaluating the safety, tolerability, and preliminary efficacy of UGX-201 in advanced retinitis pigmentosa (RP) patients have shown promising outcomes. The safety profile of UGX-201 demonstrated favorable results, with no serious adverse events reported and all adverse events being of grade 1 seriousness. In terms of efficacy, the first patient in Cohort 1, barely able to see finger counting at 10cm at baseline, showed improvements in visual acuity, as evidenced by their performance on the Early Treatment Diabetic Retinopathy Study (EDTRS) chart, FrAct LogMAR and Full-field Stimulus threshold Testing (FST) results. The results suggest that UGX-201 optogenetic therapy has the potential to yield substantial improvements in visual function for patients with advanced RP, even for those with extremely low baseline visual acuity. Further data from the completion of follow-up for remaining patients in Cohort 1 and assessment of Cohort 2 will provide more comprehensive information on the safety and efficacy of UGX-201. Conclusion: Initial findings of this IIT indicate that UGX-201, the optogenetic therapy using the AAV vector to deliver and express recombinant opsin in RGCs, could be a safe and effective treatment for enhancing visual function in individuals with advanced RP, even those with extremely low baseline visual acuity.

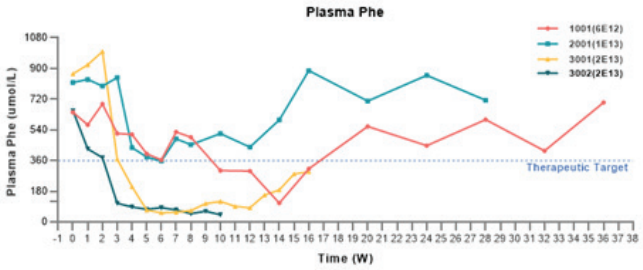
More comprehensive data on the safety and efficacy of UGX-201 will be obtained upon completion of follow-up for the remaining patients in Cohort 1 and the full enrollment and assessment of Cohort 2.

1410. Safety and Efficacy of NGGT002 (rAAV8-hPAH) in Adults with Phenylketonuria (PKU): Results from an Investigator Initiated Study

Huan Zhou[1], Xiaoli Li[1], Gang Wang[2], Zhimin Shi[2], Yiting Liu[3], Guang Qu[2,3], Lixin Jiang[2,3], Hongju Wang[1]

[1]National Institute of Drug Clinical Trial, The First Affiliated Hospital of Bengbu Medical College, Bengbu, Anhui, China,[2]NGGT (Suzhou) Biotechnology Co., Ltd., Suzhou, Jiangsu, China,[3]NGGT, Inc, Walnut Creek, CA

Introduction: Phenylketonuria (PKU), also known as Phenylalanine hydroxylase (PAH) deficiency, is a rare autosomal recessive inherited disorder of phenylalanine (Phe) metabolism, most commonly caused by pathogenetic variants in the PAH gene. Hepatic PAH is responsible for the conversion of Phe to tyrosine (Tyr). Deficiency of PAH activity results in accumulation of Phe and lack of Tyr in the blood and brain, and subsequently abnormal brain development in childhood, psychiatric and cognitive problems in adults. The outcomes of current dietary restriction treatment are still suboptimal and pharmacological approaches have drawbacks. NGGT002 is an AAV8-based vector carrying the optimized expression cassette for human PAH (hPAH) and being investigated for treatment of PKU. Here, we present the safety and efficacy results of 4 adults with PKU who received a single intravenous (IV) infusion of NGGT002 in an investigator initiated trial (IIT). **Methods:** In the dose-exploring IIT study (NCT06061614), NGGT002 (rAAV8-hPAH) was administered at three ascending doses into adult PKU patients who had confirmed PAH mutations and displayed baseline serum Phe levels of ≥600 μmol/L. The primary endpoints included the incidence and severity of treatment-related side effects and the proportion of patients achieving a sustained blood Phe concentration of ≤360 μmol/L, the therapeutic goal, at 12,24,52 weeks after infusion. All participants will be followed for up to 5 years. **Results:** Four participants were enrolled in the study. As of the cut-off date (January 13, 2024), 1 participant dosed at 6.0×10^{12} vg/kg was followed up for 36 weeks, 1 at 1.0×10^{13} vg/kg for 26 weeks and 2 at 2.0×10^{13} vg/kg with 1 for 16 weeks and the other for 10 weeks. The most common treatment-related adverse events (AEs) reported in all subjects were transient and asymptomatic elevations in liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which were well-managed by a tapering course of corticosteroid treatment. No severe liver dysfunction or long-term AEs on liver function was observed. All other AEs were identified as grade 1 or 2. All 4 subjects responded to NGGT002 treatment as demonstrated with a reduction in blood Phe level. 2 subjects dosed at 2.0×10^{13} vg/kg showed more rapid and significant decreases in blood Phe, reaching the normal level of ≤120 μmol/L. These subjects maintained a stable Phe level below the therapeutic goal of 360 μmol/L for at least 13 weeks as of data cut-off (Figure 1). **Conclusion:** A single infusion of NGGT002 gene therapy is well tolerated in adults with PKU. Encouraging evidence of significant and stable efficacy has been observed. These results support further clinical development of NGGT002. Patients in this study will continue to be followed. Updated efficacy and safety results will be reported.



1411. Long-Term Safety and Urea Cycle Function Following a Phase 1/2 Trial of DTX301 in Adults with Late-Onset Ornithine Transcarbamylase (OTC) Deficiency

Cary O. Harding[1], Tarekegn Geberhiwot[2], Maria Luz Couce[3], Nathalie Guffon[4], Wen-Hann Tan[5], Leticia Ceberio Hualde[6], Aneal Khan[7], Laura Konczal[8], Janet A. Thomas[9], Margo Sheck Breilyn[10], Lin Zhang[11], J Lawrence Merritt[11], Eric Crombez[11]

[1]Oregon Health & Science University, Portland, OR,[2]University of Birmingham, Birmingham, United Kingdom,[3]University of Santiago de Compostela, Santiago de Compostela, Spain,[4]Reference Centre in Inherited Metabolic Diseases, HCL, Lyon, France,[5]Boston Children's Hospital, Boston, MA,[6]Biocruces Bizkaia Health Research Institute, Cruces University Hospital, Barakaldo, Spain,[7]MAGic Clinic Ltd, Calgary, AB, Canada,[8]University Hospitals of Cleveland Medical Center, Cleveland, OH,[9]University of CO School of Medicine and the Children's Hospital Colorado, Aurora, CO,[10]Icahn School of Medicine at Mount Sinai, New York, NY,[11]Ultragenyx Pharmaceutical Inc, Novato, CA

Background: OTC deficiency is an X-linked urea cycle disorder that results in dysregulation of the urea cycle and a life-long risk of episodic hyperammonemia that can lead to coma, irreversible brain damage, and death. DTX301 is an AAV8 vector containing the OTC transgene that is being investigated for treatment of OTC deficiency. We report long-term safety follow up (LTFU) of 11 adults with late-onset OTC deficiency who received DTX301 in the Phase 1/2 trial (NCT02991144) with a focus on hepatotoxicity and urea cycle function. **Methods:** The initial Phase 1/2 study lasted 52 weeks; all participants are being followed for up to an additional 8 years. Patients received a single IV infusion of DTX301 on Day 1 ranging from $3.4x10^{12}$ to $1.7x10^{13}$ GC/kg. Patients in the last cohort received a prophylactic oral steroid taper. All patients received reactive oral steroids as needed. Data cutoff was 03 February 2023 for efficacy and 08 December 2023 for safety measures. **Results:** Patients have been followed for 2.5-6 years. No treatment-related serious AEs, dose-limiting toxicities, or infusion-related events have been reported. During the primary study, six patients experienced vector-induced hepatic effects, lasting from 20-168 days with peak ALT levels from ~1.5-6.0×ULN. All resolved following corticosteroid administration. During LTFU, three patients experienced mild ALT elevations and one patient experienced hyperammonemic crises (all considered unrelated to DTX301). Corticosteroids were generally well tolerated. Five patients had non-serious corticosteroid-related AEs. Most corticosteroid-related AEs

were mild (grade 1) AEs, though one patient had grade 3 AEs. Neither prophylactic nor reactive corticosteroids were associated with increased ammonia, hyperammonemia, or hyperammonemic crisis. The rate of ureagenesis improved over time, beginning at Week 6 and was sustained through long-term follow up. The overall mean (SD) rate of ureagenesis increased from 137 (88) μmol*h/kg at Baseline to 224 (178) at Week 52 (67% increase). Increases were sustained at least through Week 182 (the last time point with more than 1 patient), with a mean (SD) of 226 (92) μmol*h/kg (n=4, 79% increase). **Conclusions:** DTX301 continues to show a manageable safety profile. Expected vector-induced effects were treated by corticosteroids and resolved. Patients tolerated corticosteroid regimens without metabolic decompensation. Improvements in ureagenesis supports restoration of urea cycle activity and is encouraging evidence of durable efficacy. Patients in this study continue to be followed. The randomized, placebo-controlled Phase 3 trial (NCT05345171) is currently enrolling.

1412. Safety and Efficacy of Delandistrogene Moxeparvovec versus Placebo in Duchenne Muscular Dystrophy: Phase 3 EMBARK Primary Results

Jerry R. Mendell[1,2], Francesco Muntoni[3], Craig McDonald[4], Eugenio M. Mercuri[5], Emma Ciafaloni[6], Hirofumi Komaki[7], Carmen Leon-Astudillo[8], Andrés Nascimento[9], Crystal Proud[10], Ulrike Schara-Schmidt[11], Aravindhan Veerapandiyan[12], Craig M. Zaidman[13], Maitea Guridi[14], Alexander P. Murphy[15], Carol Reid[15], Christoph Wandel[14], Damon R. Asher[16], Eddie Darton[16], Stefanie Mason[16], Rachael A. Potter[16], Teji Singh[16], Wenfei Zhang[16], Paulo Fontoura[14], Jake S. Elkins[16], Louise R. Rodino-Klapac[16]

[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[2]The Ohio State University, Columbus, OH,[3]The Dubowitz Neuromuscular Centre, LONDON, United Kingdom,[4]UC Davis Health, Sacramento, CA,[5]Pediatric Neurology Institute, Catholic University and Nemo Pediatrico, Rome, Italy,[6]University of Rochester Medical Center, Rochester, NY,[7]Translational Medical Center, National Center of Neurology and Psychiatry,, Tokyo, Japan,[8]Department of Pediatrics, University of Florida,, Gainesville, FL,[9]Neuromuscular Unit, Neuropaediatrics Department, Hospital Sant Joan de Déu, Barcelona, Spain,[10]Children's Hospital of the King's Daughters, Norfolk, VA,[11]Department of Pediatric Neurology, Center for Neuromuscular Disorders in Children and Adolescents, University Clinic Essen, University of Duisburg-Essen, Essen, Germany,[12]Arkansas Children's Hospital, University of Arkansas for Medical Sciences, Little Rock, AR,[13]Department of Neurology, WUSTL, St. Louise, MO,[14]F. Hoffmann-La Roche Ltd, Basel, Switzerland,[15]Roche Products Ltd, Welwyn Garden City, United Kingdom,[16]Sarepta Therapeutics, Inc., Cambridge, MA

Introduction: Delandistrogene moxeparvovec, a rAAVrh74-based gene transfer therapy designed to address absent functional dystrophin by delivering a transgene encoding engineered micro-dystrophin, is approved in the USA, UAE, and Qatar for treatment of ambulatory pediatric patients aged 4 through 5 years with Duchenne muscular dystrophy (DMD) with a confirmed *DMD* gene mutation (as of Jan 2024).We report findings from Part 1 (Week 52) of the 2-part EMBARK trial(NCT05096221). **Methods:** Key inclusion criteria: Ambulatory males aged ≥4–<8 years with a confirmed *DMD* mutation within exons 18-79 (inclusive); North Star Ambulatory Assessment (NSAA) score >16 and <29 at screening; rAAVrh74 antibody titers<1:400; stable daily oral corticosteroid dose for ≥12 weeks pre-screening. Eligible patients received delandistrogene moxeparvovec (1.33×10^{14} vg/kg) or placebo. Primary endpoint was change from baseline to Week 52 in NSAA total score. **Results:** At Week 52 (N=125), the primary endpoint was not statistically significant—least-squares mean (SE) between-group difference for delandistrogene moxeparvovec (n=63) vs. placebo (n=61) was 0.65 (0.55) points (95% CI, −0.45,1.74; P=0.24). Between-group differences for time to rise (TTR), 10-meter walk/run(10MWR), stride velocity 95th centile (SV95C), and time to ascend 4 steps favored the treatment group with nominal significance (P<0.05). The most common treatment-related treatment-emergent adverse events (AEs) were vomiting (54.0%),nausea (31.7%), and decreased appetite (27.0%), with no AEs leading to study discontinuation or death. A prespecified global statistical test on a composite of functional endpoints (NSAA, TTR, 10MWR, SV95C, 100MWR, ascend 4 steps)indicated treatment benefit (P=0.004). **Conclusions:** Although EMBARK did not meet the primary endpoint, the totality of functional assessments indicated beneficial modification of disease trajectory. The safety profile was manageable and consistent with prior experience.

Thursday Posters: Cell Therapy and Cell-Based Gene Therapy Trials

1413. Consistency of Outcomes with Injectable Human Corneal Endothelial Cell Therapy Across Multiple Clinical Trials

Arnaud Lacoste

Aurion Biotech, Seattle, WA

Purpose: To compare the safety and efficacy outcomes of injectable human corneal endothelial cells from three clinical trials across different geographies in subjects with corneal edema secondary to endothelial dysfunction. **Methods**: Clinical trials conducted in Japan and El Salvador evaluated a single intracameral injection of human corneal endothelial cells in combination with ROCK inhibitor in subjects with corneal edema. Comparative retrospective analysis of outcomes was conducted between 65 subjects treated in the first-in-human investigator-initiated trial, 16 subjects treated in the exploratory IOTA trial, and 22 subjects treated in the Escalón trial. These clinical trials included multiple surgeons and subjects were followed for 12 months. **Results**: Similar safety and efficacy outcomes were observed in the Japan, IOTA and Escalón clinical trials. At 12 months, mean improvement in BCVA was 0.79 LogMAR, 1.00 LogMAR, and 0.66 LogMAR; mean CCT improved by 25%, 24%, and 19%, and percentage 3-line improvement in BCVA was 89%, 100% and 89% among each trial respectively. There were no treatment related systemic or ocular SAEs. **Conclusion**: A single intracameral injection of human corneal endothelial cells in combination with ROCK inhibitor was generally well tolerated and demonstrated comparable and repeatable efficacy outcomes out to 12 months as measured by BCVA and CCT in subjects

with corneal endothelial dysfunction across different geographies involving multiple surgeons. **Study Design**: *First-in-Human (Japan)* - Open Label; Investigator Initiated Trial (IIT)*IOTA Exploratory (El Salvador)* - Open Label; Sponsor Initiated; Proof of Concept*Escalón (El Salvador)* - Phase 1; Randomized; Masked. **Précis:** Retrospective analysis of three clinical trials was conducted to assess safety and efficacy of human corneal endothelial cells in subjects with corneal edema across different geographies. Safety outcomes measured by TEAEs were consistent across all clinical trials, with no treatment related systemic or ocular SAEs. Efficacy outcomes included both functional assessment (BCVA) and anatomical assessment (CCT), demonstrating consistent improvements in visual acuity and corneal edema. **Background Statement:** Corneal endothelial dysfunction is a progressive disorder causing visual impairment. To date, 130 subjects have received corneal endothelial cell therapy. A comparative analysis of these subjects was conducted across multiple clinical trials. About Arnaud Lacoste, MBA, PhD. As chief scientific officer of Aurion Biotech, Arnaud Lacoste leads the company's research and development of advanced cell therapies for ocular diseases. Previously, Arnaud spent 12 years at Novartis where he directed cell and gene therapy programs and built iPSC, CAR-T and ophthalmology cell therapy platforms. At Novartis, Arnaud and his team were responsible for advancing clinical candidates from early discovery to clinical stages. Prior to Novartis, Arnaud was the director of Rockefeller University's stem cell facility, and co-founder and chief scientific officer of Inseron, which developed genetic biomarkers for drug discovery and pathway profiling. Arnaud Lacoste holds an MBA from Boston University, a PhD in Life Sciences from the Université Pierre et Marie Curie (Paris VI), and an undergraduate degree from the University of Bordeaux.

1414. Deep Serological Profiling of Autoimmune Patients Treated with Anti-CD19 CAR-T Cells

Daniel Nunez[1], Darshil Patel[1], Jenell Volkov[1], Steven Wong[1], Dan Thompson[1], Zachary Vorndran[1], Georg Schett[2], Samik Basu[1]

[1]Cabaletta Bio, Philadelphia, PA,[2]Friedrich Alexander University Erlangen-Nürnberg (FAU) and University Hospital Erlangen, Erlangen, Germany

Anti-CD19 chimeric antigen receptor (CAR) T cell therapy is a ground-breaking emerging treatment modality for severe refractory systemic lupus erythematosus (SLE) and has shown early promise in other diseases, including myositis and systemic sclerosis. Initial clinical data demonstrate that adoptive transfer of anti-CD19 CAR-T cells induce a durable long-term remission in SLE patients. However, the mechanisms underlying remission are unclear. Our aim was to elucidate serological factors that are associated with responses in SLE, myositis, and systemic sclerosis patients following treatment with anti-CD19 CAR-T cells. Sera were collected from 8 SLE, 2 myositis, and 3 systemic sclerosis patients prior to and following anti-CD19 4-1BBz CAR-T cell therapy (1x106 CAR-T cells/kg following preconditioning with cyclophosphamide 1000 mg on day -3 and fludarabine 25 mg/m2/day on days -5 to -3 as described in Mackensen et al., Nat Med 2022). All patients had severe refractory disease prior to adoptive T cell transfer and were in remission off-therapy with up to 29 months of follow-up post-treatment. Sera were evaluated for 25 cytokines and chemokines by electro-chemiluminescence immunoassay (MSD). Sera were also evaluated for 17 SLE-associated antibodies, 28 myositis-associated antibodies, and 14 infectious disease-associated antibodies using a custom developed Luminex-based immunoassay. Serum levels of the inflammatory cytokines IL-10, IL-6 and TNFα were decreased in most patients post-anti-CD19 CAR-T cell infusion relative to pre-infusion. Levels of IL-7 and BAFF increased between 2 and 7 months post treatment. SLE associated antibodies decreased substantially in most patients following anti-CD19 CAR-T cell infusion. In one patient, SLE associated antibodies either remained stable or increased mildly despite resolution of clinical disease. Both myositis patients observed a decrease in Jo1-associated autoantibodies. Infectious disease associated antibodies typically remained stable or changed minimally following anti-CD19 CAR-T cell infusion. Together, these data suggest that anti-CD19 CAR-T cell therapy leads to the reduction of systemic inflammation and a drop in disease-associated antibodies without impacting pre-existing humoral immunity in a diverse set of autoimmune diseases. These studies support the continued exploration of anti-CD19 CAR-T cell therapy in autoimmune disease.

1415. FT819-102: Clinical Translation of Off-the-Shelf TCR-Less CD8ab+ Anti-CD19 CAR-T Cells for the Treatment of B Cell-Mediated Autoimmune Disorders

Lilly Wong[1], Amanda Sims[1], Alec Witty[1], Nick Brookhouser[1], Allison Aguilar[1], Christopher D'Angelo[2], Amitkumar Mehta[3], Umar Farooq[4], Anil Bagri[1], Rebecca Elstrom[1], Bahram Valamehr[1]

[1]Fate Therapeutics, Inc., San Diego, CA,[2]Division of Oncology and Hematology, University of Nebraska Medical Center, Omaha, NE,[3]Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL,[4]Division of Hematology, Oncology, Blood & Marrow Transplantation, University of Iowa Hospitals and Clinics, Iowa City, IA

Autoreactive B cells are key drivers of various autoimmune diseases such as systemic lupus erythematosus (SLE), lupus nephritis, rheumatoid arthritis (RA), and scleroderma. Management of autoimmune disease often includes prolong use of immune suppressants and B cell directed agents. Rituximab, an anti-CD20 antibody, has demonstrated the ability to deplete peripheral blood B cells. Despite this fact, the clinical efficacy of rituximab is limited and varies broadly among different autoimmune diseases due to incomplete clearance of autoreactive B cells in secondary lymphoid tissues. A single dose of autologous CD19-directed chimeric antigen receptor (CAR) T-cell therapy has shown results in sustained depletion of peripheral B cells and durable remission in refractory SLE patients (Mackensen, 2023). However, for CAR T-cell therapy to be a promising modality for the treatment of autoimmune diseases, several obstacles hampering its range of application including the challenge of controlling the T-cell product consistency, the inherent variability of manipulating a heterogenous cellular population and the inability to create the quantity of CAR T cells have to be overcome to support a wide patient base. FT819 is an off-the-shelf, anti-CD19 CAR T-cell product candidate derived from a renewable master iPSC line that may be ideal for broad patient access in the management of autoimmune diseases. The unique ability to mass produce uniform immune effector cells offers distinct advantages over patient- and donor-derived therapeutic approaches, enabling on-demand administration. FT819,

administered following lymphodepleting chemotherapy, appears safe and well tolerated with evidence of anti-tumor activity, including complete responses in aggressive lymphomas with no ICANS or GVHD, and low incidence of CRS (Mehta, 2022; NCT04629729). Here, we present preclinical and translational data that supports the development of FT819 in B cell-mediated autoimmune disorders. Preclinically, FT819 co-cultured with PBMCs from five heathy donors demonstrated potent and specific CD19+ B cell killing in an in vitro cytotoxicity assay. Similarly, PBMCs derived from SLE and RA patients exhibited durable elimination of CD19+ B cells in the same assay. Biodistribution studies in immunodeficient mice illustrated the ability of FT819 to be well distributed throughout the mouse, including in tertiary tissues that are implicated in autoimmune diseases, such as bone marrow and spleen. As further evidence of the translatability of FT819 to autoimmune disorders, in the Phase 1 lymphoma study (NCT04629729), blood and tumor samples were obtained from patients and evaluated for FT819 mediated B cell effects by flow cytometry and IHC. After FT819 infusion,peripheral blood CD19+ B cells were undetectable in patients with sustainedsuppression at the end of the treatment cycle, Day 29. Importantly, B cell depletion was also observed in lymph node biopsies illustrating the ability of FT819 to reach secondary tissues. Recovery of B cells in the periphery occurred 1-3 months after the treatment cycle. Based on itsestablished clinical and translational profile in B-cell lymphoma, coupled withpreclinical data in CD19-mediated autoimmunity, FT819 may be an idealoff-the-shelf cell therapy with the potential to reset the immune system ofpatients with autoimmune diseases.

1416. Preliminary Safety Data of a Phase 1/2 Clinical Trial to Support the Use of High Dose Intrathecal AAV9/AP4M1 for the Treatment of Patients with SPG50 Disease

Souad Messahel[1], Xin Chen[2], Terry Pirovolakis[1], Sydney Cooper[2], Andrea Boitnott[2], Kalayarasan Srinivasan[2], Alagar Muthukumar[2], Keith Gottlieb[1], Darius Ebrahimi Fakhari[3], Steven J. Gray[2], James Dowling[4], Susan Iannaccone[2]

[1]Elpida Therapeutics SPC, Los Angeles, CA,[2]UT Southwestern Medical Center, Dallas, TX,[3]Boston Children's Hospital, Boston, MA,[4]Hospital for Sick Children, Toronto, ON, Canada

Hereditary Spastic Paraplegia Type 50 (SPG50) is a rare, childhood-onset autosomal-recessive disease caused by bi-allelic loss-of-function variants in the *AP4M1* gene, which impacts adaptor protein complex 4 function. This results in a progressive neurological syndrome characterized by progressive spastic paraplegia, global developmental delay, secondary microcephaly and seizures, with onset typically in early childhood. The majority of children become non-ambulatory and full-time wheelchair users. We present preliminary safety data of four subjects, up to 24 months post treatment in a phase 1/2 clinical trial using targeted gene replacement of the AP4M1 gene across the CNS. No deleterious immune responses have been noted, and the treatment has been well tolerated across all 4 subjects. There has been no evidence of DRG toxicity and nerve conduction has been shown to be stable following gene transfer. Our recombinant vector is designed to achieve life-long expression of AP4M1 and broad CNS transgene distribution. Our approach utilizes early intervention and higher intrathecal AAV doses than are used in most other intrathecal AAV clinical trials. Doses given to 3 subjects aged 3-5 years were $1x10^{15}$ vg (total dose per patient), and $4x10^{14}$ vg to a 5-month-old presymptomatic infant. APM41 treatment is challenging as it does not benefit from cross correction or amenable to enzyme replacement therapy. We speculate that the self-complementary design of the vector contributes to its potential efficacy, and the relatively modest strength UsP promoter contributes to its safety. Immune management was designed to allow inclusion of both CRIM(-) and CRIM(+) patients, also thought to be contributing to the overall safety of patients. Interim data from the trial, including efficacy outcomes, will be presented.

1417. Hurdles in Cell and Gene Therapy Trials: Interpreting Clinical Holds Between January 2020 to January 2024

Harishwar Venkatesh, Eunjoo Pacifici

Department of Regulatory and Quality Sciences, University of Southern California, Los Angeles, CA

Clinical development of cell and gene therapy trials contain added layers of intricacy compared to small molecule drugs, making them increasingly more demanding and costly. The characteristics of these therapies also render them particularly vulnerable to clinical holds, which typically last an average of 6.2 months, causing substantial hindrances to the advancement of clinical trials. Examining the nature of clinical holds could provide helpful insights into common pitfalls that could help researchers improve the conduct of clinical studies associated with cell and gene therapies. The objective of this study was to investigate cell and gene therapy trials in order to identify challenges and risk mitigation strategies and, ultimately, streamline the clinical trial process. A comprehensive analysis of clinical holds, FDA warning letters, and relevant literature from January 2020 to January 2024 was conducted to identify factors contributing to challenges in conducting cell and gene therapy trials. In four years, press releases and public disclosure statements from over 75 biotechnology and pharmaceutical companies were observed, revealing a total of 94 publicly disclosed clinical holds. An emphasis was placed on identifying preventable causes of clinical holds. Among all the clinical holds, 43% belonged to therapies treating oncological conditions, while 18% belonged to neurological conditions, 14% to genetic conditions, 10% to blood-related conditions, and 9% to infectious conditions. The remaining therapies treated a variety of unique conditions. Additionally, 69 of the 94 therapies were classified as biologics, the remainder being various small-molecule drugs. Across the period studied, the number of holds each year varied, ranging from 17 to 28. Forty of the clinical holds resulted from varying grade adverse events, the remaining 54 holds had causes ranging from insufficient preclinical data to manufacturing concerns. Results indicate that clinical holds have remained consistent over the last four years. The actions taken by the Food and Drug Administration (FDA) in response to these novel therapies may reflect a cautious approach aimed at prioritizing the safety of trial subjects. While adverse events are inevitable, a thorough understanding of expectations and compliance with regulations while submitting an Investigative New Drug Application (IND) may reduce the likelihood of a therapy being placed on a clinical hold.

1418. Targeting Infections Post Alternative-Donor Hematopoietic Cell Transplantation with Pathogen-Specific T Cells

Anastasia Papadopoulou[1], Ioannis Kyriakou[1], Zoe Boussiou[1], George Karavalakis[1], Chrysa Pantazi[1], Ioanna Vallianou[1], Maria Giannaki[1], Fani Stavridou[1], Maria Liga[2], Ioulia Mavrikou[1], Kiriakos Koukoulias[1], Apostolia Papalexandri[1], Fotini Kika[1], Eleftheria Sagiadinou[2], Ioannis Batsis[1], Alexandros Spyridonidis[2], Ioanna Sakellari[1], Evangelia Yannaki[1]

[1]George Papanikolaou Hospital, Thessaloniki, Greece,[2]University Hospital of Patras, Patra, Greece

The rising trend in alternative-donor hematopoietic cell transplantation (HCT) has brought out an increasing incidence of opportunistic infections. Adoptive immunotherapy with pathogen-specific T cells (pSTs) holds promise as it targets precisely the infected cells circumventing the systemic toxicity of traditional drug therapies, is less prone to resistance and provides a long-lasting immune memory. We here present combined results from two phase I/II studies evaluating the safety and efficacy of trivalent-pSTs targeting cytomegalovirus (CMV), Epstein Barr virus (EBV), and BK virus (BKV) and pentavalent-pSTs targeting additionally, adenovirus (AdV) and Aspergillus fumigatus (AF). GMP-grade pSTs were developed from donor blood mononuclear cells, stimulated with pepmixes of EBV (LMP2/EBNA1/BZLF1), AdV (Hexon/Penton), CMV (pp65/IE1), BKV (Large-T/VP1) and AF (Gel1/SHMT/Crf1) antigens. After 10 days in culture, the cells reached a 7fold increase resulting in a mean cell count of 222±30x10^6 cells. Expanded pSTs were predominantly CD3+ (93.2%) with balanced distribution of CD4+ (50.9±3%) and CD8+ (41.7±3%) subsets and displayed central (40.4±4%) and effector (43.5±4%) memory markers. Patients, excluding those with graft-versus-host disease (GvHD) requiring >0.5mg/kg methylprednisolone, received a single infusion of pSTs (2x10^7 cells/m^2) to control post-HCT infections from the targeted pathogens. In total, 29 patients received pSTs for single (CMV=8/ EBV=6/ BKV=6), double (BKV+EBV=3/ BKV+CMV=2/ EBV+CMV=1) or triple infection (CMV+EBV+BKV=3). Infusions were well-tolerated, with no related toxicity or GvHD occurrence/ exacerbation. 29 patients post haplo- (n=27) or volunteer unrelated-donor (n=2) HCT, received pSTs for CMV, EBV and BKV infections. Out of a total 41 infections at infusion, including 21 viral diseases (BKV cystitis/nephropathy, CMV gastritis/colitis/pneumonia, EBV colitis/encephalitis/PTLD), 35 (85.4%) showed complete response, 5 (12.2%) partial and 1 (2.4%) no response. In the latter case, a patient with triple infection, received a dual-specificity (EBV & BKV) product from a CMV-negative donor and controlled all infections except CMV. Importantly, 16/41 infections including one EBV-PTLD case and 3 BKV cases previously unresponsive to standard treatment, were successfully resolved with minimal (9/41) or no (7/41) antiviral medication. Clinical and viral load responses coincided with circulating pST expansion. Over 6 months post infusion, 17/29 patients experienced 22 new viral reactivations from which, 11 were controlled by the expanded pSTs, requiring minimal or no use of antiviral drugs. The other 11 reactivations occurred mostly in patients with relapse or increased immunosuppression at reactivation (7/9) and required pharmaceutical intervention or/and a second pST infusion (5/9). Nine of these 11 reactivations, including one EBV-PTLD case, were completely resolved, one responded partially, and a single CMV reactivation persisted due to ongoing immunosuppression. In summary, pSTs can serve as a powerful, sustainable, and safe alternative therapy for opportunistic infections post-allo-HCT, significantly reducing or even eliminating the need for drug therapy by effectively managing both current and future reactivations. Clinical Trial Registry: EudraCT2020-004725-23, NCT05471661/ EudraCT2014-004817-98.

Friday Posters: AAV Vectors - Virology and Vectorology

1419. Strategies for Large-Scale Plasmid DNA Complexation and Delivery to Support Commercial-Scale AAV Production

Kenneth W. Thompson[1], Collin Rasmussen[1], Natalie Miller[1], Siva Sai Krishna Dasa[1], Emily Jackson-Holmes[2], Chao Yan Liu[1], Jonathan Zmuda[1]

[1]Thermo Fisher Scientific, Frederick, MD,[2]Thermo Fisher Scientific, Carlsbad, CA

While AAV gene therapy has entered the commercial landscape, achieving scalable and robust manufacturing processes for consistent high-quality and high-yield AAV production remains a challenge. To address these shortcomings, we developed the Gibco™ CTS™ AAV-MAX Helper-Free AAV Production System, an all-in-one, chemically defined, suspension based AAV production system that allows for scalable, high-titer production of AAV viral vectors in a non-293T cell lineage. Recently, we developed processes for AAV production in single-use stirred tank reactors up to the 1000L production scale. During this work, we identified two critical aspects of the workflow required to ensure consistent, high titer, and high quality AAV production: 1) the health of the cells at the time of transfection, and 2) efficient and consistent complexation and delivery of plasmid DNA at large volumes. Because GMP-grade plasmid DNA can account for >50% of the entire cost of a clinical or commercial-grade production run, an in depth understanding of the critical parameters of this process is of the utmost importance to minimize production risks. In the present study, we utilized dye-based studies as well as actual large-scale (50-100L) complexation reactions to identify the critical parameters enabling efficient and consistent complexing of plasmid DNA and transfection reagent in bioprocessing bags and stirred tank mixers as well as strategies for delivery into large scale reactor vessels. For bioprocessing bags, the parameters evaluated included headspace, rocking angle and speed, orientation of the bag on the rocker, mixing time, complexation volume and pump flow rates. For stirred tank mixers, parameters tested were mixing time and top vs. bottom addition of components to the reactor as well as power input. The data presented indicate the key aspects of large-scale plasmid DNA complexation and delivery critical for clinical and commercial scale AAV processes. We rank a hierarchy of factors that should be evaluated and controlled to provide maximum consistency in AAV production at large scales using the Gibco™ CTS™ AAV-MAX Production System.

1420. Optimization of Transient rAAV Production via HEK293 Cell Line Generation, Clone Selection and Production Process

Ulrike Jung[1], Yi-hsuan Huang[1], Sven Mathias[1], Pia Brinkert[2], Kathrin Teschner[3], Sandra Klausing[3], Suneetha Nunna[4], Magda Tomala[4], Stefano Boi[1], Kristin Thiele[1]

[1]Sartorius Stedim Cellca GmbH, Ulm, Germany,[2]Sartorius Xell GmbH, Schloß Holte-Stukenbrock, Germany,[3]Sartorius Xell GmbH, Bielefeld, Germany,[4]Sartorius Stedim Biotech GmbH, Göttingen, Germany

Recombinant adeno-associated viruses (rAAV) are currently the predominant viral vector for *in vivo* gene therapy in clinical studies. The initial phase of therapy development focuses on increasing the yield of rAAV. This process commonly involves transient transfection in HEK293 cells. An essential component of this step is therefore the development of a HEK cell line that generates highly potent doses of viral vectors and the optimization of the production process. Commonly a proprietary cell line is used, and a pre-existing set of plasmids fine-tuned for the plasmid ratio and amount per cell, using a specific growth medium. In some cases, feeds are also tested. We've developed a single cell cloning approach that allows automated high throughput screening of 400 clones for rAAV productivity (vg/L and TU/L). While yield is highly dependent on the medium, feeds and plasmid ratios, we show that even the impact of the plasmid system varies greatly depending on cell clone or pool or even culture conditions. Accordingly, optimization of the setup and selection of the best combination of parameters is a highly complex task and requires a high throughput approach for best results.

1421. High-Fidelity Maintenance of Heterologous DNA Sequences Stably Integrated in Recombinant Baculovirus Under Repeated Amplification Conditions

Chingyi Ho, Platon Selemenakis, Moeed Ahmad, Favour Malumi, Min Chen, Haifeng Chen

Virovek, Inc., Houston, TX

The baculovirus expression system has been widely used in biopharmaceutical industries for the production of various proteins, including vaccines, therapeutic proteins, enzymes, viral-like particles (VLPs) and adenovirus associated virus (AAV) vectors. However, baculovirus harboring heterologous DNA sequences is genetically unstable and productivity is lost after a series of amplifications or passages in insect cells due to the accumulation of defective interfering particles (DIPs) and loss of the heterologous DNA sequences. A new method of maintaining foreign DNA sequences stably integrated in the recombinant baculovirus (rBV) despite of repeated amplifications or passaging is urgently needed. We discovered that the genetic stability of rBV was dramatically increased by linking a drug resistant gene expression cassette to the heterologous DNA sequence(s) and adding drug selection despite repeated amplifications. After the rBVs were generated without plaque purification, they were used to infect fresh Sf9 cells overnight and then selection drug was added. After 3 to 4 days of infection in the presence of selection drug, specific rBV to total rBV ratio was determined with QPCR method using primer sets corresponding to the heterologous DNA sequence and to the wild type baculovirus DNA sequence (gp64). The results indicate that the heterologous DNA sequences were quickly deleted after several passages without drug selection, whereas the rBV retained the heterologous DNA sequences stably integrated even after passaging 10 times in the Sf9 cells under drug selection conditions. The results clearly demonstrate that under selection pressure, the recombinant AAV genome and the Rep and Cap expression cassettes were maintained stably in the rBV despite repeated amplifications even without plaque purification. We used passage 1 and passage 10 rBVs respectively for AAV productions and compared their AAV yields. The QPCR titer results indicate that at least 1.25E+14 vg purified AAV vectors were obtained from one liter of passage 10 rBV infected Sf9 cells. SDS-PAGE and agarose electrophoresis analyses indicate that AAV vectors produced by the passage 10 rBVs had the same VP1/VP2/VP3 ratio and DNA genome size as those produced by the passage 1 rBVs. Further experiments to analyze the AAV infectivity, sequence the AAV genome and the Rep-Cap regions are undergoing. This high-fidelity method of maintaining heterologous DNA sequences stably integrated in the rBVs should pave the way to commercial scale biologics manufacturing without compromising the productivity and integrity of the products.

1422. A Highly Efficient PCR-Based Approach to Isolate Novel Naturally-Occurring AAVs

Emanuela Pone[1,2], Antonella Ferrara[2], Elena Marrocco[2], Arjun Padmanabhan[2], Vittoria Iovino[1], Zoltán Zádori[3], Ivana Trapani[2,4], Alberto Auricchio[2]

[1]Department of Translational Medicine, Federico II University, Napoli, Italy,[2]Telethon Institute of Genetics and Medicine (TIGEM), Pozzuoli (NA), Italy,[3]Institute for Veterinary Medical Research,, Centre for Agricultural Research, Hungarian Academy of Sciences, Budapest, Hungary,[4]Department of Advanced Biomedicine,, Federico II University, Napoli, Italy

Adeno-associated virus (AAV) vectors, renowned for their exceptional efficacy and safety, stand as the leading platform for in vivo therapy. To uncover new AAV variants with distinctive biological features, PCR-based amplification of AAV capsid sequences from biological samples has been mostly focused on short variable regions of the Cap gene (here called Dg PCR for diagnostic PCR).Here we describe an efficient PCR-based method that amplifies in a single step the entire Cap coding sequence (here called Full PCR) thus allowing to identify novel variants with differences across the whole capsid which we used to screen a collection of porcine liver samples. Using the traditional Dg PCR which targets two/three hypervariable regions (VRs) in the CAP gene, 36% (n=98/273) of liver samples were found to contain AAV sequences. Amino acid sequence alignment of these fragments revealed 7 novel AAV variants among the positive AAV samples. When the liver samples were analyzed with Full-PCR, 25% (n=69/273) of all samples were positive for AAV presence, however 17 of them were novel variants (including the 7 identified by DgPCR) based on aminoacid differences across the whole CAP and not limited to the hypervariable regions. All 17 variant CAP sequences were vectorized resulting in high titer AAV vector preps. We conclude that Full PCR is more effective than Dg PCR for the identification of novel AAV variants from biological samples allowing to isolate in a single step the whole CAP sequence for further rapid vectorization.

1424. Orientation of Plasmid Origin of Replication and Its Impact on Inverted Terminal Repeat Stability

Syam Pillai[1], Judy Jan[1], Alexei Saveliev[2], Ying Chen[2], John Bowen[1], Brian Tomkowicz[3]

[1]Plasmid Operations, SK pharmteco - US, King of Prussia, PA,[2]Next Generation Sequencing, SK pharmteco - US, King of Prussia, PA,[3]Viral Vector Research & Development, SK pharmteco - US, King of Prussia, PA

The observations that Adeno-associated virus 2 (AAV2) could be rescued and replicated from pro-viral plasmids and that the *cis* oriented AAV ITRs were the only sequences essential for rescue, replication, and packaging of viral genomes underscores the importance of these DNA sequences for successful vector manufacturing. AAV2 ITRs are unique nucleotide sequences that support cross-packaging of *cis*-plasmids across all AAV natural or engineered serotypes. These GC-rich segments of DNA are ~145 nucleotides long, with the first 125 palindromic nucleotides able to form a T-shaped hairpin structure, which flanks the entire AAV genome and provides critical recognition sequences that are required for directing the AAV Rep and host cell proteins to replicate and encapsidate the viral DNA into pre-assembled AAV capsids. We sought to address the impact of the plasmid Ori orientation and its proximity to ITR on the stability of both ss- and scAAV ITR-containg plasmids. Towards that end, we employed short-read Illumina MiSeq Next-Generation Sequencing technology to identify the mutation frequency across multiple plasmid propagation conditions. Our results indicate that across the different bacterial strains tested (DH5α & Stbl3), culture media (LB, TB & proprietary media), propagation temperature (30°C vs 37°C), or colony size (small vs large), the predominant variable that directly impacted ITR stability was the orientation of the Ori in plasmid constructs. When the orientation of the Ori was adjacent to scAAV ITRs, significant deletions were detected in the proximal ITR, resulting in complete proximal ITR deletion in 33% of clones in the condition using Stbl3 at 37°C. However, all other conditions resulted in a 22 bp B loop deletion in the same scAAV ITR in 6.41 to 91.63% of alleles. For ssAAV ITR plasmids, the B loop deletions and C loop deletions were observed in 7.21 to 50.53% and 6.15 to 18.55% of alleles, respectively, across the treatments. When the Ori was orientated in the direction opposite the ITR, separated by the antibiotic resistance cassette, no significant deletions were detected in either the left or right ITR. Moreover, no deletions were observed in the distal ITR, regardless of Ori orientation. These results provide significant insight into plasmid ITR stability to ensure high quality plasmids and viral vectors for AAV-based gene therapies.

1425. A Comparison Study of Orthoganol Assays, Mass Photometry, SV-AUC, Absorbance, ELISA and dPCR to Estimate Empty Full AAV Particles

Srivanya Tummala, Khan Umaer, Ngan Trinh, Kelly Geosits, Marguerite Campbell, Brian Tomkowicz

Viral Vector Research & Development, SK pharmteco - US, King of Prussia, PA

Mass photometry is a relatively new analytical technology that can measure the mass of individual AAVs in solution. The method uses light-based interference microscopy to determine quality attributes of AAV samples, including empty/full/partial capsid ratios, sample purity, aggregation, and titer. To date, the gold standard for measuring these attributes has been Sedimentation Velocity - Analytical Unltra-Centrifugation (SV-AUC), a technique which uses the buoyant density between particles of various sizes in a heterogenous mixture. While SV-AUC offers unparalleled resolution of various AAV particles (empty/full/partial/overfilled) in a mixture, it is a laborious technique that requires extensive expertise to execute both from an assay set-up and data interpretation perspective. To ensure that robust process parameters are being met, in process sample testing during vector manufacturing needs to be timely, cost effective, and easily achieved, yet still be reliable in providing accurate information. Towards that end, we compared several commonly employed analytical techniques used in the determination of empty/full capsids: ELISA (dPCR VG/ELISA VP), Stunner (dPCR VG/Stunner VP), Absorbance (A_{260}/A_{280} ratio), SV-AUC, and Mass Photometry. Our results indicate that on purified AAV particle, A_{260}/A_{280}, SV-AUC, and mass photometry equally predict similar % Full AAV particles. On the contrary, we found that ELISA-based methods were inadequate and equally were not accurate in % full estimations. Employing Absorbance and mass photometry methods for particle characterization are simple methods that require very little training yet offer outstanding resolution and accuracy. These metrics for assay performance are highly desirable in large scale manufacturing where test sample quantities and volume require the need for significant speed and cost-savings.

1426. Assessing the Impact of Adeno-Associated Virus Production, Residual DNA Packaging, and Potency Between ms-DNA vs Plasmid DNA Formats

Khan Umaer[1], Srivanya Tummala[1], Ying Chen[2], Alexei Saveliev[2], Roderick Slavcev[3], Alvaro Amorrortu[3], Nafiseh Nafissi[3], Brian Tomkowicz[1]

[1]Viral Vector Research & Development, SK pharmteco - US, King of Prussia, PA,[2]Next Generation Sequencing, SK pharmteco - US, King of Prussia, PA,[3]Mediphage Bioceuticals, Inc., Toronto, ON, Canada

Ministring DNA (msDNA™) is a novel, non-viral gene therapy technology that forms a linear, covalently closed (LCC) DNA vector containing only the region of interest. For AAV production, this includes the Gene-of-interest, Rep2Cap, and pHelper sequences. Unlike conventional plasmid-based systems, msDNA™ format is completely devoid of backbone sequences that are undesirable contaminants in cell and gene therapy products. Hence, the msDNA™ format confers a selective advantage for viral vector and mRNA-production platforms. Herein, we comprehensively examined AAV viral vectors produced using conventional pDNA vs LCC ministring DNA formats. Our results demonstrate that AAV vectors produced using msDNA™ showed a considerable reduction in aberrant backbone DNA incorporation by Next-gen sequencing, improved full particle yields, as well as increased *in vitro*- and *in vivo*-based potency. Because msDNA™ has a strong safety profile due to the absence of immunogenic sequences, comes with an in-built safety switch that prevents undesired chromosomal integration, and is easy to manufacture, this platform provides an attractive alternative for improved safety for gene therapy products.

1427. Topoisomerase Poisons Alter AAV Transduction and Integration Targeting

Aradhana Kasimsetty[1], Young Hwang[1], John K. Everett[1], Alexander G. McFarland[1], Aoife M. Roche[1], Denise E. Sabatino[2,3], Frederic D. Bushman[1]

[1]Department of Microbiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[2]The Raymond G. Perelman Center for Cellular and Molecular Therapeutics, The Children's Hospital of Philadelphia, Philadelphia, PA,[3]Department of Pediatrics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA

Introduction: Adeno-associated virus (AAV) vectors are a promising but sometimes inefficient transgene delivery system, motivating the development of small molecule modulators to improve efficiency and reduce vector dose. Some of these modulators are associated with DNA damage response (DDR), which has been implicated in AAV viral replication and in some cases, AAV integration. Integration events can result in genotoxicity but can also promote vector persistence in dividing cells. One category of small molecule modulators is topoisomerase poisons, which are reported to increase production from vector transgenes in short-term transduction experiments. Topoisomerase poisons stabilize topoisomerase complexes post DNA cleavage, freezing single-strand (Topoisomerase I poisons) or double-strand (Topoisomerase II) DNA breaks. Thus, we investigated the effects of multiple topoisomerase poisons on AAV transduction and integration. **Methods:** We evaluated the transduction effect of three topoisomerase poisons, camptothecin, doxorubicin, and etoposide, in several cell models. We demonstrated dose-dependent and cell line-dependent increases in transgene expression for an AAV-GFP vector. Transduction and transgene persistence were monitored by measuring GFP fluorescence. We then isolated DNA for integration site analysis (ISA). We selectively amplified AAV integrants with ITR-primed, sonication-based, ligation-mediated PCR. Amplified integrants were sequenced with Illumina short-read sequencing. Integration sites (IS) were mapped with the AAVengeR software pipeline. Integration frequency and clonality were quantified by sonicated fragment lengths and unique molecular identifiers (UMIs). Simulated random matched controls were used to compare IS distributions to distributions of selected histone markers and transcription factors associated with topoisomerase binding. **Results:** Both dividing and induced non-dividing cell lines demonstrated an increase in GFP expression in response to all three drugs, with primary cell lines having higher tolerability for the drugs and greater transgene expression. While all three drugs showed an increase in expression at early time points, as reported previously, longer-term time points showed that the samples treated with the topoisomerase I poison camptothecin did not show increased transduction, while samples treated with topoisomerase II poisons doxorubicin and etoposide retained higher transgene expression compared to controls. ISA results showed similar levels of IS frequency across the control and camptothecin-treated cells, whereas the doxorubicin and etoposide-treated cells demonstrated increased integration frequencies, up to 4-fold higher than baseline. Preliminary integration distribution mapping supports a change in IS distribution with an increased association with topoisomerase binding sites in type II topoisomerase poisoned cells. **Conclusions:** ISA of cells treated with topoisomerase poisons indicate that these small molecules can influence both AAV transduction and integration. While DDR likely contributes broadly to an increase in early transgene expression, the variation in integration effects between camptothecin and doxorubicin/etoposide implies that the nature of the DNA damage protein targeted plays a role in promoting and targeting integration. This study demonstrates that small molecules may be used to redirect AAV integration targeting. These effects should be considered when evaluating modulators for enhancing AAV transgene expression.

1428. Structural Insights into Porcine Adeno-Associated Viruses

Austin Nelson, Mario Mietzsch, Paul Chipman, Julia Eby, Robert McKenna

University of Florida, Gainesville, FL

Current clinical trials using adeno-associated virus (AAV) vectors exclusively rely on capsids derived from primate origin. However, pre-existing neutralizing antibodies in a large percentage of the human population, from prior exposure to naturally circulating AAVs, can lead to vector inactivation and loss of treatment efficacy for many of the primate AAVs. Advances in DNA sequencing technologies have led to the discovery of AAVs from a wide range of non-primate animals including numerous isolates from pigs (AAVpo.1 - AAVpo.8). The capsid amino acid sequence identity of these viruses can vary by up to 50% to the primate AAV serotypes. In this study, the capsids of AAVpo.1 and -po.6 were selected for structural characterization due to their low viral protein (VP)1 sequence identity compared to AAV2, AAV4 and AAV5, as well as to one another. The porcine AAV vectors were produced using the standard triple transfection system of HEK293 cells utilizing the AAV2 rep gene to package AAV2-ITR vector genomes. The resulting vectors were purified by Iodixanol density gradient ultracentrifugation, revealing the typical incorporation of VP1, VP2, and VP3 into the AAV capsids. Here we present the capsid structures of AAVpo.1 and AAVpo.6 determined by cryo-electron microscopy. As the porcine AAVs have been described to infect human cells, and AAVpo.6 being able to cross the blood brain barrier (BBB), structural comparisons to the existing AAV capsids structures, including those of AAV9 and AAVrh.10, will be conducted. These comparisons will aid in the identification of potential receptor utilized by these capsids. Furthermore, the reactivity to human antibodies will be analyzed to determine the value of porcine AAV capsids as alternatives to the AAV vectors currently used in clinical trials.

1429. The AAVone Single-Plasmid AAV Production System Effectively Generates Large-Size AAV Vectors in Suspension HEK 293 Cells with Bioreactor System

Pauline Schorter[1], Huan Hu[2], Zhen Shi[2], Elisa Berera[1], Julie Regnery[1], Yuling Dai[2], Rongze Yang[2], Yuyan Wang[2], Leming Wang[2], Jiarui Liang[2], Daozhan Yu[2], Quentin Bazot[1], Qizhao Wang[2]

[1]ABL, Lyon, France,[2]AAVnerGene Inc., Rockville, MD

We have recently introduced AAVone, a novel, single-plasmid AAV production system. This streamlined system incorporates the Ad helper genes (E2A, E4orf6, and VA RNA), AAV helper genes (rep and cap) and the AAV vector genome into one compact plasmid, named

pAAVone, which has a 13 kb backbone plus the AAV genome. By simply transfecting host cells with the pAAVone plasmid, efficient AAV vector production is achieved. We previously reported that AAVone shows a 2 to 4-fold increase in AAV yield compared to the traditional triple plasmid system for a payload size of 2.2 kb or 2.8 kb. To test the effectiveness of the AAVone system for packaging larger AAV vectors, we created pAAVone constructs with varying AAV genome sizes from 2.2 kb to 4.7 kb with plasmid sizes from 15.2 kb to 17.7 kb which encodes enhanced green fluorescence protein (EGFP). The AAVone system efficiently packages AAV9 vectors with these varying genome sizes in suspension cells. In suspension HEK 293T cell culture using flasks, the yield for 4.4 kb AAV9 vectors was approximately 2.27e12 vg/ml, a ~38% reduction compared to the 3.72e12 vg/ml yield with 2.2 kb genomes. The yields for 4.6 kb and 4.7 kb AAV9 vectors were around 2e12 vg/ml. Using suspension HEK 293 cells in flasks, we achieved over 1e12 vg/ml crude titer with both FectoVIR-AAV and Transporter 5 as transfection reagents, with Transporter 5 yielding higher crude and full particle ratios in the AAVone system. A decrease in productivity of 28% was noted when comparing a 4.4kb payload to a 2.2kb genome. In suspension HEK 293 cells cultured in Ambr250 bioreactors, the crude AAV9 reaches 1.34e12 vg/ml of genome titer(dPCR) and 5.56e12 vp/ml capsid titer (Elisa) for 2.2 kb genomes. AAV9 particles produced with 2.2kb payload are 38.8% full, either with 1 copy (29.4%) or 2 copies (9.4%) of genomes, as measured by mass photometry. Packaging efficiency test for construct with 4.4kb payload size in HEK293 cells cultured in a bioreactor is ongoing and is expected to reach ~1e12 vg/ml based on observations made in flask cultures. In conclusion, while larger AAV genomes lead to a slight decrease in overall genome titer, the productivity for those larger payload size is significantly higher than triple plasmid system with the same payload size. The AAVone system remains highly effective in producing large-size AAV vectors.

1430. The AAVone Single-Plasmid System Efficiently Generates High-Quality AAV Vectors

Yu Liu[1], Yuyan Wang[1], Rongze Yang[1], Yuling Dai[1], Zhen Shi[1], Leming Wang[1], Cuiping Chen[1], Haniya Zaidi[1], Jiarui Liang[1], Ngoc Tam Tran[2,3], Daozhan Yu[1], Phillip WL Tai[2,3], Qizhao Wang[1]

[1]AAVnerGene Inc., Rockville, MD,[2]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[3]Department of Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA

We previously developed an all-in-one, single-plasmid AAV production system, named AAVone. In this system, the Ad helper genes (*E2A*, *E4orf6*, and *VA RNA*), AAV helper genes (*rep* and *cap*), and the AAV vector genome are consolidated into a single compact plasmid. The pAAVone plasmid design, has a 13-kb 'backbone' (obligate plasmid sequences required for AAV production), which is significantly larger than AAV's packaging capacity. With this system, an AAV vector can be efficiently generated by simply transfecting the corresponding pAAVone plasmid into host cells. We show that AAVone has a two- to four-fold increase in AAV productivity compared to what is achieved by the original triple plasmid system. A comparative analysis revealed that AAV vectors produced by different systems exhibited similar AAV capsids, genome content, and in vitro infectivity. The integrity of the AAV genome payload in AAV vector is a critical quality attribute for clinical applications. By leveraging the capabilities of single molecule, real-time (SMRT) sequencing, we are able to analyze AAV genomes at the single molecule level. The pAAVone-AAV2-*CMV-EGFP* plasmid used in AAVone system and pAAV-*CMV-EGFP* plasmid used in the triple plasmid system have the same 2,730 bp AAV-*CMV-EGFP* genome, and harbor a 130-bp L-ITR(15 bp shorter than the wtITR) and a 119-bp R-ITR[PT1] (an extra 11 bp deletion in the C arm). AAV genomes from the two systems have nearly the same genomic read-length profiles, showing a major 2,771 nt peak, which is the intact AAV genome size harboring two 145 nt intact ITRs(2730 nt+15 nt*2+11 nt). *The* majority of ITRs in the vectors produced by either systems are 140-150 bp in length at both the L-ITR and R-ITR, and displayed similar flip:flop ratios. The presence of ITRs missing 11 bp of the C arm, constituted only about 1.5% of the total reads, implying that the deletion was largely repaired during vector genome replication. Despite their similarities, we observed some intriguing phenomena between the AAV vectors produced by AAVone and triple plasmid systems. The AAVone system's AAV vectors exhibited a slightly higher percentage of the intact AAV genome, a notably higher read coverage at the sequences close to R-ITR, fewer non-ITR truncations, and lower percentage of snap-back genomes. Taken together, our data support the notion that vectors produced by AAVone have nearly identical capsid and genome components compared to vectors produced by triple plasmid transfection; and they perform equally well. These similarities underscore the efficacy and reliability of the AAVone system as a promising alternative for AAV vector production, with potential benefits, such as increased intact genomes, reduced non-ITR truncations, improved vector genome homogeneity, which all contribute to improved vector quality and therapeutic potential for gene therapy applications.

1431. Impurities Analyses of AAV Vectors Produced by the Novel AAVone Single-Plasmid AAV Production System

Yuyan Wang[1], Yu Liu[1], Yuling Dai[1], Zhen Shi[1], Rongze Yang[1], Leming Wang[1], Cuiping Chen[1], Haniya Zaidi[1], Jiarui Liang[1], Ngoc Tam Tran[2,3], Daozhan Yu[1], Phillip WL Tai[2,3], Qizhao Wang[1]

[1]AAVnerGene Inc., Rockville, MD,[2]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[3]Department of Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA

We previously developed an all-in-one, single-plasmid AAV production system, named AAVone. In this system, the Ad helper genes (*E2A*, *E4orf6*, and *VA RNA*), AAV helper genes (*rep* and *cap*), and the AAV vector genome are consolidated into a single compact plasmid. The pAAVone plasmid design has a 13-kb 'backbone' (*i.e.*, obligate plasmid sequences not meant to be packaged into the AAV vector), which is significantly larger than the AAV packaging capacity. In this system, an AAV vector can be efficiently generated by simply transfecting the corresponding pAAVone plasmid into host cells. The platform shows a two- to four-fold increase in AAV productivity compared to what is achieved by the original triple plasmid system. Residual input plasmid DNA is one potential concern with the AAVone system, as it harbors the *rep*, *cap*, *E2A*, *E4orf6*, *VA RNA* and AAV genomes in one plasmid. To address this concern, we employed single molecule, real-time (SMRT) sequencing to thoroughly examine potential impurities originating from the AAVone plasmid system. First, we confirmed that the two

ITRs are the primary *cis* elements responsible for AAV packaging, as the majority of the reads are mapped to sequences between the ITRs. Second, we found that the frequency of detecting backbone sequences located outside of the ITRs are primarily determined by their proximity to the elements. In other words, sequences in close proximity to the ITRs typically exhibit higher read coverages, whereas those situated further away, tend to have lower read coverages. Third, we observed an asymmetrical distribution of impurities for both AAV vectors produced by AAVone and triple-plasmid systems. A higher abundance of reads was found mapping closer to the left (L)-ITR than mapping to the right (R)-ITR. Within the AAVone system, the three most prominent contaminating elements were those associated with *VA RNA* (located near the L-ITR), followed by the *cap* (0.48%, positioned near the L-ITR), and the bacterial backbone (0.34%, situated near the R-ITR). In contrast to the AAVone system, the predominant contaminants in the triple plasmid systems were the bacterial backbone of pGOI. Analysis of genomic contamination within the AAVone system reveals that a substantial portion of these contaminants comprised of sequences containing ITRs. For example, a majority of VA RNA sequences (99.47%) appear to be contiguous with the GOI. Likewise, a considerable proportion of *cap* sequences (93.28%) and bacterial backbone sequences (94.61%) also contained the GOI, suggesting that they are composite sequences consisting of the GOI and the *cap* or bacterial backbone regions. Overall, there were no notable differences in host cell (hc) DNA contaminants among the AAV vectors produced by the AAVone and triple plasmid systems, each showing approximately 3% of the total sequences. In summary, SMRT sequencing of vectors generated by the AAVone platform demonstrated significantly lower levels of bacterial DNA contaminants and antibiotic resistance genes, comparable levels of *rep*, *E2*, and *E4* contaminants, but a substantial increase in *VA RNA* and *cap*, compared with the triple plasmid system. Despite this shift in the nature of contaminants, the overall total genomic contamination in the AAVone system does not exhibit a general increase in undesired genomic contaminants, and may even show a decrease in frequency. This underscores the effective management of total genomic contaminants by the AAVone system, a critical factor in ensuring the production of high-quality and safe AAV vectors.

1432. Optimization of AAVone system to Further Increase AAV Packaging Efficiency and Reduce Impurities

Zhen Shi, Yuling Dai, Yuyan Wang, Yu Liu, Rongze Yang, Cuiping Chen, Leming Wang, Haniya Zaidi, Daozhan Yu, Qizhao Wang

AAVnerGene Inc., Rockville, MD

Our previously developed single-plasmid AAV production system, AAVone, offers streamlined AAV vector generation via transfection of a single compact plasmid into host cells. In this system, the Ad helper genes (E2A, E4orf6, and VA RNA), AAV helper genes (rep and cap), and the AAV vector genome are consolidated into a single compact plasmid, pAAVone, which has a 13-kb 'backbone'. In this system, replacing the ATG start codon with ACG for Rep expression resulted in decreased AAV production, highlighting the importance of efficient translation initiation. Conversely, incorporating a more potent intron donor significantly boosted AAV yields by enhancing splicing efficiency intron between P40 promoter and Cap ORF. Moreover, a novel P5 promoter, P5I, was developed to drive Rep expression, which demonstrated the capability to enhance AAV packaging efficiency by 40% when compared to original AAV2 P5 promoter. Notably, while the dual P5 design(P5-Rep-Cap-P5) in the triple plasmid system enhanced AAV packaging efficiency, excision of the additional P5 after Cap from pAAVone revealed its dispensability for AAV packaging. Additionally, our research also found that VA RNA II is non-essential for AAV production, unlike the crucial role played by VA RNA I. Intriguingly, removing the additional P5IEE and VA RNA II resulted in a 36% increase in AAVone system productivity. We observed the asymmetrical distribution of plasmid related impurities in AAVone system with the primary dual P5 design, which showed higher abundance in the sequences closer to L-ITR compared with R-ITR. We hypothesize that the presence of the additional P5 after Cap might be contributing to this phenomenon, as its functional similarity to ITRs. Elimination of the additional P5 and VA RNAII not only increase AAV productivity, but also reduced plasmid related impurities closed to L-ITR, making the impurities are almost equal for the sequences out of L-ITR and R-ITR. These results demonstrated the potential for further optimizing the AAVone system design to enhance both packaging efficiency and reduce plasmid related impurities.

1434. Genome Length Dissection: A Comparative Analysis of AAV Variants for Enhanced Gene Delivery Efficiency

Javier Alcudia[1], Jasper Cantrell[1], Wan-Jung Chang[2], Matthew Embury[1], Jonathan Haldeman[2], Han Jiang[1], Soohong Kim[1], Bo Li[1], Patrick Wai-Lun Li[1], Jilin Liu[1], Alexander Mastrokyriakos[1], Steven Pennock[1], William L. Perry[2], Thomas Wechsler[1]

[1]J&J, Brisbane, CA,[2]J&J, Spring House, PA

Adeno-associated virus (AAV) is a small, non-enveloped virus with a single-stranded DNA, measuring approximately 25 nm in size. The wild-type AAV genome spans about 4.7 kb and relies on a helper virus for replication. Although the packaging capacity of AAV vectors is typically thought to be under 5 kb, some research has indicated successful delivery of larger transgenes. Nevertheless, subsequent investigations have revealed incomplete assembly of particles in such vectors. To enhance the understanding of how transgene size affects AAV packaging and quality, AAV transgenes of 7 different sizes (from 3.1 to 5.1 kb) were packaged into AAV1 serotype in HEK293 suspension cells by triple transfection. Transgenes were constructed with the CAG promoter driving eGFP expression and non-coding stuffer sequences of various lengths. Downstream purification using affinity chromatography and anion-exchange chromatography (AEX) was performed for all samples, and a thorough analysis was conducted to assess vector purity, integrity, and the percentage of fully assembled capsids. No significant differences in AAV titers were observed for transgenes below 4.8 kb, however a slightly decreased genome titers were observed for vectors above 5 kb. The transgene size had no effect on VP ratios by SDS-PAGE gel, however transgenes above 4.8 kb showed a truncated or smaller genome size as observed on an alkaline gel. TEM-based analysis remains qualitative and was used to monitor capsid aggregation and non-AAV protein contamination. Neither were observed in any of the vectors with different genome length.

Additionally, 3 of the 4 orthogonal methods used for determining full percentage indicate a significant decrease in the percentage of full capsid above 4.7 kb, and a consequent increase in partially packaged capsids as observed by AUC. These results were reasserted by NGS AAV content analysis where the encapsulated AAV genome fraction was consistently above 90% in all the samples from 3.1 to 5.1 kb lengths. However, the percentage of particles carrying the full-length payload dropped slightly then significantly at 4.8 and 5 kb, respectively. In conclusion, we demonstrated here that overpacking AAV beyond 4.7 kb led to decrease in fully packaged capsid and full-length payload.

1435. Early Characterization of Research-Grade AAV6 Productions Allows for Manufacturing Alignment During Candidate Nomination

Amira D. Rghei, Shona Robinson, Lisa Santry, James Fasano, Jay Cai, Daniel Kennedy, Theresa Dao, Omar Matalka, Azam Hassaninasab, Will Arnold, Adriana Geldart, Debaditya Bhattacharya, Bojiao Yin, Mercedes Segura, Stacie Seidel, Cherylene Plewa
ElevateBio, Waltham, MA

Adeno-associated virus (AAV) is the most popular vector in the gene therapy space, with extensive applications for treatment and cure of monogenic disorders. There are currently hundreds of active clinical trials for recombinant AAV (rAAV) therapeutics, and a handful of EMA and FDA-approved modalities. Though there have been large strides in the gene therapy field, effective characterization (primarily packaging efficiency and manufacturability) prior to larger scale-up of rAAV vectors remains an ever-evolving challenge in the industry. These larger scale process development (PD) efforts can include costly and labor-intensive optimization of production, purification, and post-purification processes. Unfortunately, the characteristics of the final scaled-up products may not match with material used for the initial R&D candidate characterization, resulting in longer and more tedious pre-clinical development timelines to reconcile the differences. There is a clear need for early and well-defined characterization of therapeutic candidates at the R&D level that aligns with production, purification, and characterization methods across both upstream and downstream PD platforms. Here we describe a process to streamline our lead therapeutic candidate selection process for research and development (R&D)-grade rAAV6 vectors, using a high throughput workflow preceding large scale-up efforts. Four AAV vectors expressing therapeutically relevant transgenes, with genomes between 4.1-4.7kb in length, were packaged in AAV6 using our R&D scaled-down method. A series of analytics were used to characterize R&D material and larger scaled-up material, including vg titers (ddPCR), viral protein ratios (Maurice, automated capillary electrophoresis system), % full analysis (Mass photometry and analytical ultracentrifugation), followed by 3rd Generation Sequencing. These results show that our R&D grade vectors had crude titers >1e11 vector genomes (vg)/mL, as well as >30% full by Mass photometry and AUC. When compared to PD 2L bioreactor scaled-up AAV material, purified by AKTA (high performance liquid chromatography) chromatography, the R&D and PD material were demonstrated to have comparable characteristics. This early characterization of rAAV6 candidates has allowed for faster, and more efficient testing of multiple construct designs, and cost-efficient movement towards manufacturing of lead therapeutic candidates from our R&D to PD team.

1436. Characterization of Residual Contaminants in AAV Vectors Leveraging Long-Read Sequencing

Kiran Adhikari[1], Bob Bruccoleri[2], Serena Dollive[1]
[1]Oxford Biomedica (US) LLC, Bedford, MA,[2]Congenomics, LLC, Glastonbury, CT

An ideal Adeno-associated virus (AAV) vector would consist of a full-length construct packaged within the capsid. However, existing vectors are not 100% pure as they contain residual contaminants. The types and amount of these residuals can vary between vector production conditions and vector genomes. Proper characterization of residuals is important to make the AAV vectors more reliable and safer. Here, we have developed an assay to characterize the residuals present in the vector sample by taking advantage of PacBio long-read sequencing. With the sequencing reads capable of spanning the whole length of the genes, we were able to quantify the amount of residual antibiotic resistance genes, Adenovirus genes E1, E2A, and E4 as well as Rep and Cap present on the vector sample. We were also able to characterize whether these genes are full length or partial. This method could help improve product quality by more robustly characterizing residual contaminants.

1437. An Improved Helper Plasmid Containing E2a and E4 Gene Deletions Results in Increased AAV Productivity

Laura van Liehout, Stacy Ota, Eli Wiberg, Annie Adusei, Katrina Costa-Grant, Marissa Stanvick, Ify Iwuchukwu, Diane Golebiowski, Jin Yin
Oxford Biomedica, Bedford, MA

Adeno-associated virus (AAV) vector production typically involves transfection of an adenovirus helper plasmid to provide the helper genes E2a, E4 and VA RNA, which are required for AAV replication and gene expression. These helper plasmids tend to be large and can be difficult to manufacture, leading to higher costs at GMP scale. We sought to reduce the size of our helper plasmid by introducing deletions into the E2a and E4 genes as they represent the majority of the plasmid sequence. Within the E2a gene, there is approximately 3 kb between the E2a promoter and the start codon. Five different E2a deletions were made in this region ranging from 0.5-2.0 kb. The three largest deletions failed to produce comparable vector genome (VG) titers to the control. Both the 0.5 kb and 0.9 kb E2a deletions demonstrated comparable productivity to the control helper plasmid, therefore we selected the larger 0.9 kb deletion for additional studies. The E4 gene is composed of at least six open reading frames (ORF) of which E4ORF6 is thought to be critical. Three E4 deletions were tested; the first removed 1.2 kb (ORFs 1-3), the second removed 1.5 kb (ORFs 1-4) and the third removed 2.0 kb (ORFs 1-4, 6/7). All three constructs kept ORF6 intact. Each of the E4 deletions generated comparable VG productivity to the control helper. There was an advantage to keeping both ORF6 and ORF6/7 intact, therefore we selected the 1.5 kb E4

deletion for follow up. In combination with removal of 1 kb from the plasmid backbone, the size of the deletion helper plasmids was reduced from 12 kb to 10 kb for the E2a deletion and 9.5 kb for the E4 deletion. We also evaluated a combination of the E2a and E4 deletions resulting in a plasmid 8.5 kb in size. Interestingly, the E4 deletion and E2a+E4 deletion helper plasmids both resulted in VG and capsid productivity that was 2-3X higher than the control helper. The E2a deletion alone did not result in any increases and appears inconsequential. Using RT-qPCR, Rep and Cap transcripts increased in the E4 deletion and E2a+ E4 deletion helpers, which is also supported by an increase in Rep and Cap protein by western blot. The E2a deletion alone did not show an increase in Rep and Cap transcripts or protein. RT-qPCR analysis for E2a transcripts shows minimal impact from any helper deletion including the E2a deletion, suggesting that this particular E2a deletion does not impact E2a expression. However, E4orf6 RT-qPCR indicates there is a reduction in ORF6 transcripts with the E4 deletion and E2a+E4 deletion helpers but not with the E2a deletion. This could suggest that the optimal expression level of E4ORF6 is lower than what is generated from an intact E4 gene and that lower E4ORF6 levels allow for enhanced Rep and Cap expression, leading to higher VG and capsid productivity. The E2a+E4 deletion helper has resulted in increased productivity for a number of capsid serotypes including AAV2, AAV5, AAV6, AAV8 and AAV9 and across five genomes with multiple promoters and transgenes ranging from 2.3-4.2 kb. Finally, the increase in E2a+E4 deletion helper productivity has been observed for both pOXB dual and traditional triple transfection, demonstrating the wide-ranging utility of this improved helper plasmid for various AAV applications.

1438. Impact of Sample Treatment on DNA Length Distribution Analysis by Duplex ddPCR

Marina Magerl, Felicia Thoennissen, Sophie Beer, Rebecca Feiner, Sonya Schermann, Markus Hoerer, Sabine Geiger

Ascend, Planegg-Martinsried, Germany

Introduction Recombinant adeno-associated virus (rAAV)-encapsidated plasmid impurities are an undesirable byproduct of vector manufacturing consisting of heterogeneous non-vector genome fragments of DNA arising from the production plasmids. Horizontal gene transfer of manufacturing plasmid-derived resistance genes might be a potential risk when they are packaged as full-length genes. Thus, regulatory authorities require thorough characterization of these DNA impurities. We developed a novel duplex ddPCR method for assessing the length distribution of encapsidated nptII (kanamycin resistance) gene fragments. Here we describe how typical sample treatments used to disrupt virus capsids prior to droplet formation can impact fragment length analysis performed by duplex ddPCR. **Methods** To assess full-length nptII gene species, two duplex ddPCR approaches were developed. In the first one, two primer-probe sets with different fluorophores positioned 795 bp apart were used so that droplets positive for both fluorophores indicate full-length sequences. However, this approach carries the risk of overestimating full-length DNA species if two smaller nptII gene fragments are co-packaged in the same rAAV capsid, creating double-positive droplets. Thus, a second approach was developed using two primer-probe sets with different fluorophores spanning approximately 400 bp fragments at each side of the gene, minimizing the risk of false-positive full-length results (Figure 1). Sample treatments typically performed to reduce re-annealing of plus and minus rAAV strands after capsid disruption such as the use different buffers and heat treatments were tested in both methods. Additionally, the impact of mechanical processing (e.g. vortex mixing) on nptII length distribution analysis was assessed. Acceptance criteria for the assays included recovery of >90% of a linearized plasmid control, confirmation of dilutional linearity for plasmid controls and samples, and coefficients of variation <15% for replicate measurements. **Results** Plasmid and rAAV samples which were not subjected to a specific treatment prior to ddPCR showed the expected results, while a dramatic decrease in full-length species was observed for samples which were treated with heat for 30 minutes. The impact on DNA integrity seemed to be time-dependent since heat treatment for shorter time periods caused less fragmentation. Interestingly, samples subjected to mechanical manipulation (vortexing) for different amounts of time did not show any signs of DNA fragmentation. **Conclusion** While the first method described here (using two primer/probe sets positioned 795 bp apart) might lead to an overestimation of full-length nptII impurities due to co-packaging of two smaller gene fragments in one capsid, the second approach we developed (using primer/probe sets spanning approximately 400 bp at each side of the gene) can be considered fit for the purpose of assessing full-length nptII gene fragments in rAAV batches. However, care must be taken during sample treatment since commonly used methods such as heat treatment can quickly result in fragmentation of DNA, leading to an underestimation of full-length impurity species. Separation of intact capsids into droplets prior to heat disruption ensures that DNA fragments are distributed into individual droplets in one piece.



Figure 1. Schematic representation of the assay principle of duplex ddPCRs for nptII

1439. Impact of the Genome Length of Adeno-Associated Viral Vectors on Yield and Quality Parameters

Rupert Derler, Martina Ohme, Elisabeth Schweigert, Florian Dunker, Kathrin Breunig, Florian Sonntag, Markus Hoerer, Andreas Schulze

Ascend, Planegg, Germany

Wild-type adeno-associated virus (AAV) contains a single-stranded DNA genome with a length of approximately 4.7 kb. Yet even packaging of close-to wildtype sized transgene cassettes can lead to lower yields compared to smaller transgene cassettes. Production of rAAV with vector genomes in the range of the wild-type genome length is therefore challenging concerning vector yields and quality parameters. We have developed a robust modular suspension platform process based on our proprietary HEK293 cell line and split two plasmid system, that is optimized towards yield at best possible quality with full scalability. The platform has already provided successful manufacturing of several distinct capsids. We continue to expand our data to underline the platform's universal application across both natural and engineered

capsids. We present here the impact of a wild-type genome length cassette (4.7 kb) in comparison to a medium-size vector genome (approx. 3.0 kb) in context of AAV9 produced using our suspension platform process. We analyzed yield and quality parameters that are known to be strongly impacted by the design and choice of the biological starting materials and the upstream process. A panel of analytical methods was applied to enable a comprehensive and detailed comparison of many different attributes. Vector genome and capsid yields in a comparable range were observed independent of the vector genome length. Increased levels of mispackaged plasmid- and host cell DNA-derived sequences were detected for the wild-type genome length in comparison to the medium-size vector genome using the same process parameters for both setups. Integrity of all vector genomes was demonstrated by alkaline gel electrophoresis. Our results demonstrate the applicability of our suspension platform rAAV9 production process for challenging vector genome lengths at the rAAV packaging limit. We continuously invest in further CMC innovation to optimize yield and quality for all relevant genome lengths across capsids.

1440. Development of a Water-Based Hypotonic Cell Lysis Method for AAV Vector Production

Sujit Pujhari, Ronald Smithwick

Pharmacology, Physiology and Neuroscience, School of Medicine, University of South Carolina, Columbia, SC

Gene therapy has emerged as a promising approach for treating genetic diseases, and adeno-associated viral (AAV) vectors have gained widespread use due to their safety and efficacy. More than 200 gene therapy trials using rAAV vectors are currently underway or completed in the USA. rAAVs are produced in human embryonic kidney cells (HEK293T) and, more recently, using insect cells (sf9 cells). AAV being a cell-associated virus, the cells must be lysed to purify. Commonly used methods of cell lysis, including freeze-thaw (FT), sonication, or chemical lysis, are time-consuming and expensive. Therefore, new methods are needed to purify AAV to ensure readily accessible, cost-effective, and safe AAV treatment. To overcome this obstacle, this study has developed a novel water-based hypotonic (WH) method for AAV production that is faster, simpler, and more cost-effective than the traditional freeze-thaw (FT) water lysis method. This study used the AAV6 serotype expressing GFP visual marker protein. The quality of AAV6 expressing GFP produced using the WH-lysis method was comparable to those produced using the FT-lysis method. Furthermore, the transduction efficiency of virus preparations by measuring GFP expression in HEK293T, Huh7.5, Cos-1, and Vero cells and found that the WH-lysis method outperformed the FT-lysis method. Overall, this proof of concept study demonstrates that the WH-lysis method is a fast, convenient, and effective method for producing high-quality AAV vectors for various gene therapy applications. The use of this method could significantly reduce the cost and time required for AAV vector production, making gene therapy more accessible and affordable to patients.

1441. Development of a Single Clonal Suspension Cell Line PCS3.0 for High Yield AAV Production

Xin Swanson[1], Longxiang Sheng[1], Paul Li[2], Ye Bu[1], Yunyi Chen[1], Weixiao Huang[1], Zexin Zhou[1], Liying Yang[1]

[1]PackGene Biotech INC, Houston, TX,[2]PackGene Biotech, Worcester, MA

Cell substrates are among the most crucial raw materials in the AAV manufacturing process. The monoclonal characteristics of HEK293 cell lines mitigate genetic heterogeneity among cell pools, enabling stable and efficient AAV production using triple-plasmid transfection. To generate highly homogeneous monoclonal cells, we utilized a Dispencell in-situ cell dispenser for single-cell sorting of the parental HEK293 cell pool, and D0 cells were imaged using CloneSelect Imager for assurance of clonality. Sorted single cells were cultured, expanded, and screened by AAV production yield and Population Doubling Time (PDT). The promising high-yield candidate clone underwent a series of gradient-decreasing serum cultures to obtain suspension cell clones. To enhance AAV productivity stability, suspension cell clones underwent two rounds of single-cell sorting and screening based on the triple-plasmid transfection platform. The top clone with the highest AAV productivity was selected and confirmed as the PCS3.0 cell line, with a systematic characterization of growth curve, PDT, and metabolic dynamics during AAV production. PCS3.0 cells were assessed with a production test on different AAV serotypes, including AAV2, AAV5, AAV8, and AAV9, using a therapeutic Gene of Interest (GOI) on shake flask and 3 L stirred bioreactor scales, showing stable production capacity with lysate genome titer ranging from 1E+11 vg/mL to 5E+11 vg/mL. Host cell DNA (HCD) and empty capsid ratio in DS were further evaluated. HCD can be controlled below 100 ng/E+13 vg, and the empty capsid ratio can be controlled below 10%. In conclusion, we successfully constructed a suspension single clone cell line, PCS3.0, enabling high AAV production capacity, stability, and quality, providing strong support for industrial-scale AAV manufacture in IND/BLA applications.

Friday Posters: AAV Vectors - Capsid Engineering

1442. Rationally Designed Chimeric AAV Capsids Demonstrate Enhanced Muscle Transduction and Reduced Liver Tropism in Both Rodents and Non-Human Primates

Paul Li[1], Yue Pan[1], Yujian Zhong[2], Youwei Zhang[2], Zhiyong Dai[2], Huan Chen[2], Keqin Tan[2], Rui Duan[3], Min Guan[4], Ye Bu[2]

[1]PackGene Biotech, Worcester, MA,[2]PackGene Biotech, Houston, TX,[3]South China Normal University, Guangzhou, China,[4]Chinese Academy of Sciences, Shenzhen, Guandong, China

The recombinant adeno-associated viral (rAAV) vector stands out as a pivotal technique within the gene delivery arsenal. However, its potential clinical application remains restricted by insufficient target

transduction and undesirable side effects, notably liver toxicity. To address these limitations, we employed sequential evolution of the AAV capsid via a robust pipeline, which starts with a direct comparative analysis of pre-selected AAV capsids screened from a high-throughput library and ends with functional validations in both rodents and NHP models. First and foremost, we rationally designed three chimeric capsids: AAV.Zero1, AAV.Zero2, and AAV.Zero3, which were verified to dramatically narrow down their tropisms in multiple major organs, especially in the liver. Further modification confirmed AAV.Zero3, a good choice of myotropic capsid backbone for a peptide-displaying mutant called AAV.eM5, exhibits superior efficiency and specificity in the musculature, including skeletal muscle and the heart, following systematic delivery cross-species. This holds great potential for muscle-related gene therapy. We envision that AAV.Zero1 and AAV.Zero3 are universal backbones that may not only be useful in targeting muscles but also be broadly applied to other organs or cells with specific peptide insertions. Moreover, our comprehensive serotype screening methodology (π-Icosa) is compatible with any capsids, targets, and species, and will thus facilitate and accelerate the development of optimal AAV vectors for human gene therapy.

1443. Novel Insertion Peptide AAV Capsids Exhibit Enhanced Transduction in Muscle and Ocular Tissue Across Different Serotypes

Trailokyanath Panigrahi[1], Ruchita Selot[1], Ashish Khaparde[1], Veluchamy A. Barathi[2], Arkasubhra Ghosh[1]

[1]GROW Research Laboratory, Narayana Netralaya Foundation, Bangalore, India,[2]Translational Pre-Clinical Model Platform, Singapore Eye Research Institute, Singapore, Singapore

Despite the success of recombinant AAV (Adeno-associated viral vectors) across numerous clinical trials, many studies have documented sub-optimal results with various AAV serotypes. This challenge has spurred development of capsids with enhanced efficiency, employing diverse strategies such as site-directed mutagenesis, rational design, directed evolution, and insertion of RGD peptides identified from a library-based approach. Here, we have developed a set of new insertion peptide containing AAV capsids incorporating RGD peptides (7-mer RGD integrin binding sequence, AAV-TIP) selected via rational design from pre-existing information regarding sequence affinity to integrin subsets expressed on human cells and compared that with a published, best performing RGD sequence (7-mer RGD containing sequence AAV-CIP). Further, since comparative studies on insertion of the peptides across different serotypes and application in tissue types are lacking, we tested three distinct clinically applicable serotypes (AAV2, AAV8, AAV9) in muscle and retina. The capsid sequences from AAV2, AAV8 and AAV9 were used for insertion of two different RGD peptides at positions 588, 589 and 589 respectively. The novel capsids were packaged by standard triple transfection method followed by purification via affinity chromatography. Subretinal and intramuscular injections were administered into 6-week-old C57BL/6J male mice at 6x10^8vg/eye and 1x10^9vg/TA muscle respectively. Six weeks post injection, muscle tissues were analysed for GFP expression using imaging, Q-PCR, and immunoblotting. Retinal structural changes and spread of GFP expression across all groups were assessed weekly by funduscopy, FFA and PS-OCT imaging. The viral genome quantification for wild-type and mutant vectors ranged from 7.04x10^11vgc/mL to 5.2x10^12 vgc/mL. In HEK293T cells and differentiated myotubes, AAV9-TIP.GFP and AAV9-CIP.GFP transduction exhibited higher expression levels compared to AAV9-WT.GFP (~20-fold, $p<0.05$ and 14-fold, $p<0.05$ respectively). Expression from AAV2 mutants was similarly high compared to AAV2-WT, but AAV8 mutants showed a lesser difference with AAV8-WT compared to other serotypes. This suggests that novel AAV-TIP peptide improves transduction at par with comparator. Analysis of GFP expression in mouse muscle revealed approximately >25-fold ($p<0.05$) higher expression from both AAV9-CIP.GFP and AAV9-TIP.GFP compared to AAV9-WT. Although AAV2 transduction overall was very low in muscles, we observed 3-fold ($p=0.051$) increase in GFP gene expression with AAV2-TIP and no significant change with AAV2-CIP. Interestingly, in muscle tissues, we observed much lower fold differences between AAV8-WT and AAV8 mutants compared to AAV9, which requires further validation. Subretinal injection of AAV9-TIP.GFP demonstrated early onset of expression and widespread distribution of GFP, showing ~10-fold increase in expression from both mutants across all serotypes compared to its wild-type ($p<0.05$ when comparing mutants with respective WT). Subtle differences in patterns of expression between TIP and CIP mutants were seen specific to the tissues type and serotypes were observed. However, AAV9-TIP vectors showed the highest overall transduction in retina as well as muscle. Interestingly in retina, both AAV2 CIP and TIP showed significantly higher expression than WT ($p<0.05$). The mouse in vivo data demonstrates significant superiority of new TIP vectors in the context of all three serotypes and non-inferiority to comparator CIP. We anticipate that these novel capsids will help improve AAV vector performance in clinical studies and reduce vector dosage.

1444. Selection and Characterization of AAV Capsids with Improved Tropism for Ocular Tissues in NHP

Bingfu Jiang, Dianyang Chen, Lumei Xiao, Huimin Kong, Peilu Li, Bing Wu, Hao Luo, Jinxin Deng, Zheng Luo, Yue Zhang, Shurong Zuo, Yanjun Shen, Hui Liu, Carol Huang, Jay Hou, Yanqun Shu

Skyline Therapeutics, Cambridge, MA

Adeno-associated virus (AAV) is considered as the most attractive vector for therapeutic gene delivery, AAV2 and AAV2 derived capsids have proven especially useful for vector construction for treating ocular disease. Intravitreal injection (IVI) is a simple and promising delivery route, but it often fails to achieve desired efficacy owing to inferior efficiency of vector to cross retina inter limiting membrane (ILM). To obtain capsids with high efficiency across ILM and improve tropism for the cells in retina, AAV2 based capsid library screening was carried out in non-human primates (NHP). Several capsids with significantly increased transduction for retinal cells were attained from the screening, and after packaging GFP and luciferase reporter transgene into a group of selected capsids, we thoroughly characterized them in NHP. The individual vector was injected via IVI into the animals and the vector genome copy numbers, transgene mRNA levels and expressed protein levels were measured using qPCR, RT-qPCR and luciferase assay. Retina wholemount and immunohistochemistry was also performed to detect which type of cells in the retina were transduced 3 weeks post injection. Our results indicated that most

of the novel vectors outperformed the benchmark wildtype AAV2 in biodistribution and transduction of retinal cells. Genome copy numbers were correlated with reporter gene expression well. In addition, the selected novel capsids showed notably higher levels of staining for retinal cells compared to wildtype AAV2. All the data suggested that the identified capsids gained biochemical properties that enabled them to cross ILM and penetrated different layers in the retina to effectively transduce the cells. We further evaluated the capsids in rodent via IVI injection, the results were consistent with that from NHP. All in all, the data indicates that the promising novel AAV2 capsid variants can be used to efficiently target retina; the vectors generated with the novel capsids can be potentially used in gene therapy for the treatment of a variety of human ocular diseases.

1445. AI-Aided AAV Capsids Design for Efficient Retina Transduction in NHP

Shuyuan Chen, Xiaodong Pang, Changpeng Wan, Zhongwan Li, Weiwei Wu, Runyuan Zhang, Xing Lan, Guanlin Li, Zhenhua Wu, Shaoyong Li, Guo-jie Ye
Exegenesis Bio Inc., Lower Gwynedd, PA

The success of adeno-associated virus (AAV) vector-based gene therapy depends on efficient delivery of the vector to the target cells. Most ocular diseases are due to the impairment of the development or function of photoreceptors, and/or cells of the RPE. Delivery of genes to these and other retinal cells presents a challenge because of the physical barriers of the retina. While subretinal administration provides the most direct access to photoreceptors and the RPE, it is more invasive, with a risk of damage to the retinal tissue. Intravitreal (IVT) injection is a safe, non-surgical procedure and have been widely used for delivery of protein therapeutics to treat common retinal diseases. Thus, more effective IVT delivery of AAV-based gene therapeutic products for retina is an unmet need for ocular gene therapies. Here, we reported two novel AAV capsids (AVT260 and AVT239) engineered with our AI-aided AAVarta® platform. In NHP, the efficiency of retina transduction of AVT239 and AVT260 delivered by intravitreal injection are 307-fold and 119-fold higher than that of AAV2, and 5-fold and 2- folds higher than AAV2.7m8, an intravitreal benchmark AAV capsid, respectively. The screening process relies on the next generation sequencing (NGS) of the target mRNA recovered from the retina and driven by a photoreceptor-specific promoter. Data obtained from each round of screening in NHP was used to train our regression models which will direct the design of AAV capsid library of next generation. After AAVarta® enrichment ranking analysis, 95% of top 100 capsid variants were shown to originate from our AI platform. Selected candidates were individually produced for validation with GFP or mCherry as reporter genes. Several variants exhibited widespread and high-level retinal transduction in NHP. Especially, both AVT260 and AVT239 showed significantly better transduction efficiency in the retina over AAV2.7m8 in Cynomolgus macaque (*Macaca fascicularis*). These results demonstrate that our designed variants with high efficiency in retinal transduction offer promise for IVT retinal gene therapies and how we can employ the AI power in capsid design.

1446. Criticality of Manufacturability Assessment in Supporting Discovery of Novel AAV3B and AAV8 Variants

Amanda Zhang, Azadeh Bazarchi, Bonnie Wu, Nadia Gantt, Thuy Bui, Ayda Mayer, Craig Malzahn
REGENXBIO Inc., Rockville, MD

Our institution has been undertaking the effort to engineer capsids for improved transduction efficiency and minimized off-target effects for certain routes of administration. To achieve this goal, we developed a directed evolution platform NAVIGATE (Novel AAV Vector Intelligent Guided Adaptation Through Evolution) for new capsid discovery, and this is accomplished by generating diverse libraries with randomized 7-mer peptide insertions in the AAV VR-IV and VR-VIII surface loop regions. For one of the projects, we applied NAVIGATE to AAV8 and AAV3B capsid optimization in the context of suprachoroidal space (SCS) delivery to ocular tissues. During library screening phase, we used various high-throughput techniques for material generation, such as transfecting with enhanced plasmid ratio or purifying mixed AAV variants using ultracentrifugation technology, which resulted in production of ~300 runs and >10000 peptide variants. The AAV library material produced went through multiple rounds of selection and the biological activity data generated showed differences in folds of improvement for these novel capsids over the AAV8 and AAV3B parental controls. Subsequently, a panel of variants were selected at 0.25L production scale for preliminary manufacturability assessment. Manufacturability assessment is crucial in helping to identify the suitability of a therapeutic molecule, to evaluate its fitness to a platform, and to reduce the process development effort due to unexpected results. For the initial manufacturability screening study, we used an in-house developed high throughput chromatography technology that can simultaneously purify 12 runs, at both the capturing and polishing chromatography steps. We were able to quickly observe the process differences between these novel SCS capsids. While the harvest titers, analyzed by digital droplet PCR (ddPCR), were all acceptable, there is a ~2.5 fold difference between the lowest and highest harvest titers for this panel of tested variants. The purification steps also demonstrated differences in the step yields and elution patterns for certain AAV8 and AAV3B variants. The polishing steps showed that the AAV8 variants all eluted earlier and that AAV3B variants all eluted later when compared to AAV8 and AAV3B controls. We further narrowed down the more promising novel SCS capsids to produce at 10L scale for a more complete manufacturability assessment, and the process profiles matched that of the smaller scale high throughput screening. The final produced material all had similar product qualities based on capsid purity, genome integrity, and percentage of full capsids present in solution, but the thermos-stability testing showed major differences, up to ~30°C, in melting temperatures. This information in combination with earlier biological activity data helped to determine the desirable capsids for further NHP studies. In summary, via our high throughput toolbox we ensured optimal production efficiency to provide material for our NHP studies, while swiftly discovering the potential process and product differences to better understand manufacturability for these variants. This has aided in selecting the novel SCS capsids that have optimal biological activity profiles but also possess the desirable manufacturability attributes, thus saving time, cost, and effort related to potential product redesigning.

1447. Grafting of an Enhanced VP1u Region onto Multiple Serotypes of AAV to Boost Baseline Transduction of Vectors for Gene Therapy

Mengtian Cui, Jackson McGowan, Jialing Yang Liang, Xiupeng Chen, Mitchell Yip, Suk Namkung, Tapan Sharma, Fang Zhang, Thomas Leland, Dan Wang, Jun Xie, Guangping Gao, Phillip Tai
UMass Chan Medical School, Worcester, MA

Adeno-associated virus (AAV) vectors are currently the most utilized viral vehicle for gene therapy. AAV capsid engineering efforts to boost transduction and safety have been in high demand. We have discovered an AAV2 capsid modality within the VP1 unique (VP1u) region, which has been named "MC1". The VP1u sequence is located at the N-terminus of the VP1 protein and initially resides within the lumen of the capsid. The region is then hypothesized to be externalized during its conformational change in late-endosomal/lysosomal compartments. VP1u is believed to participate in endosomal escape and trans-Golgi network (TGN) trafficking steps. We have shown that the MC1 modification AAV2 (AAV2.MC1) outperforms the gold-standard retinotropic capsid AAV2.7m8 for mouse retinal transduction following intravitreal (IVT) injection. AAV2.MC1 showed wider transduction of the retina in cynomolgus macaques than achieved by AAV2 following IVT administration. Interestingly, AAV2.MC1 externalizes its VP1u region at lower temperatures than AAV2 (45-50 °C versus 55 °C) as revealed by dot blot assays using a VP1u epitope-targeting antibody (A1). Using an intact capsid-specific antibody (A20), we found that AAV2.MC1 exhibited an overall higher thermostability than that of AAV2 (70-72 °C versus 68 °C), which was consistent between vectors packaged with either single-stranded or self-complementary genomes. These phenomena are also assessed by differential scanning fluorimetry (DSF). These findings reveal a role for the VP1u region in impacting AAV stability. Further studies in this regard are in process. Since VP1u is conserved among different AAV serotypes and shares similar functional domains with other parvoviruses, we hypothesized that the MC1 modality can increase the transduction efficiencies of other serotypes when grafted onto other capsid variants. Grafting MC1 onto AAV.7m8 further boosts its transduction. Unexpectedly, different serotypes showed distinct tolerances for the MC1 graft (*i.e.*, do not compromise vector packaging/production). AAV8, and AAV9 tolerated the graft, but only when purified by iodixanol gradient ultracentrifugation. Purification by CsCl gradient ultracentrifugation for these capsids caused >10-fold reduction in titers. Since CsCl purification requires higher centrifugation speeds compared with iodixanol purification, we hypothesize that the MC1 graft onto AAV8 and AAV9 may lower their stabilities. In contrast, AAV3b tolerated the MC1 graft, likely because of the high conservation between AAV2 and AAV3b. We have found that the MC1 graft increases AAV3b transduction *in vitro* and AAV1 transduction of mouse muscle. Grafting of MC1 onto AAV8 (AAV8.MC1) showed a two-fold greater transduction of mouse liver than achieved by grafting wildtype AAV2 VP1u onto AAV8. Grafting of MC1 onto AAV1, AAV5, AAV9, and subsequent *in vivo* testing is ongoing. Our results show that both AAV stability and transduction can be significantly impacted by VP1u, a domain that has largely remained unexplored by AAV capsid engineering efforts. G.G. and P.T. are corresponding authors.

1448. Intravitreal Delivery of an AAV2-Based and Cell-Penetrating Peptide Grafted Novel Capsid Produces Enhanced Panretinal and Photoreceptor Transduction with Reduced Inflammation

Jianghui Wang, Mengtian Cui, Hao Liu, Dan Wang, Phillip WL Tai, Guangping Gao
UMass Chan Medical School, Worcester, MA

Inherited retinal diseases (IRDs) are attractive therapeutic targets at the forefront of recombinant Adeno-associated virus (rAAV)-based gene therapy. The rapid development in this field has resulted in the first gene therapy product approved by FDA, Luxturna. To efficiently transduce the major target cells in IRDs, photoreceptors, rAAV needs to be administered via invasive subretinal injection. Due to the clinical and technical limitations of the subretinal injection, this approach entails an invasive procedure and only achieves local therapeutic effects and significantly reduces the efficiency of gene therapy. Thus, increasing efforts are dedicated to engineering novel capsids that enable rAAV penetrating to photoreceptors following intravitreal (IVT) injection. In this study, we aimed to graft cell-penetrating peptides onto AAV2 capsid and select resulting capsids for enhanced penetrating capability through vitreous space and retinal structures. To this end, we designed a moderately diverse capsid library with over 1000 cell-penetrating peptides (CPP) at various length (3-42 mer) inserted at position N587 of AAV2 VP3. The resulting AAV2 library were intravitreally injected in mice, and mRNA were subsequently prepared from retina and RPE complex one week post-IVT, followed by Next Generation Sequencing to examine the abundance of each capsid variant in the tissues. One CPP candidate, tentatively named as CPP1, was selected after one round of selection based on its highest enrichment in the retina at the transcriptional level. To validate our engineering strategy, we produced a single-stranded AAV2.CPP1.EGFP (ssAAV2.CPP1) and dosed mice by IVT injection ($2x10^9$ vgs/eye) along with AAV2 (ssAAV2) and 7m8 (ss7m8), an engineered AAV2 variant capable of transducing photoreceptor efficiently via IVT. Our preliminary data revealed that ssAAV2.CPP1 resulted in more robust (>2-fold) panretinal transduction than both ssAAV2 and ss7m8. Moreover, compared to the other two vectors, ssAAV2.CPP1 demonstrated enhanced GFP expression in all inner retinal cell types. Most importantly, ssAAV2.CPP1 exhibited 7- and 2.4-fold increase in photoreceptor transduction compared to ssAAV2 and ss7m8, respectively. As rAAV-induced immune responses and inflammation are major barriers in ocular gene therapy, we examined the expression of several inflammatory cytokines at mRNA levels in the eyes of the mice treated with different rAAV. We found that ss7m8 is prone to increased expression of these inflammatory cytokines, particularly Interleukin 6 (IL-6), while ssAAV2.CPP1 generates significantly lower IL-6 expression compared to ss7m8. Furthermore, there were significantly fewer microglial cells, a local immune cell type, identified in the inner plexiform of the mouse retina receiving ssAAV2.CPP1 compared to those receiving ss7m8. Altogether, our preliminary data suggest that our CPP-engrafting

capsid engineering strategy can potentially generate novel capsids that outperform 7m8 capsid in retinal transduction in both inner retinal cells and photoreceptors with reduced inflammation, potentially expanding our toolbox for retinal gene therapy applications through IVT. Given the restricted rounds of selection in this pilot study, further evolution and examination of this library are underway. J-H.W., M.C. and H.L. are considered as co-firstauthors.

1449. Engineering Novel AAV Capsids through Directed Evolution of the VP1u Region

Mengtian Cui, Jiang-Hui Wang, Jackson McGowan, Mitchell Yip, Phillip WL Tai, Guangping Gao
UMass Chan Medical School, Worcester, MA

Adeno-associated virus (AAV)-based vectors are currently the most used viral vectors for clinical gene therapy. As the number of clinical gene therapy trials has increased, there is a strong demand for safe and effective capsids with specific tropism profiles. To this end, we previously discovered two natural AAV2 variants isolated from human tissues that showed strong transduction of mouse and NHP retinas following intravitreal (IVT) injections. Interestingly, these variants only differ from AAV2 in the VP1 unique (VP1u) region, a poorly investigated sequence at the N-terminus of the VP1 protein. These findings hint at the potential for engineering capsids at the VP1u region. As the majority of capsid engineering is made in VP3, we hope to expand rational design efforts through identifying positive VP1u mutations that can likewise enhance transduction. However, sparse knowledge of VP1u limits our confidence in rationally modifying the region. To overcome this issue, we employed a directed evolution, a proven approach to assist the identification of tissue-tropic and highly efficient capsids, at the VP1u region. We randomly introduced mutations into the entire VP1u by error-prone PCR, followed by NGS analysis to estimate variant diversity and enrichment. In the first round of the AAV2 VP1u library, 4.93E5 unique capsids (approximately 95.5% variants and 0.5% AAV2) were identified, and the library was intravitreally injected in mice, followed by mRNA preparation from the retina and RPE complex one-week post-injection. The VP1u variants were amplified and packaged into a second AAV library consisting of 4.2E5 unique capsids (approximately 85% variants and 15% AAV2). Following the injection of this library, the unique capsid variants found in the tissue were reduced to 5.4E4. The enrichment score (ES) from the 2nd round of screening indicates the relative tissue abundance of each capsid normalized to library gDNA. Based on this ES metric, there were 10 candidates found to outperform AAV2. Interestingly, the top candidates differed from the AAV2 VP1u sequence by single amino acid mutations located in poorly described regions. A third round of screening will be performed to further enrich the leading candidates.To validate our engineering strategy, we selected one of the ten aforementioned candidates, named v113, to evaluate the potential of these capsids. This variant was individually packaged with an *Egfp* expression cassette and then evaluated by IVT injection in mice. v113 was found to transduce the mouse retina with two-fold greater efficiency than AAV2. It showed a relatively better photoreceptor transduction. These preliminary results demonstrated the potential of AAV VP1u library screening as a means of identifying effective capsids. This exploration has encouraged us to individually evaluate more highly enriched candidates following additional rounds of selection. Moreover, the knowledge gained from this work may provide invaluable insights into the AAV field and enable us to better characterize the under-researched functional domain of VP1u, expanding our capsid engineering approaches. **M.C., J-H.W. are considered as co-first authors.**

1450. SNAC: Single-Nuclei Atlas of Capsid Distribution for AAV Capsid Selection for Ocular Gene Therapies

Eugenia Lyashenko[1], Rachna Manek[1], Yinyin Huang[2], Jeremy Huang[2], Margaret Hennessy[1], Michael Ryan[1], Jasmine Bloom[1], Tess Torregrosa[1], Pavitra Ramachandran[1], Andre Kurlovs[2], Virginia Savova[2], Giorgio Gaglia[2], Sourav R. Choudhury[1]
[1]Genomic Medicine Unit (GMU), Sanofi, Waltham, MA,[2]Precision Medicine Computational Biology (PMCB), Sanofi, Cambridge, MA

Neurodegenerative ocular diseases lead to irreversible loss of vision and affect up to 300 million people worldwide. Because the eye is a relatively small and contained anatomical structure, AAVs can be delivered locally. Therefore, ophthalmic gene therapy can potentially avoid systemic immune responses and provide therapeutic benefit at low capsid doses, making ophthalmology an excellent candidate for the gene therapy approach. Recent advances in genome-wide association studies (GWAS) and single cell sequencing have allowed the genetic targets of disease to be mapped to specific cell populations. However, cell-type specific gene therapies for ocular indications are not yet available due to lack of cell-type specific capsids to deliver the therapies. To address this unmet need in gene therapy, we have developed a novel methodology, SNAC (Single-Nuclei Atlas of Capsid Distribution), to screen and select AAV capsids targeting specific cell-type populations in NHP retinas by generating a single-cell resolution transduction profile of a library of 24 natural AAV capsids. First, we manufactured and injected the pooled GFP-encoding capsids into NHP eyes via intravitreal route of administration. From nuclei isolated from peripheral and central/macula regions, we generated and deeply sequenced transcriptomic and GFP barcode libraries. Using these sequences, we matched the transcriptomes of individual nuclei to the list of GFP barcodes contained in the injected library. Next, using CellBridge (a software package pipeline published by Sanofi) and data- and literature- derived marker gene sets from NHP and human retinal studies, we identified major cell types and quantified individual AAV capsid transduction profiles of specific cell populations. As a result, we detected all major retina cell types specifically, photoreceptors (rods and codes), bipolar cells, amacrine cells, horizontal cells, retinal ganglion cells (RGCs) and non-neuronal subtypes (Muller glia, microglia, astrocytes, endothelial cells, pericytes). We validated the internal consistency of the data by comparing the cell type proportions from different regions (peripheral compared to macula) to benchmark retinal cell type proportions from the literature. Finally, we quantified the percentage of AAV transduced cells by cell type, finding that AAV2, AAV6, and AAV1 are the top three capsids transducing RGCs, amacrine cells, horizontal cells, and Muller glia. This confirms previous observations that AAV2, the current AAV capsid of choice for ocular gene therapies in the clinic, transduces the eye at high levels. To validate these findings, we conducted bulk RNAseq of retinal samples from the same animals and found that the ranking of capsids detected by SNAC

agrees with that from bulk RNA sequencing of the entire tissue. In conclusion, we have developed SNAC, a method which allows screening barcoded AAV libraries to detect cell-type specific transduction in tissues. We demonstrated and validated this method on a library of 24 known natural AAV capsids in NHP retinas for ocular gene therapy indications. SNAC now can be applied for high-throughput single-cell resolution profiling of retina transductions by kilo-scale AAV libraries. In the future, this methodology can be applied to other tissues where precise cell-type specific targeting of AAV capsids is needed.

1451. Development of Mutant AAV-DJ Capsids with Better Transduction Efficiency for Local and Systemic Delivery

Ozgun Firat Duzenli[1], Zhang Lianwen[2], Kah-Whye Peng[2], George Aslanidi[1]

[1]The Hormel Institute, University of Minnesota, Austin, MN,[2]Mayo Clinic, Rochester, MN

The success of desired therapeutic benefit with AAV vectors requires the use of high vector doses which inevitably increase the risk of dose associated adverse events such as host immune response and liver toxicity. Therefore, it is critical to develop novel AAV vectors which are able to deliver therapeutic genome load at lower doses. To this end we produced several capsid-optimized AAV.DJ vectors with point mutation of critical amino acid residues to obtain novel vectors with improved transduction efficiency. These mutations included: N664V located in HI loop and involved in virus escape from the endosome; I648V and T710I reduce binding affinity for neutralizing antibodies; G506R and Y707C are glycan-binding residues which can reduce undesired liver accumulation while maintaining broad biodistribution; T493V, Y706F, and Y732F, which are proven to improve intracellular trafficking of AAV vectors. We also combined several single mutations to test the advantages of the synergetic effect of targeting multiple mechanisms of AAV life cycle and host interactions. These double mutants include T493V+N664V, G506R+Y707C, and Y706F+Y732F. The packaging plasmids encoding mutant capsids were generated by site-directed PCR by using the plasmid encoding wtAAV.DJ as a template. A single-stranded DNA cassette encoding firefly luciferase - yellow fluorescent fusion protein under the chicken β-actin promoter was packaged to each single and double mutant AAV.DJ and compared with wtAAV.DJ. The packaging efficiency of the most capsid-mutants was 2-3 times lower than wtAAV.DJ, while vectors harboring the mutations Y707C and G506R+Y707C were packaged with about 1-log lower titer. We further evaluated the transduction efficiency of the wt and mutant AAV.DJ vectors in HEK293 cells by using *in vitro* luciferase assay. AAV vectors were used at different MOI consisting of 2.000, 5.000, and 10.000 vg/cell. The bioluminescence signal was measured 48 hours post-infection. We observed that the mutants N664V, I648V, and Y732F showed 3-6 fold superior transduction compared to the wt AAV.DJ capsid, while infectivity of all double mutants was similar to wtAAV.DJ. To test the *in vivo* bioactivity of wt- and mutant AAV.DJ vectors, six- to ten-week-old male C57BL6 mice were intramuscularly injected with $1x10^9$ vg/mice with AAV vectors. Expression was monitored weekly with IVIS Lumina S5 Imaging System for two weeks. Consistent with *in vitro* results the *in vivo* luciferase assay showed that only AAV.DJ-N664V mutants have superior *in vivo* activity compared to wtAAV.DJ vector and all other capsid compositions displayed similar or lower transduction. We are currently testing capsid-modified AAV.DJ vector activity by non-invasive *in vivo* imaging technique with a sodium iodide symporter (NIS) from minke whale (*Balaenoptera acutorostrata scammoni*) for precise tracking of vectors spread and tissue transduction. Our results might lead to the development of superior AAV vectors for local and systemic administration by manipulating critical regions of viral capsids. **Acknowledgments:** This project is supported by FDA/CBER R01FD007483 funding.

1452. Evaluation of Cross-Species Expression Across Four Species and Cellular Tropism of VCAP-102, an Engineered Blood-Brain Barrier-Penetrating AAV Derived Capsid from TRACER Platform Screens

Hamza Khalid[1], Anupriya Kulkarni[1], Munachiso Egbuchulam[1], Alexandra Tsolias[1], Amy Bruce[1], Rong Zhao[1], Tyler Moyer[2], Nilesh Pande[1], Chanchal Sadhu[1], Mathieu E. Nonnenmacher[2]

[1]Translational Research, Voyager Therapeutics, Inc., Cambridge, MA,[2]Capsid Discovery, Voyager Therapeutics, Inc., Cambridge, MA

The blood-brain barrier (BBB) prevents therapies from engaging targets within the central nervous system (CNS) resulting in the use of invasive delivery methods of adeno-associated virus (AAV) vectors. The TRACER (Tropism Redirection of AAV by Cell-type-specific Expression of RNA) platform has enabled the generation of engineered AAV variants that have greater BBB crossing efficiency and liver detargeting compared to parental AAV capsids using intravenous (IV) administration. Previously, we have shown that VCAP-102 was superior to AAV9 in crossing the BBB after IV administration and had widespread CNS transduction and expression in Cynomolgus macaques. To evaluate the efficacy of VCAP-102 for preclinical studies in rodents and show potential translatability into humans, we performed VCAP-102 biodistribution and transgene expression studies after IV administration in mice, marmosets, African Green Monkeys (AGM), and Cynomolgus macaques. We observed higher biodistribution and widespread transgene expression of VCAP-102 in all the tested species as compared to AAV9. Further, using immunofluorescent staining, we confirmed that VCAP-102 mediated transgene expression in both neurons and astrocytes in the cortex of mice, AGM, and Cynomolgus macaques. Our data confirm that VCAP-102 is superior to AAV9 in crossing the BBB across multiple species after IV administration, thus suggesting that VCAP-102 can be used for preclinical studies and may be useful for CNS-targeting indications.

1453. GUAAVA: Generation of Ultra-Manufacturable Adeno-Associated Viruses by Artificial Intelligence

Jason Wu[1], Yumeng Cao[2], Yu Qiu[2], Tess Torregrosa[1], Margaret Hennessy[1], Michael Ryan[1], Mehmet Takar[1], Eugenia Lyashenko[1], Sourav R. Choudhury[1]

[1]Sanofi, Waltham, MA,[2]Sanofi, Cambridge, MA

Gene therapy is transforming human health by addressing rare diseases with single dose-administration of therapeutic adeno-

associated viral (AAV) vectors. However, the exorbitant financial cost of these treatments limits widespread adoption of gene therapies. Manufacturing cost is a major contributor to Cost of Goods Sold (COGS) for genomic medicines. Improving manufacturability of AAV capsids can lead to the reduction of COGS for AAV-based gene therapies, therefore allowing for increased patient accessibility to gene-therapy products. Taking advantage of recent advancements in artificial intelligence (AI) and falling costs of DNA synthesis, we applied machine learning (ML) methodologies to rationally design super-manufacturable AAV capsids. Here, we developed a generative AI framework, GUAAVA (Generation of Ultra-manufacturable Adeno-Associated Viruses by Artificial Intelligence), to derive high manufacturability AAV mutant sequences. First, we developed a state-of-the art ML model to predict the manufacturability of AAV capsid mutants based on the amino acid sequence of the capsid monomer, viral capsid protein 1 (VP1). This model is trained on capsid fitness data of AAV2 VP1 single point mutants and embeds sequences in the space of a massive protein language model. Then, using the predictive ML model as an oracle, we built a generative framework, GUAAVA, that focuses on exploring the sequence space around wild-type AAV2 sequence. Finally, we validated the GUAAVA model experimentally by comparing the manufacturability of the *in-silico* designed sequences to a set of randomly generated AAV2 mutants. To do this, we synthesized a pool of viral capsids encoding GUAAVA-generated sequences and randomly generated AAV2 mutants and measured relative production of each viral capsid by NGS in a pooled library. We found that the manufacturability of the best GUAAVA-generated capsid is more than 1000-fold that of parental AAV2 capsid. Furthermore, when we ranked individually packaged capsids by manufacturability, we found that GUAAVA-generated capsids accounted for the top 20 capsids. In addition, we show that the top individually packaged GUAAVA-generated capsids have x200 fold higher production than wild-type AAV2 in a titer assay. In summary, we have established GUAAVA, a generative AI-based platform and used it to improve the manufacturability of AAV capsids which can increase the economic viability of AAV gene therapies. This generative model framework is extensible; with proper training data, it can apply to other critical features of AAV gene therapies, such as immunogenicity or tissue tropism, bringing AAV-based genomic medicines to a wider patient population.

1454. High Throughput Production, Purification and Screening Pipelines for AAV Capsid Variants

Krithika Vaidyanathan[1], Adam Rosenbaum[1], Olga Allen[1], Natalia Sprofera[1], Simran Kunder[1], Anisha Nandy[1], Brittney Butler[1], Lexie Cui[2], Siqi Zhao[1], Sarah Boswell[1], James Kennon Smith[1], Sury Somanathan[1], Narendra Maheshri[1]

[1]Ginkgo Bioworks, Boston, MA,[2]Ginkgo Bioworks, Cambridge, MA

The Adeno-associated virus (AAV) field has advanced tremendously over the past 60 years and the virus has quickly become a preferred vehicle for in vivo gene therapies. Novel capsid development via engineering and high-throughput screening has demonstrated the value of isolating variants more highly specific to specific target indications and applications. Existing approaches range from shuffling of the entire capsid sequence to randomization of discrete, functional regions of the AAV's surface variable loops, producing millions of capsid sequences requiring downselection. Ginkgo Bioworks is building high-throughput, arrayed transfection and purification pipelines that allow for the construction of large libraries of pre-selected capsids for in vivo and in vitro applications.Here we developed and evaluated automated AAV triple transfection workflows at 100uL, 500uL, and 1mL plate scale formats to produce 100-1000 combinations of different AAVs with modified capsids and barcoded ITR payloads in an arrayed format. Cell lysates were pooled before being subjected to density gradient centrifugation, size-exclusion or affinity chromatography (AAVX) purification workflows. These barcoded AAV capsids variant libraries were then characterized via amplicon sequencing after purification as well as after administration in vivo to study biodistribution. Alternatively, cell lysates were processed in arrayed workflows where individual well lysates were not pooled and instead subjected individually to high throughput IMCS tip- or magnetic bead- based AAVX purification. These arrayed libraries were then deployed in high-throughput, singleplex, in vitro applications. Both pooled and arrayed approaches yielded sufficient vectors for downstream applications. The flexibility of using this dual approach allows the interrogation of a pooled library of constructs in vivo or evaluation of arrayed individual constructs using high throughput screening platforms in vitro to pre-select vectors with desired preclinical phenotypes.

1455. Nanobody-Mediated Retargeting of a Modified AAV Enhances Distinct and Highly Specific Transduction of Tumor Tissues

Olaniyi Olarewaju[1], Franziska Held[1], Pamela Curtis[2], Cynthia Kenny[2], Tadas Panavas[2], Udo Maier[1], Francois du Plessis[1]

[1]AAV Gene Therapy Group, Research Beyond Borders, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riß, Germany,[2]Biotherapeutics Discovery, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, CT

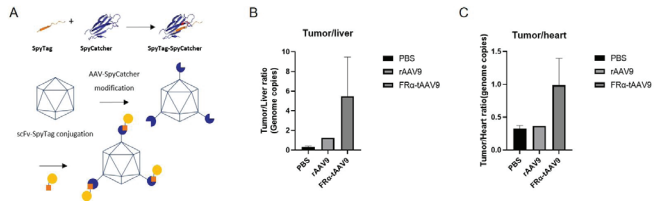
Background: Despite the recent clinical and commercial successes of adeno-associated virus (AAV)-based gene therapies, the platform is still poorly explored for targeted delivery of therapeutic agents to tumor cells, due to the refractiveness of tumor cells to AAV transduction. In this study, we harnessed a nanobody (VHH) in combination with AAVs by engineering a capsid-surface modified AAV decorated with a single domain antibody, VHH. The aim was to establish a proof-of-concept by targeting the fibroblast activating protein (FAP) which is a distinctly expressed cell surface receptor in tumor stroma and fibrotic tissues, but rarely expressed in normal tissues. Thus, creating a versatile AAV vector platform for targeting tumor cells. **Methods**: An α-FAP VHH (α-FAP-VHH) was displayed on the capsid surface of an AAV2 vector with the HSPG-binding motif ablated ($AAV2^{KO}$). To display approximately 1-5 copies of the VHHs per capsid, the VP1 start codon was mutated on the AAV2 Rep2/Cap2 plasmid ensuring only the production of Rep proteins, VP2 and VP3 capsid proteins respectively. The VP1 capsid protein was then supplied in *trans* on an additional plasmid with the nanobody integrated at the GH2/GH3 loop of the modified VP1. The modified $AAV2^{KO}$ displaying α-FAP-VHHs were packaged with the nanoluciferase (NLuc) transgene. The generated AAV was functionally validated for specific targeting of

human FAP receptor (huFAP) expressing cells *in vitro* in a human FAP receptor-expressing HT1080 fibrosarcoma cell line (HT1080-huFAP), as well as a HT1080-huFAP cell-derived xenograft (CDx) mouse model. **Results**: Our *in vitro* data shows that the AAV2^{KO}_α-FAP-VHH vector selectively transduces HT1080-huFAP cells very efficiently (23 to 80-fold) compared to the negative control non-huFAP expressing HT1080 cells. Moreover, the activity of AAV2^{KO}_α-FAP-VHH can be competitively inhibited by blocking the FAP-receptor on the HT1080-huFAP cells by an α-FAP antibody, indicating a specific and direct interaction of the vector with the FAP receptor via the integrated VHH. Following our *in* vitro experiments, the HT1080-huFAP cells were engrafted in NXG immunodeficient mice to generate a CDx huFAP expressing mouse tumor model. Transduction of the HT1080-huFAP CDx mice with AAV2^{KO}_α-FAP-VHH resulted in an enhanced transduction (>6-fold) of HT1080-huFAP tumors compared to wild-type (WT) AAV2, combined with a strong detargeting (=10-fold) of AAV2^{KO}_α-FAP-VHH from the liver compared to WT AAV2. To broaden the versatility of such a high-fidelity platform, which is hugely necessitated due to the limited nanobody repertoire, a high throughput yeast display library screen was performed to identify novel α-FAP nanobodies. We successfully identified and characterized several novel α-FAP nanobody candidates with binding affinities to FAP ranging between 0.1 nM to >100 nM. These new VHH binders will be used to further investigate the epitope and affinity influence for antibody based AAV targeting. **Conclusion**: This study offers a new opportunity for exploring the application of AAVs as a veritable vehicle for highly selective delivery of therapeutic agents to the tumor stroma. Furthermore, we utilized an adaptable high throughput yeast display library screening platform for the identification of novel single domain antibodies for the functionalization of AAV capsids. Beyond tumor targeting, this platform is applicable for selective delivery of AAVs to specific target cells or tissues of interest. This can help overcome the challenges posed by AAVs broad tropism, which often lead to the need for high vector dose administration to achieve therapeutic level of transgene expression in target tissues.

1456. The Development of Tumor Targeting AAV

Lei Zhao, Chuyuan Chen
Cure Genetics, SuZhou, China

Introduction: Cancer immunotherapy requires many modalities such as mono-clone antibody, oncolytic virus, immune agonist cytokine, chimeric antigen receptor immune cell, antibody-drug conjugate, tumor vaccine, etc. based on different MOAs (mechanism of action) and drug targets. The new modality, novel MOA and promising combination will give more solutions for clinical requirements. Cytokine armored oncolytic virus is one of the valuable therapy which designed both to lyse tumor cells by oncolytic virus and to regulate immune responses by secreting of cytokines. However, the high immunogenicity of oncolytic virus requires intratumoral (IT) administration and multiple dosing, somehow limited the application in clinical trials. Here, we developed a tumor targeted AAV vector (t-AAV) which delivered specific cytokine to solid tumor. This new modality can 1) use intravenous (IV) administration instead of IT; 2) secrete pro-inflammatory cytokine in tumor microenvironment (TME) to reduce the side effect from systemic administration of cytokine; 3) long-term treatment by single dose of AAV. This t-AAV is engineered from wtAAV which carried SpyCatcher modified VP1 in site of 453. This viral particle was then conjugated with SpyTag modified tumor cell targeting scFv (anti folate receptor alpha, FOLR1 or FRα) (Fig. A). This t-AAV (AAV9 based) infected human SKOV3 cell line (FRα+) with appx. 15-20% infection rate while rAAV9 only represented less than 5%. Biodistribution study also confirm that t-AAV can specific target solid tumor in compare with rAAV9. Results and Discussion: AAV9 and AAV6 are selected as basic AAV serotype for SpyCatcher engineering. The plasmid of VP1 has been isolated from Rep-Cap plasmid for protein engineering. The SpyCatcher003 (variant of SpyCatcher) was inserted in the site of 453 of VP1 with the replacement of 453-459. The titer after 4-plasmid AAV packaging and purification is comparable with triple-plasmid packaging of rAAV9. The viral vector was then conjugated with SpyTag003 (variant of SpyTag) modified anti-FRα scFv. The conjugation yield is very high and the ratio of VP1 and VP2 is 1:1 before and after conjugation. The transduction activity after conjugation of t-AAV is monitored by infection of SKOV3 cell line. The AAV9 based T-AAV has 15-20% infection in different MOIs activity while rAAV9 only has less than 5% infection. The in vivo biodistribution of t-AAV has been tested in mice. In compare with rAAV9, t-AAV represents strong solid tumor targeting effect while also showed liver and heart tropism in mice (Fig. B-C.). This result indiates the potential IV administration of t-AAV. The secretion of cytokine was studied in both SKOV3 and MC38 (both with FRα+) cell lines. Both cell lines represent very good IL-12 secretion with maximum 200-600 pg/mL. Conclusions: Tumor targeted AAV (t-AAV) based on AAV9 serotype represents high target cell selectivity in vitro and in vivo. The delivery and expression of cytokine with t-AAV has been also confirmed in vitro. It is a potential vector for cancer immune therapy.

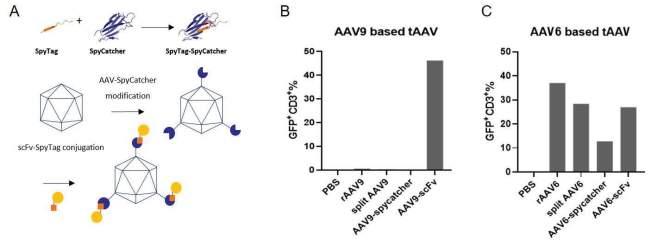


1457. The Development of T Cell Targeting AAV

Lei Zhao, Yongji Jiang
Cure Genetics, SuZhou, China

Introduction: Chimeric Antigen Receptor T-Cell (CAR-T) is one of most potent therapy in hematological cancer treatment. However, the disadvantage of the autologous cell therapy, non-off-the-shelf productivity and the potential risk of new cancers after treatment with CAR-T therapies limited its application. Although the allogeneic cell therapy offers advantages over the autologous counterpart, it still remains the new cancer risk from gene integration by transduction of CAR with lentivirus (LV) or retrovirus (RV). Nowadays, in vivo CAR-T, which directly dose the vector with CAR gene to patient, is developed as another option with allogeneic cell therapy. The vectors such as LV, adenovirus (AD), lipid nanoparticle (LNP), virus like particle (VLP) etc. had been developed for T cell targeting properties. Here, we developed a T cell targeting adeno-associated virus (T-AAV) vector for in vivo CAR-T therapy. The T-AAV is engineered from wtAAV which

carried SpyCatcher modified VP1 in site of 453. This viral particle was then conjugated with SpyTag modified T cell targeting scFv (Fig. A). This T-AAV (AAV9 based) infected human T cell in hPBMC with appx. 40-50% infection rate while rAAV9 only represented less than 1% (Fig. B-C). This T-AAV is potentially in vivo CAR-T vector without the risk of genome integration. It also generates the long-term gene expression in T cell compare with RNA technologies. Results and Discussion: AAV9 and AAV6 are selected as basic AAV serotype for SpyCatcher engineering. The plasmid of VP1 has been isolated from Rep-Cap plasmid for protein engineering. The SpyCatcher003 (variant of SpyCatcher) was inserted in the site of 453 of VP1 with the replacement of 453-459. The titer after 4-plasmid AAV packaging and purification is comparable with triple-plasmid packaging of rAAV9. The viral vector was then conjugated with SpyTag003 (variant of SpyTag) modified anti-CD3 scFv (OKT3). The conjugation yield is very high and the ratio of VP1 and VP2 is 1:1 before and after conjugation. The binding affinity of T-AAV and CD3 has been confirmed by ELISA, T-AAV represented appx. 5-10 folds increased affinity than recombinant scFv which correlated with number of scFv (VP1) in one T-AAV nano-particle. The transduction activity conjugation of T-AAV is monitored by infection of hPBMC. The infection of CD3+ T cell has been used for criteria of T-AAV activity. The AAV9 based T-AAV has 45% infection activity while rAAV9 only has less than 1% infection. However, AAV6 based T-AAV does not improve the T cell specificity in compare with rAAV6. Thus, AAV9 based T-AAV has been chosen as the vector for in vivo CAR-T therapy. The in vivo biodistribution of T-AAV has been tested in mice. In compare with rAAV9, T-AAV also showed liver tropism in mice but with appx. 10-fold lower infection activity. This result represents the potentially safety profile with T-AAV. Conclusions: T-AAV based on AAV9 serotype represents high T cell selectivity in vitro assay and reduced liver tropism than rAAV9 in mice. It is a potential vector for in vivo CAR-T or other T cell therapies.



1458. T-Lymphocyte Specific *In Vivo* Gene Delivery with CD8-Targeted AAV2 and AAV6

Muhammed Burak Demircan[1], Luca Zinser[1], Alex Michels[1], Mar Guaza-Lasheras[1], Fabian John[1], Samuel Arthur Theuerkauf[1], Dorothee Günther[1,2], Dirk Grimm[3], Petr Chlanda[4], Frederic B. Thalheimer[1], Christian J. Buchholz[1,5]

[1]Molecular Biotechnology and Gene Therapy, Paul-Ehrlich-Institut, Langen, Germany,[2]Molecular Biotechnology and Gene Therapy, Ernst Strüngmann Institute for Neuroscience in Cooperation with Max Planck Society, Frankfurt Am Main, Germany,[3]Heidelberg University Hospital, Heidelberg, Germany,[4]Department of Infectious Diseases/Virology, Medical Faculty, University of Heidelberg, BioQuant, Heidelberg, Germany,[5]Frankfurt Cancer Institute, Goethe University, Frankfurt am Main, Germany

A major challenge for *in vivo* gene therapy is the development of vectors capable of mediating highly selective gene transfer specifically into therapy-relevant cells. Among these, subtypes of T lymphocytes, particularly CD8+ T cells, represent a highly relevant target cell population not only in patients, but also in mice to address basic immunological research questions. Among various strategies, receptor-targeted approaches emerge as ideal for specifically targeting CD8+ T cells. Here we present DARPin-targeted AAVs (DART-AAVs), a second-generation receptor-targeted AAV, exhibiting improved DARPin display specific for human and murine CD8. The insertion of DARPins into the GH2-GH3 loop of viral protein 1 (VP1) in both AAV2 and AAV6 resulted in high selectivity for CD8-positive T cells, while maintaining robust gene delivery efficiency. Cryo-electron tomography enabled imaging of DARPins protruding from particle surfaces. Importantly, the capsid core structure was unaltered from that of the parent capsid core at a resolution of 3.3 Angstrom. In complex primary cell mixtures, i.e. in donor blood and in mice, CD8-targeted AAVs were by far superior to unmodified AAV2 and AAV6 in terms of selectivity, target cell viability and gene transfer rates. *In vivo*, up to 40% of activated CD8+ T cells were hit upon a single vector injection into humanized mice. By inserting a murine CD8-specific DARPin into the GH2-GH3 loop of the AAV2 VP1, we achieved >99% gene transfer selectivity on CD8+ murine splenocytes, however, the gene transfer rates were low under the non-activated conditions. Remarkably, after *in vivo* conditioning of mice with T cell-activating compounds (anti-CD3/IL2 complex), gene transfer rates dramatically increased, leading to up to 20% transduction of CD8+ T cells in murine blood with a single vector injection into immunocompetent mice. In all settings, selectivity for CD8+ T cells was close to absolute with exceptional detargeting from liver. These findings demonstrate that the capsid insertion of DARPins enables highly selective and efficient gene transfer into both mouse and human T lymphocytes. Consequently, the CD8-AAVs described in this study broaden the array of strategies available for immunological research and enhance the scope of *in vivo* gene therapy options.

1459. Mucin-Type *O*-Glycosylation in Viral Protein 2 Indirectly Affects the Transduction Efficiency of Recombinant Adeno-Associated Virus Serotype 6

Yuki Yamaguchi[1], Kentaro Ishii[1], Sachiko Koizumi[2,3], Hiroaki Sakaue[4], Takahiro Maruno[1,5], Mitsuko Fukuhara[1,5], Risa Shibuya[1], Yasuo Tsunaka[1], Aoba Matsushita[1], Karin Bandoh[1], Tetsuo Torisu[1], Chie Murata-Kishimoto[2], Azusa Tomioka[4], Saho Mizukado[4], Hiroyuki Kaji[6], Yuji Kashiwakura[7,8], Tsukasa Ohmori[7,8], Atsushi Kuno[4], Susumu Uchiyama[1]

[1]Graduate School of Engineering, Osaka University, Osaka, Japan,[2]GlycoTechnica Ltd., Yokohama, Japan,[3]Precision System Science Co. Ltd., Chiba, Japan,[4]Cellular and Molecular Biotechnology Research Institute, National Institute of Advanced Industrial Science and Technology, Ibaraki, Japan,[5]U-Medico Inc., Osaka, Japan,[6]Institute for Glyco-core Research (iGCORE), Nagoya University, Aichi, Japan,[7]Department of Biochemistry, Jichi Medical University School of Medicine, Tochigi, Japan,[8]Center for Gene Therapy Research, Jichi Medical University, Tochigi, Japan

Recombinant adeno-associated viruses (rAAVs) are promising vehicles for target genes in gene therapy. Glycosylation of biopharmaceuticals is known to influence on their safety and efficacy, and rAAV capsids may undergo glycosylation as post-trans modification during manufacturing process. Some previous studies reported glycosylation of rAAVs; however, the types of glycan that attach to rAAVs are still controversial. In this study, we extensively studied on rAAV serotype 6 (rAAV6) to explore whether rAAV is glycosylated, and if so, what types of glycans are actually attached to the capsid. For deep and precise characterization, we attempted to isolate the glycosylated rAAV6 particles. After testing more than 20 lectins, the fractionation of rAAV6 using *Agaricus bisporus* agglutinin (ABA), which is one of the *O*-glycan-recognizing lectins successfully captured the rAAV6 particles which accounted for only 1% of total particles. LC-MS/MS analysis for those fractions AAV6 was modified with two types of *O*-glycans: the mucin-type glycans, *O*-GalNAc (Tn-antigen) and mono- and di-sialylated Galβ1-3GalNAc (T-antigen) with at S156, T162, T194, and T201 in viral protein (VP)2, and *O*-GlcNAc at T242 in VP3. Interestingly, mucin-type glycans in VP2 were only observed in by ABA-bound particles, whereas *O*-GlcNAc in VP3 was only detected in ABA-unbound particles. Considering the reported three-dimensional structure of rAAV6, the *O*-GlcNAc at T242 in VP3 region is located at inner side of the capsid, whereas although the structure of VP1 and VP2 has not been elucidated, mucin-type *O*-glycan clusters at in VP2 should be located at the position which is accessible by ABA. Physicochemical and biological analyses were performed for the fractionated rAAV6 to reveal the impact of the mucin-type *O*-glycosylation on particle size distributions, VP ratio, in vivo and in vitro transduction efficiencies. Surprisingly, the mucin-type *O*-glycosylated rAAV6 particles encapsidated full-length genome and showed significantly lower VP1 ratio than rAAV6 without mucin-type *O*-glycans, resulting in lower transduction efficacy both in vitro and in vivo.In summary, rAAV6 contains 1% of ABA-bound particles, namely mucin-type *O*-glycosylated rAAV6 particles with lower VP1 ratio which resulted in reduced transduction efficiency than that of rAAV6 without mucin-type *O*-glycans, suggesting that mucin-type *O*-glycosylation of rAAV indirectly influence on transduction efficiency. This report provides us the conclusively proof that rAAV is *O*-glycosylated and in-depth insights of glycosylation for rAAV-based therapeutics.

1460. Computational Design and Validation of Extended Rigid Hairpins on the Surface of AAV

Samantha A. Yost[1], Sigmund Tejada[1], Elad Firnberg[1], Emre Firlar[2], Olivier F. Danos[1], Ye Liu[1], Andrew C. Mercer[1], Jason T. Kaelber[2]

[1]Research and Early Development, REGENXBIO, Inc., Rockville, MD,[2]CryoEM & Nanoimaging Facility, Institute for Quantitative Biomedicine, Rutgers, The State University of New Jersey, Piscataway, NJ

Adeno-associated virus (AAV) capsid variants with enhanced gene transfer utility are highly sought after. Flexible loops in variable region eight (VR-VIII) and more recently VR-IV have been used as targets for capsid engineering by peptide library insertion but appear to be limited to small peptides and may interfere with the native folding and function of capsid (VP) proteins. Larger proteins have been successfully incorporated with varying degrees of success by using very long, flexible linkers, fusion with the N-terminus of VP2, or as an additional covalently linked moiety; however, these methods can raise concerns about manufacturability, homogeneity, and batch-to-batch variability. Here we present computationally designed, rigid linkers in VR-VIII to create a stabilized hairpin that protrudes from the capsid surface. We hypothesize that these would function as an extended hinge, allowing for larger insertions without compromising VP folding and increasing ligand accessibility. Hairpin constructs were initially based on a tryptophan zipper motif or streptococcal protein G hairpin before applying hallucination methods to refine sequences within the context of AAV9. An additional mutation of Q590 to proline, a conserved residue found in several other serotypes in the adjacent position in structural alignments (e.g. AAV8 P593), was included in some constructs to increase stability. All six stable hairpin design constructs packaged AAV genome successfully by ddPCR evaluation with titers ranging from 38-105% of wild type AAV9. Structures were evaluated for three constructs via cryo-electron microscopy to confirm the predicted conformation and support further refinement. Previously described muscle targeting peptides incorporated at the end of a hairpin loop were tested in differentiated human myoblasts to verify transduction capabilities via reporter protein expression. Myoblasts transduced with AAV9 displaying a stable hairpin and the inserted peptide exhibited 300-fold higher fluorescence intensity from expressed tdTomato over cells treated with wild type AAV9. Insertion of larger targeting moieties such as 16kDa designed ankyrin repeat proteins (DARPins) with inclusion of a stabilized hairpin provided a 3-fold improvement in transduction of cells expressing the DARPin target over GS linker. We hypothesize that the utility of these hairpin linkers may extend to other AAVs, and further evaluation is ongoing.

1461. mRNA Enrichment Enables High-Throughput Directed Evolution for AAV Capsids

Sigmund K. S. Tejada, Sungyeon Cho, April R. Giles, Samantha A. Yost, Andrew C. Mercer, Xu Wang, Ye Liu, Elad Firnberg

REGENXBIO Inc., Rockville, MD

AAV capsid library studies ranging from pools of <100 individually barcoded capsids up to large libraries containing >10^8 variants can suffer from high data noise and variability due to poor sampling of barcoded transcripts measured with Next Generation Sequencing (NGS). Reliable NGS data from transduced tissue is dependent on measuring large numbers of transcripts to reduce library sampling biases. The NAVIGATE (Novel AAV Vector Intelligent Guided Adaptation Through Evolution) platform overcomes this potential problem by including an mRNA enrichment process using magnetic poly-dT oligo beads to capture mRNA from extracted total RNA or directly from the transduced tissue lysates without a separate RNA extraction step. This technology, when paired with the automatic high-throughput Apex purification system (ThermoFisher), and subsequent use of a one-step RT-PCR kit can accelerate the tissue-to-NGS processing to one day instead of several days. This methodology was optimized to be used in multiple non-human primate (NHP) tissues including retina, muscle, liver, and brain. The NAVIGATE platform was first employed to discover improved muscle capsids by harvesting tissues three weeks post-intravenous administration of AAV libraries in NHPs. During early platform development, traditional column

RNA extraction and RT-PCR was done on skeletal and cardiac tissues that resulted in no amplification of library transcripts; by comparison, mRNA enrichment after pooling the same tissue types provided robust amplification and sequencing of high library diversity providing highly enriched AAV variants on the target tissue. NAVIGATE was also used to identify ocular capsids that would improve retina transduction post-suprachoroidal space (SCS) administration. To determine on which template capsid to build a peptide insertion library and select for improved-retina transduction post-SCS delivery, a pool of 118 diverse capsids packaged with a barcoded CAG.eGFP transgene was administered to NHPs at a dose of 7.2E11 genome copies per eye via SCS administration. Retina was collected and total RNA was extracted and processed, resulting in poor PCR amplification of the library target sequence, low-quality NGS reads, and detection of only 73 out of 118 barcodes with an average standard deviation of 0.016. In contrast, performing mRNA enrichment provided strong amplification of library target sequence, high-quality NGS reads, and detection of 99 out of the 118 barcodes with an average standard deviation of 0.008. The mRNA-enriched dataset identified AAV3B and AAV8 as potent SCS capsids which could be used as backbones for further directed evolution. The mRNA enrichment workflow, and subsequent mRNA DIRECT workflow developed for high throughput processing, provides high-quality mRNA transcript recovery and robust library of NGS data for evaluating AAV library in various NHP tissues.

1462. Evaluating Antibody Binding Affinities to AAV by Charge Detection Mass Spectrometry

Lohra Mickelle Miller[1], Junping Zhang[2], Benjamin E. Draper[1], Weidong Xiao[2], Roland W. Herzog[3], Klaus-Ingmar Pfrepper[4], Martin Jarrold[1]

[1]Megadalton Solutions, Bloomington, IN,[2]Indiana University, Indianapolis, IN,[3]IU School of Medicine, Indianapolis, IN,[4]PROGEN, Heidelberg, Germany

Recombinant adeno-associated virus (rAAV) is a non-pathogenic virus that has shown great success as a gene therapy vector. However, the interaction of the viral vector with host antibodies can result in neutralization and reduced transduction efficiency. The techniques that can be used to characterize interactions between rAAV and antibodies are ensemble methods that are limited in their ability to determine stoichiometry. Charge detection mass spectrometry (CD-MS) is a single particle technique that is routinely used to resolve and quantify empty, partial, and full AAV particles. Here, CD-MS is used to determine the stoichiometry of ligand binding, using AAV8 capsids as a test system with murine monoclonal antibody ADK8. Both the intact antibody (mAb) and fragment antibodies (Fabs) were investigated. With intact mAbs, the number of bound antibodies increased with increasing mAb concentration but did not reach saturation. Instead, the capsids agglutinated forming dimer, trimer and tetramer complexes that were directly observed by CD-MS. Fc depleted $F(ab')_2$ fragment antibodies also did not saturate and showed increased agglutination compared to the intact mAbs. On the other hand, increasing Fab concentration led to saturation at around 60 Fabs bound per AAV8 capsid, confirming expectations that there are 60 sites available for Fab binding. For all ligands the CD-MS mass measurement allowed for the distribution of bound species to be quantified as a function of initial ligand concentration. Binding curves were determined for Fab binding to AAV8. The results showed strong positive cooperativity, where the binding affinity increased substantially with Fab coverage. While this work focuses on AAV8 and ADK8, the method is robust and can be used to characterize the binding of any ligand to any serotype of interest.

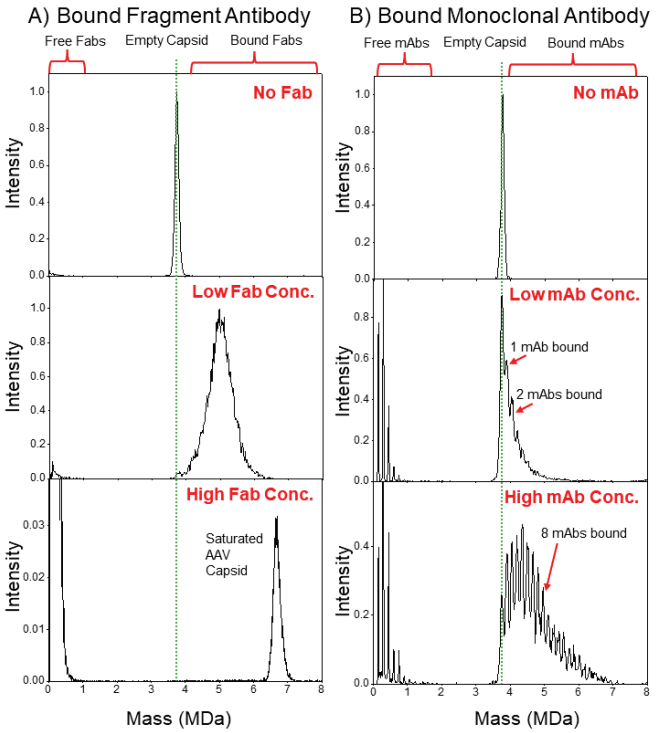


Figure 1. AAV8 empty capsid bound to fragment antibodies until saturation. B) AAV8 empty capsid bound to monoclonal antibodies at two mAb concentrations.

1463. Characterization of Adeno-Associated Virus Capsids by a Novel Capillary Isoelectric Focusing-Based Fractionation Method and Charge-Detection Mass Spectrometry

Jiaqi Wu[1], Benjamin E. Draper[2], Chris Heger[1]

[1]Bio-Techne, San Jose, CA,[2]Megadalton Solutions, Bloomington, IN

Adeno-associated viruses (AAVs) are small, non-pathogenic, ssDNA packaging viruses that have become widely used for gene therapy [1]. AAVs are comprised of three capsid proteins (VP1, VP2, and VP3) that form a 60-mer capsid in an approximately 50:5:5 VP3:VP2:VP1 ratio [2]. In addition to possessing different molecular weights, these proteins exhibit charge heterogeneity from both sequence differences and post-translational modifications [3]. Charge heterogeneity is also conferred to the intact capsid. The charge heterogeneity of the intact capsid is influenced by ssDNA packaged in the particles [4], random incorporation of VP subunits [5], and post-translational modifications of the capsid proteins, for example, deamidation of VPs [3]. Imaged capillary isoelectric focusing (icIEF) [6] is a powerful tool for protein charge heterogeneity characterization. We have reported that AAVs exhibit charge heterogeneity that can be observed using imaged cIEF (icIEF) [7,8]. A new CE instrument was recently released (MauriceFlex™, Bio-Techne) that can fractionate protein charge variants

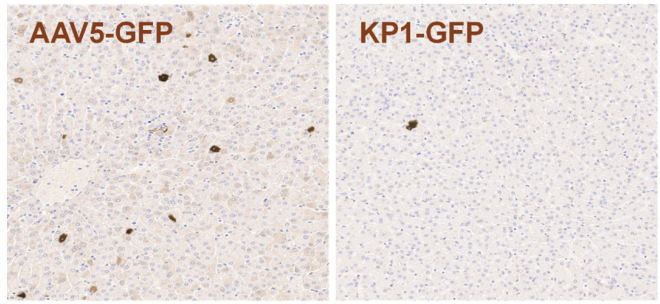
for downstream analysis and is expected to provide a clearer insight into these charge variants. Here we show for the first time, a seamless workflow to fractionate intact AAV capsids with comprehensive downstream analysis using both capillary electrophoresis and charge detection mass spectrometry (CD-MS). CD-MS measures the mass of intact AAV particles to characterize capsid protein average viral protein ratio and DNA packaging heterogeneity. [9,10] Through this analysis, we demonstrate that not only do we fractionate intact particles, but we can isolate subpopulations based on various features of the virus including capsid ratio, content status, and post-translational modifications. [1] Naso et al., BioDrugs. 2017 Aug;31(4):317-334. [2] Xie et al., Proc Natl Acad Sci U S A. 2002 Aug 6;99(16):10405-10. [3] Giles et al., Molecular Therapy, 26, 2018, 2848-2862. [4] Heldt et al., Langmuir. 2023 Apr 25;39(16):5641-5648. [5] Wörner et al., Nat Commun. 2021 Mar 12;12(1):1642. [6] Wu et al., TrAC. 2022 150, 116567. [7] Wu and Heger, Green Analytical Chemistry 2022 Volume 3, 100027. [8] Ramirez et al., Electrophoresis 2023;44:1258-1266. [9] Pierson et al. Anal. Chem., 2016, 88, 6718-6725. [10] Werle et al. Mol. Ther. Meth & Clin. Dev. 2021, 23, 254-262

1464. AAV KP1 Efficiently Transduces Human Cell Lines *In Vitro* and Mouse Liver but Not Non-Human Primate Liver After Intravenous Injection

Zhong-Dong Shi, Hongbin Xu

Frontera Therapeutics, Bedford, MA

To date, seven AAV gene therapy drugs have been approved including Glybera (2012), Luxturna (2017), Zolgensma (2019), Upstaza (2022), Roctavian (2022), Hemgenix (2022), and ELEVIDYS (2023). Despite the remarkable achievements, limited tissue transduction has prevented AAV from broader application expansion. To realize the full potential of AAV vectors, a lot of work has been focused on improving cell transduction by modifying the capsids or screening for novel capsids. However, oftentimes novel capsid variants screened from in vitro cell models or mouse models cannot be translated to human patients. NHPs are the closest animal models to humans regarding genetics, physiology, and behavior, and have been frequently used to evaluate novel capsids in recent years. The goal of this study was to evaluate whether a novel AAV capsid, KP1, is better than AAV5 for liver targeting. KP1 capsid was identified by directed evolution in human pancreatic islets in vitro and has been shown to transduce both mouse and human hepatocytes very efficiently in mice with humanized livers (Pekrun et al., 2019). To evaluate whether KP1 is a better liver-targeting capsid than AAV5, we compared transduction efficiencies of the bac/Sf9 system-produced AAV KP1 and AAV5 in cultured cells, in mice and NHPs (African green monkeys). For in vitro cell and NHP studies, the transgene for both KP1 and AAV5 is CBA-GFP (PA091, scAAV); for mouse study, the transgene is HLP-FVIII-SQ (PA103, ssAAV). In vitro, at MOI = 1E4, the KP1-GFP vector transduced 94.6% of 293T cells while AAV5-GFP transduced 9.02% of 293T cells; when transduced a human hepatoma cell line Huh-7 at MOI = 1E4, the transduction efficiencies were 80.3% and 0.26% for KP1 and AAV5, respectively. In wild-type mice, ~150% of human FVIII-SQ antigen was detected at 4 weeks post-injection of 5E12 vg/kg of KP1-PA103, to get a similar level of human FVIII-SQ antigen expression from AAV5-PA103, the dose had to be at 6E13 vg/kg, while 2E13 vg/kg of AAV5-PA103 only produced ~50% of FVIII-SQ. The data suggest that KP1 is much more potent than AAV5 when targeting mouse liver. Lastly, we compared KP1 and AAV5 in NHPs by systemically injecting 4E12 vg/kg of two AAV-GFP vectors in African green monkeys and collected animal tissues 4 weeks after injection. The bioassay data showed that NHPs injected with the KP1-GFP vector had much lower GFP expression in the liver based on IHC staining compared to animals injected with the AAV5-GFP vector. Vector genome quantification showed that AAV5 and KP1 have ~4.43E6 and ~3.06E5 copies/ug DNA in the liver, respectively. GFP expression levels in the liver correlated well with the vector genome data. In summary, KP1 capsid transduces exceptionally well in both human cell lines in vitro and mice liver. However, it shows very poor liver targeting in NHPs (African green monkey) compared to AAV5 capsid when injected intravenously. Our observation is consistent with other reported studies in rhesus and cynomolgus monkeys (Pekrun et al., 2022; Catalyst Biosciences, 2020). The underlying cause of low transduction in NHP liver remains to be understood and whether KP1 can efficiently transduce human hepatocytes in human patients remains unknown.



Representative GFP IHC staining shows significantly higher and broader GFP protein expression in the African green monkey liver injected with AAV5-GFP than KP1-GFP. Brown staining and blue staining are GFP protein and nucleus, respectively.

1465. Expanding the Serotype Frontier: Design of Synthetic AAV Capsids with Artificial Intelligence

Sam Sinai, David Brookes, Jeffrey Chan, Alex Mijalis, Alex Kern, Neha Prasad, Theodore Sternlieb, Farhan Damani, Kathy S. Lin, Jamie Kwasnieski, Adrian Veres, Eric Kelsic

Dyno Therapeutics, Watertown, MA

As a substantial percentage of human sera neutralize naturally circulating Adeno-associated viruses (AAVs), patients can find themselves ineligible to receive life-saving gene therapies due to previous capsid exposure. One conceptual approach to remedy these concerns is to leverage natural sequence diversity to reduce the likelihood of recognition by the immune system. However, the number of naturally occuring AAV capsids is limited and the process of discovering and characterizing new natural serotypes with clinically relevant properties is slow. In contrast, there are more possible AAV sequences than atoms in the observable universe. Hence, novel synthetic serotypes can provide an evergreen source of diversity that may address the critical concern of pre-existing immunity to gene delivery vectors. The catalog of known serotypes is a survey of

naturally occurring Dependoparvoviruses among host species, derived from sequencing human and vertebrate viromes. These canonical AAV serotypes (between 6 to 345 mutations apart) have seeded directed evolution campaigns where recombinant capsids with a low number of new mutations are optimized for better clinical properties, without drastic changes to the original sequence. However, new source serotypes with relevant in vivo delivery properties are now less frequently being discovered-in other words, for a decade or more the serotype frontier has been mostly stable. The advancement of machine learning and artificial intelligence (AI) in recent years opens a new route to expanding the AAV serotype repertoire. Key developments, such as highly-accurate protein structure prediction, structure to sequence methods, and data-driven generative models of function, have made it possible to design increasingly complex proteins while maintaining control over their structure and function. However AAV capsids are multi-domain proteins that perform several functions, which are far more challenging to engineer than the protein design tasks these AI methods have been validated on (typically single-domain, single-function, and under 250 amino acids). Here we show that it is possible to design families of capsids that are far away in sequence space from the closest serotype without getting closer to any other known serotypes. That is, by applying and adapting the AI techniques (mentioned above) in tandem, we generate a type of diversity that is impossible to create by natural recombination or DNA reshuffling. We show that these methods are capable of introducing many novel mutations in highly-conserved and easily destabilized regions. For instance, with a single experiment we successfully introduce 45 edits to a largely buried and mutation-sensitive segment of AAV9, changing more than half of the residues of an integral architectural part of the VP monomer. We also generate 4 distinct families of capsids with mutations in different structural segments, ranging between 37- 50 mutations to the AAV9 backbone. Testing high-edit distance variants for in-vitro transduction efficiency on HEK293 cells, we also observe up to 60-fold improvements over AAV9. We prove that the combination of current cutting-edge AI tools can push the frontier of sequence diversification beyond available natural diversity while maintaining or even improving upon productivity and in-vitro transduction efficiency. Our results anticipate a fertile ground for development of novel serotypes, far beyond those found by natural or in-lab evolution.

1466. AAV(Lung)s: A Class of Lung Tropism AAV Capsids with Improved Specificity and Transduction Efficiency

Bing Wang, Cheng Xu, Yu Liu, Yuyan Wang, Leming Wang, Zhen Shi, Daozhan Yu, Qizhao Wang
AAVnerGene Inc., Rockville, MD

Developing a lung-specific AAV (Adeno-Associated Virus) capsid is a significant focus in gene therapy, particularly for treating lung-related diseases such as cystic fibrosis, chronic obstructive pulmonary disease, and genetic disorders affecting the lungs. Different AAV serotypes (AAV2, AAV5, AAV9) and engineering variants (AAV6.2FF, AAV2H22, AAV2.5T and AAV9.452sub.LUNG1) have been reported to transduce in lung cells, organoids, and tissues with different animal models. With our ATHENA I platform, we confirmed the AAV9 performs better than other AAV variants at RNA level in the lung of C57B6 mice after systematically injection. However, AAV9 demonstrates significant transduction in the heart, kidney, brain, and liver tissues as well, indicating that it is not a lung-specific capsid. Interestingly, while AAV9 exhibits high expression in the lung, it simultaneously shows a low DNA copy number in the same tissue. Following the results from the ATHENA I evaluation, we systematically designed a series of AAV variants, which we have named AAV(lung)s. Our preliminary findings consistently show that certain AAV(lung)s achieve an approximately 500-fold increase in DNA accumulation in the lung compared to AAV9. AAV(lung)s demonstrated similar RNA levels compared with AAV9, but has 10-100 folds higher than AAV2.5T and AAV2H22. Significantly, AAV(lung)s exhibit high RNA expression exclusively in lung tissues, with negligible expression in other organs. Additionally, the DNA levels of AAV(lung)s are markedly lower in the brain, heart, and kidneys, compared with AAV9. Overall, we have developed a series of AAV capsids specific to mouse lungs, which will be beneficial for future lung gene therapy applications. Further research will focus on evaluating the efficiency and specificity of these findings in non-human primates.

1467. Exploring the Inner Capsid of the Parvoviridae Family with Multiscale Computational Techniques

Heather A. Noriega[1], Bing Wang[2], Qizhao Wang[2], Daozhan Yu[2], Xiang Simon Wang[1]
[1]Artificial Intelligence and Drug Discovery Core Laboratory for District of Columbia Center for AIDS Research (DC CFAR), Department of Pharmaceutical Sciences, College of Pharmacy, Howard University, Washington, DC,[2]AAVnerGene Inc., Rockville, MD

The Parvoviridae family, particularly adeno-associated viruses (AAVs), plays a significant role in the field of gene therapy and gene delivery. Parvoviridae viruses are characterized by their small size and structurally complex capsids. These capsids are made up of protein subunits that form a tight, geometrically precise arrangement. Understanding this structure at a detailed level is crucial for insights into how these viruses infect cells and how they can be adapted for gene delivery. The external surface is responsible for the initial interaction with the host cell and has recently received much attention in structure research. However, little is known about the inner capsid structures. The inner capsid structure is vital for several functions, including the stability of the virus, its ability to infect host cells, and the packaging of its genetic material. This investigation utilizes AlphaFold2, a deep-learning-based program to build accurate 3-D structures of Parvoviridae viral proteins including both capsids and the inner region. With pLDDT (≥70), pTM (≥0.7), PAE, and RMSD (<1) scores, our structures were found to be sound and accurate. The capsids were assembled using a fit-to-map method that resulted in high scores of ≥0.7 and subjected through a wide range of algorithms to analyze the structural-based (i.e., secondary structures, SASA, etc.), sequence-based (i.e. composition, transition, distribution, etc.), and physiochemical properties (hydrophobicity, polarity, charge, etc.). With them, we were able to generate over 2000 protein descriptors. These data, with additional inputs from Cryo-EM studies (VIPER database) and X-ray structures (Protein Data Bank, PDB), were used to create a machine learning model in which the Random Forest algorithm was applied with 10-fold cross-validation. We succeeded to predict the package capacity of Parvoviridae viruses and stoichiometric assemblies with a high F1 score of 0.923. Further

usage and experimental validation are underway. The outcome of this research would be critical in overcoming the package limitation of Parvoviridae viruses and thus can offer significant contributions to therapeutic gene delivery.

1468. Deployment of a Large Language Models to Design an AAV Capsid Targeting HER2

Chenrui Xu[1], Zhehao Xiong[2], Zhaoren He[1], Hui Li[2], Liang Gao[1], Zhongshan Cai[3], Li Zhao[3], Qingzhou Ji[3], Ziyao Xu[1], Vicky Qu[1], Le Song[1], Per Greisen[4], Fernando Garces[4]

[1]BioMap, Beijing, China,[2]BioMap, Suzhou, China,[3]Porton Advanced Solution Ltd., Suzhou, China,[4]BioMap, Palo Alto, CA

Introduction: With the advent of AlphaFold2, machine learning (ML)-based protein design gained in popularity. Much of that is been tested in the antibody space ranging from the de novo discovery of new binders to developability. However, these artificial intelligence (AI) task models can also be trained to successfully design sequences for other AI protein modalities including the adeno-associated virus (AAV). AAVs are small and non-enveloped virus that has the capability to deliver DNA to specific cells through protein engineering, indicating it's potential applications in gene therapy. For example, AAV2 has been authorized to treat Retinal Dystrophies caused by gene deficiency. Although there are examples of AAV design/engineering using classical directed evolution methods, such approaches are often time and resource consuming as it often relies on trial and error. Therefore, ML methods can reduce both cost and project timelines markedly, while predicting success. However, most ML-guided methods rely on prior training with experimental data, which can limit this effort altogether. **Methods:** Here we introduce a novel AAV capsid design diagram, which leverages the power of BioMap's cutting-edge foundation AI models and performs highly efficient design in a zero-shot manner. To demonstrate this method, we chose the case of the insertion of a binding motif that recognizes the tumor associate antigen (TAA) HER2. BioMap's proprietary large foundation model xTrimoPGLM, pre-trained on 1.6 billion protein sequences, ensures that AI designed AAV-HER2 capsids retain desirable characteristics including folding and expression similar to native AAV capsid. The whole design steps are finished within BioMap's multi-objective optimization (MOO) platform, which executes task models and performs iterative optimization. The AI designed library (9×10^4) was then tested for viral viability, binding and tropism using a cohort of mice bearing HER2 expressing tumor. **Results:** Initial NGS results showed that AI designed variants displayed higher viability rate when compared with those from random mutagenesis (control). Moreover, in vitro pull-down and the in-vivo experiments revealed that top variants demonstrate suitable HER2 binding and HER2-positive tumor tropism. **Conclusions:** Altogether, BioMap's AI approach achieved faster timelines and higher hit rate, suggesting a potential approach for inserting a variety of TAAs, but not limited to, into AAV capsids.

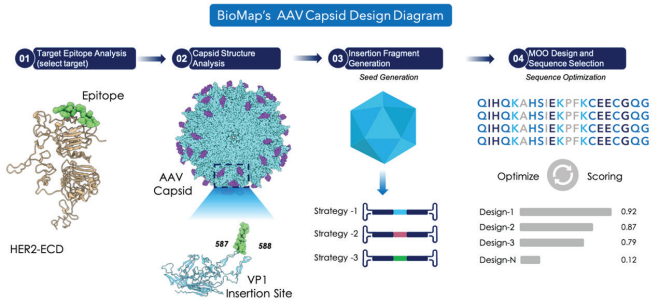


Figure1.Design Diagram

1469. Enhancing AAV Viability Prediction: A Generalizable Fine-Tuning Framework with Large Language Model xTrimoPGLM

Qirong Yang[1], Diming Zou[1], Yucheng Guo[1], Chenrui Xu[1], Damien Marsic[2], Zhongshan Cai[2], Yawen Liu[2], Ziyao Xu[1], Vicky Qu[1], Fernando Garces[3], Per Greisen[3], Qingzhou Ji[2], Le Song[1]

[1]BioMap, Beijing, China,[2]Porton Advanced Solution Ltd., Suzhou, China,[3]BioMap, Palo Alto, CA

Introduction: Machine learning (ML) techniques are revolutionizing our capacity to develop and refine proteins with enhanced and desired traits and functionalities. However, the majority of ML techniques rely on prior model training, using extensive experimental data, thus potentially limiting their overall application. Protein large language models (LLM) have emerged as powerful tools for extracting biological insights from protein sequences. Here, we developed and fine-tuned a framework based on a large-scale pre-trained language model, xTrimoPGLM (xTrimo Protein General Language Model). This framework exhibits robust transfer and generalization capabilities across various protein attributes. **Methods:** We have constructed an integrated workflow that adapted the xTrimoPGLM parameters using Low-Rank Adaptation (LoRA), and incorporated a multi-objective fusion network head for diverse predictive tasks. This workflow strategically balances the learning strengths of the model with its ability to generalize across different protein attributes. **Results:** In this work, we have successfully developed a new task model, the xTrimoAAV, and finetuned it with a folding stability dataset that enables the successful prediction of the adeno-associated virus (AAV) viability. Indeed, the model demonstrated a significant correlation 0.53 across various AAV serotypes (AAV5/AVV9) and mutation range. Moreover, by further finetuning it with known AAV experimental labelled data, the xTrimoAAV model's predictive correlation on an independent mutation dataset was enhanced to a value of 0.73. **Conclusions:** Here, the xTrimoAAV model has demonstrated outstanding performance in predicting the AAV viability. Therefore, the generalizability across different attributes supports the deployment of LLMs for the design of the next generation therapeutics such as the AAV. **Figure 1.** Pearson correlations on different task levels of Porton experimental datasets. See Table 1 for the complete set of correlations.

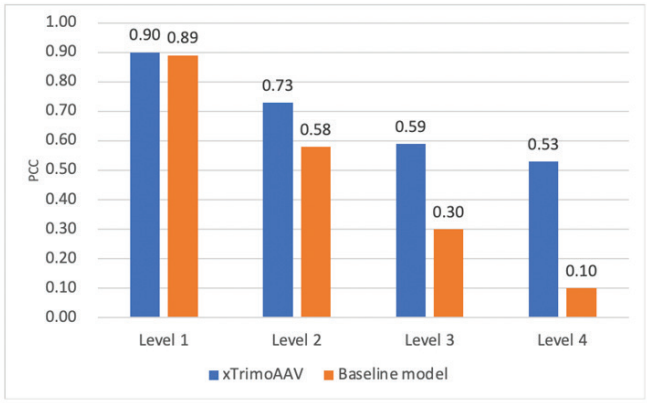


Table1. The performance of xTrimoAAV and Baseline Model on different task levels of Porton experimental datasets. **Task level**: classifications based on the difficulty level of tasks, the higher the level, the more difficult the task. **Task info**: description of task level. **Mutation info**: data mutation details e.g. AAV5 376-378 means using serotype AA5 with mutation range from 376 to 378. **PCC:** Pearson Correlation Coefficient. **Baseline Model**: xTrimoPGLM + linear regression head.

Task level	Task info	Mutation info (Train set)	Mutation info (Test set)	xTrimoAAV PCC	Baseline model PCC
Level 1	seen serotype, seen mutation region	AAV5 253-257,318-319,**376-378**,423-458,442-445,518,532-543,565-600,572-584,648-657,694-706 AAV9 **492-495**,554-557,706-718	AAV5 376-378	0.90	0.90
			AAV9 492-495	0.89	0.88
Level 2	seen serotype, unseen mutation region	AAV5 253-257,318-319,423-458,442-445,518,532-543,565-600,572-584,648-657,694-706 AAV9 492-495,554-557,706-718	AAV5 376-378	0.73	0.55
		AAV5 253-257,318-319,376-378,423-458,442-445,518,532-543,572-584,648-657,694-706 AAV9 492-495,554-557,706-718	AAV5 565-600	0.73	0.60
Level 3	unseen serotype	AAV5 253-257,318-319,376-378,423-458,442-445,518,532-543,565-600,572-584,648-657,694-706	AAV9 706-718	0.44	0.30
			AAV9 492-495	0.74	0.30
Level 4	unseen AAV data	folding stability data without any AAV	AAV5 376~378	0.54	0.10
			AAV9 492~495	0.52	0.09

Friday Posters: AAV Vectors - Preclinical and Proof-of-Concept In Vivo Studies

1470. Screening of Novel Liver-Directed Enhancers Using Pooled, Barcoded AAVs

Aditya Saxena, Yahui Lan, Perry Evans, Laura Pereira, Sean M. Armour, Daniel M. Cohen

Spark Therapeutics, Philadelphia, PA

Efficient, predictable, and safe AAV-based gene therapy relies on the delivery of expression cassettes to on-target cells through the combination of capsid tropism and promoter/enhancer cell-type specificity. Here we describe a pooled AAV approach for *in vivo* screening of cis-regulatory elements encoding hepatocyte-specific gene expression. Enhancer sequences were paired with a short hepatocyte-specific promoter with or without the inclusion of 5' UTRs. We tested different barcoding strategies and found that a 12 nucleotide random barcode can reproducibly report on promoter activity measured by next-generation RNA sequencing. Additionally, we performed a dose escalation study to understand the relationship between AAV dosage, promoter strength, as well as UMI recovery from total RNA. Based on RNA/DNA ratio, we observed enhancer-promoter pairs varying in strength by ~1000 fold, with high reproducibility between biological replicates and doses (Pearson's correlation >= 0.9). Interestingly, we identified several short liver-specific enhancers with comparable potency to ApoE-hAAT. Validation of single enhancer candidates is currently underway. Our results demonstrate the success of pooled AAV screening for selection of liver-directed cis-regulatory elements and will help enable the identification of novel, compact transcriptional units with improved potential for gene therapy, especially when paired with large payloads.

1471. A Rapid Low-Cost Method for Producing Adeno-Associated Viral Vectors Suitable for Transducing Rodent Neurons *In Vitro* and *In Vivo*

Doug B. Howard[1], Reinis Svarcbahs[2], Lana N. Gore[1], Brandon K. Harvey[2], Christopher T. Richie[1]

[1]Genetic Engineering and Viral Vector Core, National Institute on Drug Abuse, Baltimore, MD,[2]National Institute on Drug Abuse, Baltimore, MD

The use of adeno-associated viral (AAV) vectors for delivery of genetic information into the mammalian CNS remains popular, but producing in vivo-compatible vectors requires significant upfront investment (e.g., time, effort, and equipment). To address this need, we have developed a modified protocol for AAV production that does not require large capital equipment (ultracentrifugation or chromatography machines) and still produces AAV in quantities that are sufficient for testing prototypes in the rodent brain. The protocol uses a transfection-based packaging method followed by centrifugal filtration and concentration to produce a crude preparation of AAV. This "small scale" AAV production protocol has been used to successfully generate several AAV serotypes including AAV1, AAV9, AAV-DJ, and rAAV2-retro indicating the process is serotype-independent. These vectors also transduce primary rat cortical neurons in vitro. Intrastriatal injection of AAV1 or AAV9 serotyped vectors expressing EF1a-GFP-KASH into Long Evans rats demonstrated that our small scale AAV preparations produce transgene expression and inflammation patterns that are similar to those derived from injections with identical AAV vectors purified by affinity column chromatography. Overall, we describe a small-scale AAV packaging protocol that allows for an efficient means of producing multiple vectors in parallel and makes it possible to test multiple AAV prototypes (e.g., variant guide RNAs for CRISPR) simultaneously with reduced investment of resources.

1472. Development of an AAV9-Based Gene Therapy for Dermatosparaxis Ehlers-Danlos Syndrome

Abby W. McElroy[1], Jillian Gallagher[2], Toloo Taghian[2], Erin F. Hall[3], Hector R. Benatti[1], Elizabeth Parsley[4], Laura Dupont[5], Miguel Sena-Esteves[2], Alain C. M. A. Colige[5], Heather L. Gray-Edwards[2]

[1]Horae Gene Therapy Center, UMASS Chan Medical School, Worcester, MA,[2]UMass Chan Medical School, Worcester, MA,[3]UMASS Chan Medical School, Worcester, MA,[4]Cummings School of Veterinary Medicine at Tufts University, North Grafton, MA,[5]Laboratory of Connective Tissues Biology, University of Liège, Liège, Belgium

Ehlers-Danlos syndrome (dEDS) is an autosomal recessive connective tissue disorder occurring in humans, cattle, sheep, dogs, and cats. Patients present with severe skin fragility and redundant skin, spontaneous bleeding and bruising, and visceral fragility with hemorrhage. To date, there is no definitive treatment any type of EDS. This disorder is caused by a loss of function mutation in ADAMTS2 leading to impaired extracellular cleavage of the N-terminal-propeptide of type I procollagen by the procollagen N-proteinase enzyme. This results in collagen fibrils with aberrant morphology and lower tensile strength. This form of EDS is amendable to AAV gene therapy, because 1) it is caused by a loss of function of a secreted protein (ADAMTS2), 2) it is encoded by a 3.6kb cDNA, and 3) since ADAMTS2 is expressed ubiquitously throughout the body and the procollagen targeted by ADAMTS2 does not have any known function, we hypothesized that global secretion of ADAMTS2 will be efficacious in treating this form of EDS. Since ADAMTS2 functions extracellularly, our strategy is liver and skeletal muscle directed AAV gene transfer and systemic secretion of ADAMTS2 to affected tissues. Four AAV9 vectors were designed and packaged: 1) encodes for a wildtype signal peptide and codon optimized ADAMTS2 cDNA 2) encodes for an enhanced signal peptide (sp7Δ8) and a codon optimized ADAMTS2 with an HA tag at the c-terminus of the cDNA 3) a codon optimized ADAMTS2 cDNA with an HA tag located at the C-terminus of the cDNA or 4) in the middle of the cDNA. Vectors were first tested in wild type mice for expression and/or toxicity (n=4 WT 6-week-old C57BL/6 mice per group). Mice were treated via intravenous tail vein injection at a dose of 1E12 vg/mouse and were sacrificed 1-month later. No adverse clinical outcomes were observed. Tissues appeared grossly normal at necropsy. Histopathology of skin, aorta, quadriceps femoris, lung, liver, and kidney were normal. Transmission electron microscopy of the skin and aorta were unremarkable. Western blot demonstrated increased expression of an active cleavage form of ADAMTS2 plus the HA tag at approximately 75 kDa for the vector utilizing the wildtype signal peptide and with the HA tag on the C-terminus of the cDNA. Other bands were observed at approximately 45 kDa and 20 kDa, representing other cleavage products of ADAMTS2. Vector biodistribution was performed in liver, kidney, skeletal muscle, heart, aorta, and skin. A knockout ADAMTS2 mouse model was used for further therapeutic development. Two cohorts of mice were injected by intravenous tail vein injection at a dose of 1E12 vg/mouse at 8-weeks of age with three vectors containing an HA tag in the middle of the cDNA and a wild type signal peptide (n=9), an HA tag at the C-terminus of the cDNA and an enhanced signal peptide for increased secretion from liver and skeletal muscle (n=8), and a wild type signal peptide and no HA tag (n=8). Wild type untreated controls (n=7) and untreated ADAMTS_KO mice (n=4) were also evaluated. Mice were sacrificed at either 8 months or 6 months post injection. Post-mortem assessments are underway and include electron microscopy for the pathognomonic hieroglyphic collagen, histology, vector biodistribution and western blotting. In conclusion, this study outlines the development of a liver and skeletal muscle directed AAV gene therapy as a treatment for dEDS.

1473. Evaluating Tissue Specific Promoters Using Adeno-Associated Virus Based Gene Therapy

Nicole Zielinska[1], Yanlong Pei[1], Erin L. Howard[1], Brenna A. Y. Stevens[1], Melanie M. Goens[1], Elena S. B. Campbell[1], Madison E. Hughes[1], Dinghai Zheng[2], Benjamin Gordon[2], Alec Nielsen[2], Jeff Caswell[1], Luis G. Arroyo[3], Raja R. Srinivas[2], Sarah K. Wootton[1]

[1]University of Guelph, Guelph, ON, Canada,[2]Asimov, Boston, MA,[3]Clinical Studies, Ontario Vetrinary College, Guelph, ON, Canada

Background: With the rapid expansion and adoption of gene therapies, ensuring safety and addressing adverse events have become key considerations for drug product development in the gene therapy space. Many recombinant adeno-associated virus gene therapy platforms continue to use highly active promoters to drive the expression of exogenous therapeutic transgenes. Although these ubiquitous promoters are well-established and confer strong transgene expression, they present several disadvantages including off-target transgene expression which may cause compromised cell viability, inflammation, and toxicity. For instance, many gene therapy approaches have been limited by off target liver transduction, which negatively impacts drug bioavailability, therapeutic effectiveness, and increases the likelihood of adverse effects. To address these concerns, our approach involves computational mining and engineering techniques to develop a variety of tissue-specific promoters. These promoters aim to promote high level transgene expression in a specific tissue while limiting expression in any off-target tissues that are susceptible to adverse events or may increase immunogenicity of the vector. By selecting an appropriate AAV serotype and pairing it with a tissue-specific promoter, we have shown persistent transgene expression in intended tissues, and de-targeting unwanted tissues including the liver. **Methods**: Here, we use vectors AAV6.2FF, a novel triple mutant, and AAV9 to express the reporter gene human placental alkaline phosphatase (AP). This enabled the evaluation of vector biodistribution and specificity driven by tissue-specific promoters predicted and designed using Asimov's proprietary algorithm. These human promoters were identified and mined using available genetic databases, to drive expression in either the cardiac tissue or skeletal muscle thus minimizing liver transduction. AAV vectors were administered to C57BL/6 mice (n=4) using PBS as a negative control and AAV6.2FF or AAV9 with ubiquitous promoter CASI expressing AP as a positive control. Mice were transduced with $1x10^{11}$ vector genomes (vg) via intraperitoneal (IP) or intramuscular (IM) injection. Mice were euthanized 21 days post-administration and all major organs harvested and stained for AP reporter gene expression. Tissues were grossly imaged and AP activity assays were used to quantify AP expression per tissue. Histologic analysis was performed to identify the cell types where the promoters were active. **Results:** When evaluating a series of putatively cardiac- and muscle-

specific promoters, we identified three noteworthy candidates that operated as predicted by the algorithm. Denoted by number, promoters 5965 and 5970 showed precise cardiac specificity when administered via IP by either AAV6.2FF-AP or AAV9-AP vectors. Promoter 5972 showed excellent transduction in skeletal muscle when delivered by AAV6.2FF-AP via IM administration. AP assays confirmed low to undetectable AP expression in the brain, liver, nasal cavity, pancreas, spleen, and trachea as well as the heart and skeletal muscle depending on which promoter was evaluated. **Conclusions:** AAV6.2FF or AAV9 in conjunction with computationally derived promoters can efficiently transduce the cardiac and skeletal muscle while minimizing off target effects. Additionally, these promoters can achieve expression levels comparable to ubiquitous promoters all while de-targeting the liver, potentially enhancing the safety of AAV gene therapies across a variety of applications, including therapeutic interventions and advancements in biotechnology.

1474. Engineering a Targeted and Safe Bone Anabolic Gene Therapy to Treat Osteoporosis in Alveolar Bone Loss

Chujiao Lin[1], Yeon-Suk Yang[1], Jun Xie[2], Guangping Gao[2], Jae-hyuck Shim[2]

[1]UMass Medical School, Worcester, MA,[2]UMass Chan Medical School, Worcester, MA

Alveolar bone loss in elderly populations is highly prevalent and increases the risk of tooth loss, gum disease susceptibility, and facial deformity. Unfortunately, there are very limited treatment options available. Here, we developed a bone-targeted gene therapy that reverses alveolar bone loss in patients with osteoporosis by targeting the adaptor protein Schnurri-3 (SHN3). SHN3 is a promising therapeutic target for alveolar bone regeneration, because SHN3 expression is elevated in human and mouse mandible tissues with osteoporosis while deletion of SHN3 in mice greatly increases alveolar bone and tooth dentin mass. We used a bone-targeted recombinant adeno-associated virus (rAAV) carrying an artificial microRNA (miRNA) that silences SHN3 expression to restore alveolar bone loss in mouse models of both postmenopausal and senile osteoporosis by enhancing WNT signaling and osteoblast function. Additionally, rAAV-mediated silencing of SHN3 enhanced bone and collagen formation of human skeletal organoids in xenograft mice. Finally, rAAV expression was tightly controlled in the mandible via liver- and heart-specific miRNA-mediated repression or via a vibration-inducible mechanism. Collectively, our results demonstrate the clinical potential of bone anabolic gene therapy to treat alveolar bone loss in osteoporosis.

1475. *In Situ* Injection and Transduction of Human Limbal Stem Cells Using Adeno-Associated Viruses

Hyeck Soo Son[1], Albert S. Jun[2], James W. Foster[2], Wei Wang[2], Yassine Daoud[2], Gerd U. Auffarth[1], Madhuparna Roy[2]

[1]University of Heidelberg, Heidelberg, Germany,[2]Wilmer Eye Institute, Baltimore, MD

Purpose: Inherited corneal dystrophies may cause severe visual impairment. While phototherapeutic keratectomy or corneal transplantations may provide symptomatic relief, the procedures are associated with high infection and recurrence rates. Gene therapies may provide an alternative and a potentially permanent treatment. Adeno-associated viruses (AAVs) have emerged as the vectors of choice for ocular gene therapy thanks to their established safety profile. This study aimed to evaluate the efficacy of *in situ* adeno-associated virus (AAV)-mediated gene delivery into the human corneal limbal region via targeted sub-limbal injection technique. **Methods:** Human cadaveric corneal tissues were fixed on an artificial anterior chamber. Feasibility of sub-limbal injection technique was tested using trypan blue and black India ink. An eGFP encoding AAV DJ was injected into sub-limbal region. After AAV injection, corneal tissues were incubated in air-lift culture and prepared for immunohistochemical analysis. Cell survivial and expression of eGFP, stem cell markers (p63α and cytokeratin 19), and differentiation marker cytokeratin 3 (KRT3) were evaluated using confocal microscopy. **Results:** Both trypan blue and black India ink stained and were retained sub-limbally establishing specificity of the injection technique. Immunohistochemical analysis of corneas injected with AAV DJ-eGFP indicated that AAV-transduced cells in the limbal region co-express eGFP, p63α, and KRT19 and that these transduced cells were capable of differentiating to KRT3 postitive corneal epithelial cells. **Conclusions:** Our sub-limbal injection technique can target cells in the human limbus in a reproducible and efficient manner. Thus, we demonstrate that in situ injection of corneal limbus may provide a feasible mode of genetic therapy for corneal disorders with an epithelial etiology.

1476. Preclinical Development of SENS-501 as a Treatment for the Autosomal Recessive Non-Syndromic Deafness 9 (DFNB9) Using an Adeno Associated Vector-Based Gene Therapy

Rafik Boudra[1], Guillaume Olivier[1], Christophe Tran Van Ba[1], Lise Barrot[1], Julie Duron Dos Reis[1], Pierre Rambeau[1], Charlene Vaux[1], Sandra Pierredon[1], Anais Pages[1], Pauline Liaudet[1], Audrey Broussy[1], Anne-Gabrielle Harrus[1], Selma Dadak[1], Laura Gallot[1], Geraldine Petit[1], Jerome Nevoux[2], Ghizlene Lahlou[3], Natalie Loundon[4], Christine Le Bec[1], Emilie Bousquet[1], Marie-Jose Lecomte[2], Said Safieddine[1], Christine Petit[5], Arnaud Giese[1], Laurent Desire[1]

[1]Sensorion, Montpellier, France,[2]Genetics and Physiology of Hearing Laboratory, Institut de l'Audition/Pasteur, Paris, France,[3]Institut de l'Audition, Institut Pasteur, INSERM, Université de Paris, Paris, France,[4]Hopital Necker-Enfants Malades, Paris, France,[5]Institut de l'Audition/ Institut Pasteur, Paris, France

Congenital sensorineural defects heavily impact patients' life and their ability to communicate with others. Among this vast family of communicating disorders, non-syndromic autosomal recessive deafness 9 (DFNB9) is one of the most common form of congenital deafness, accounting for up to 8% of cases. This severe-to-profound auditory neuropathy is caused by biallelic loss of function in the OTOFERLIN gene (OTOF), which encodes for a calcium sensor protein involved in neurotransmitter release at presynaptic level between inner

sensory hair cells (IHC) and spiral ganglion neurons. So far, the only medical solution is cochlear implantation, which improves hearing to some degree, but still has a lot of limitations. To address this unmet medical need, we developed SENS-501, a dual-AAV (adeno associated virus) hybrid approach using two different recombinant vectors each containing one half of the OTOF cDNA. This strategy was tested on congenitally deaf DFNB9 mutant mice by injecting SENS-501 in the inner ear through the round window membrane. Hearing improvement was evaluated with multiple auditory and behavioral tests. Auditory brainstem response (ABR) recordings showed significant lowering of the thresholds along the auditive spectrum after intra-cochlear injection, with durable (one year) improvement of hearing in a dose-responsive manner. A startle reflex behavior test was performed to confirm the ability of treated DFNB9 mutant mice to integrate sound similarly to wild-type mice. We were able to detect restoration of their startle reflex ability when exposed to randomized sudden loud noises. We also evaluated vestibular function of treated mice by analyzing their exploratory behavior in an open-field arena. Data indicated that our approach did not show any significant increase in circling behavior when compared to control mice. We performed a similar set of experiments in non-human primates (NHP) after SENS-501 intracochlear injections using a surgical approach and our proprietary medical delivery device approved for clinical trial. Surgery and SENS-501 injection did not affect ABR thresholds, similar to what was observed in mice. Immunohistochemistry experiments were performed in both mice and NHP, demonstrating effective recombination and selective expression in IHCs of the full-length therapeutic protein and its flag-tagged counterpart. A 3-month GLP toxicology and biodistribution study following a single intra-cochlear injection was conducted in NHP with two doses of SENS-501. Biodistribution data indicated that the vast majority of the vectors remained in the inner ear. SENS-501 shedding in fluids showed a limited dissemination of the vectors with decreasing levels over time. The two doses of SENS-501 were well tolerated and no clinical or adverse SENS-501-related effects in the inner ear or outside the inner ear tissue in any evaluation -including immunology- were found. Altogether, our nonclinical pharmacology, safety, biodistribution, and two ongoing natural history studies support the clinical development of SENS-501 and the initiation of our phase I/II clinical trial first half of 2024.

1477. Enhanced Long-Term Efficacy of an AAV8-DX88-HSA Vector for Treating Hereditary Angioedema

Weimin Guo[1], Yufeng Guo[1], Shuai Ji[1], Shengmei Wen[1], Yiting Liu[2], Guang Qu[1,2], Lixin Jiang[1,2]

[1]NGGT (Suzhou) Biotechnology Co., Ltd., Suzhou, Jiangsu, China,[2]NGGT, Inc, Walnut Creek, CA

Introduction: Hereditary angioedema (HAE) is a rare autosomal dominant disease. HAE patients experience recurrent episodes of edema (swelling) in the hands, feet, face, intestinal wall, and airway. Airway swelling is particularly dangerous and can lead to death by asphyxiation. Attack may start since childhood and, in severe cases, with weekly episodes. HAE mainly is caused by mutations in the SERPING1 gene that result in the dysfunction of its encoded protein C1 inhibitor (C1-INH), the main inhibitor of the complement and the kallikrein-kinin pathways. Defected C1-INH increases activity of plasma Kallikrein (pKal), which in turns cleaves high molecular weight kininogen (HMWK) to form bradykinin. Bradykinin binds B2 receptors on the vascular endothelium, triggering vasodilation and increased vascular permeability. DX88 is a potent and specific inhibitor of pKal with a very short half-life which limits its effects on suppressing angioedema. Long-term expression of DX88 through AAV-based liver-directed gene therapy could be a valuable approach to treat HAE. **Methods/Results:** We developed an rAAV8-based vector to express DX88-HSA fusion protein (AAV8- DX88-HSA). In vitro, purified DX88-HSA protein produced from transfected 293F cells inhibited pKal activity in either human or monkey serum activated by 20 μg/mL Dextran sulfate (DXS), approximately at an IC(50) value of 250 nM. Sequence-optimized vectors resulted in a 10-fold increase in DX88-HSA expression level compared to WT sequence in mice. AAV8-DX88-HSA stably expressed fusion protein for at least 24 weeks following a single injection at 5E11 vg/kg. The expression level reached 2000 nM in mouse serum, 8 folds higher than the concentration required to sufficiently inhibit pKal in human or monkey serum. Next, we generated a mouse model expressing human pKal to evaluate the efficacy of AAV8-DX88-HAS in vivo. In the mouse model, the expression level of human pKal was 1.5-fold of normal human serum. The concentration of the downstream HMWK was significantly reduced compared to that in WT mice, consistent with a higher activity of pKal due to its overexpression. Following AAV8-DX88-HSA treatment, there were an increase in HMWK level and a marked decrease in pKal activity, suggestive of the active inhibitory effect of DX88 mediated by the vector. An concomitant suppression of vascular permeability was observed. **Conclusions:** In vitro and in vivo data demonstrated that AAV8-DX88-HSA robustly inhibited pKal activity. A single injection of the vector stably expressed DX88-HSA for at least 24 weeks in mice. These results support further development of expressing DX88 fusion protein mediated by AAV as a potential approach to treat HAE.

1478. Exploring Gene Complementation Therapy on Dysmyelination and Neurodegeneration in a Murine Model of Free Sialic Acid Storage Disorder

Mahin Shaimonti Hossain[1], Petcharat Leoyklang[1], Marya S. Sabir[1,2], Mary Hackbarth[3], Laura Pollard[4], Marjan Huizing[1], William A. Gahl[1], May Christine Malicdan[1]

[1]Human Biochemical Genetics Section, National Institutes of Health, Bethesda, MD,[2]University of Oxford, Oxford, United Kingdom,[3]University of Maryland School of Medicine, Baltimore, MD,[4]Biochemical Genetics Laboratory, Greenwood Genetics Center, Greenwood, SC

Introduction: Free Sialic Acid Storage Disorder (FSASD) is a rare, lysosomalstorage disorder resulting from bi-allelic variants in *SLC17A5*. Thisgene encodes sialin, a lysosomal transmembrane protein responsible fortransporting sialic acid (SA) from lysosomes to the cytosol. In additionto coarse facial features, developmental delay, organomegaly, and elevatedurinary free sialic acid, the disorder results in progressiveneurodegeneration, characterized by ataxia, spasticity, hypotonia, and profoundCNS hypomyelination. There is currently no FDA approved treatment and thepathophysiology of the disorder's

neurodegeneration and myelinationdeficits remains enigmatic. In the present study, we explore *SLC17A5* genecomplementation as a potential therapeutic strategy in mouse embryonicfibroblasts (MEFs) derived from a novel mouse model of FSASD and establishgrounds for further in vivo investigations in these mice.Methods: We generated a novel *Slc17a5*^{R39C}knock-in mouse model with the most common p.Arg39Cys (R39C) mutation seenin human patients and characterized the biochemical, histological, andneurobehavioral phenotype. To explore gene complementation, we firsttransfected *Slc17a5*^{R39C} mouse embryonic fibroblasts (MEFs)with GFP-tagged human *SLC17A5* (hSLC17A5). We then delivered thehSLC17A5 or eGFP open reading frame in an AAV vector intravenously to mice atPostnatal Day 2 (PND2); these AAV vectors were driven by the hSYN1promoter. At 6- and 12-weeks post treatment, we collected biological tissuesand fluids to perform biochemical, histological, and immunofluorescent assays. Results: The *Slc17a5*^{R39C} micerecapitulated several features of the human disease, including progressiveataxia, impaired performance on a battery of neurobehavioral tests, increasedurinary free sialic acid, and impaired egress of alkyne-labeled SA fromlysosomes. Early developmental studies revealed that *Slc17a5*^{R39C} miceexhibit decreased myelination, significant white matter astrogliosis, andPurkinje cell loss over time. Purkinje cell vacuolization precededdegeneration, which was progressive from Postnatal Day 21.Transfection of *GFP-hSLC17A5* in *Slc17a5*^{R39C}MEFs increased incorporation of alkyne-labeled SA in cells, suggestingsome recovery of sialin-mediated release of alkyne-labeled SA from lysosomes.Widespread CNS tropism of AAV containing eGFP, except for glial cells, wasconfirmed by immunofluorescence. While *Slc17a5*^{R39C} miceinjected with AAV-*hSLC17A5* showed increased retention ofPurkinje cells, these cells were vacuolated in appearance and there was minimalrecovery of myelination when compared to saline-treated counterparts.Furthermore, AAV-*hSLC17A5* treatment did not correct free sialicacid levels in biological fluids or tissues. Overall, these resultsindicate the need for local CNS delivery and/or the use of a promoter that caneffectively target glial cells.Conclusion: Our study interrogatesthe feasibility for *SLC17A5* gene complementation for FSASD. Ourfindings define key cellular targets in the pathogenesis and progression ofdisease, critical for future preclinical studies and development of noveltherapeutic strategies, and emphasize the role of glial cells such asastrocytes, oligodendrocytes, and microglia in the pathobiology of thedisorder. These results underscore the intricacy of developing genetherapy for neurodegenerative lysosomal storage disorders and pave the way forfuture studies to optimize therapeutic gene delivery to the CNS.

1479. AAV Mediated Therapy for Hereditary Spastic Paraplegia Caused by Mutations in *SPTSSA*

Ashley Glover[1], Yi Gong[1], April Qian[1], Natalie Golovanov[1], Kenneth Gable[2], Casey A. Maguire[1], Teresa Dunn[2], Florian Eichler[1]

[1]Department of Neurology, Massachusetts General Hospital, Boston, MA,[2]Department of Biochemistry and Molecular Biology, Uniformed Services University of the Health Sciences, Bethesda, MD

Sphingolipids (SLs) are lipid molecules that play essential structural and signaling functions in eukaryotes. Aberrant SL metabolism has been linked to many neurological diseases, including neuronal lysosomal storage diseases, Creutzfeldt-Jakob disease, Alzheimer's disease, and Parkinson's disease. Serine palmitoyltransferase complex (SPT) mediates the first and rate-limiting step in the de novo sphingolipid biosynthetic pathway. The larger subunits SPTLC1 and SPTLC2/SPTLC3 together form the catalytic core with a smaller third subunit either SPTSSA (small subunit A) or SPTSSB (small subunit B) propelling the catalytic efficiency and providing substrate specificity for the fatty acyl-CoA substrates. Recently, a de novo variant (p.Thr51Ile, SPTSSA^{T51I}) in SPTSSA was discovered in two female children with stagnant neurodevelopment, who both showed substantially elevated levels of several SLs. To further investigate the pathophysiology of this monogenetic disease and to explore therapeutic approaches, we developed a mouse model using CRISPR technology, assessed the sphingolipid biochemistry and performed systematic behavioral phenotyping. While the *Sptssa*$^{T51I/+}$ had only a mild phenotype up to 5 month of age, biochemical analysis did reveal elevated SL levels. Surprisingly, supplementation with 10% L-serine at 2 months of age continuously for a 2-3-month duration further increased SL levels and worsened the hind limb clasping behavior at 5months of age. Notably, mixed glial cells extracted from *Sptssa*$^{T51I/+}$ mice showed remarkable elevation of SLs and AAV9-sc-CBA-SPTSSA treatment led to robust SPTSSA protein expression and subsequently significant reduction of SL in a dose dependent manner. AAV9-sc-CBA SPTSSA delivery by retroorbital injection was able to transduce the brain and spinal cord evenly and, in a dose dependent manner. In conclusion, serine supplementation in *Sptssa*$^{T51I/+}$ mice aggravated the biochemical and motor phenotype and mimicked the patient clinical features. Our preliminary data in mixed glia cells warrant further in vivo assessment of AAV mediated gene therapy to restore the dynamic regulation of SPT and to prevent abnormal hindlimb clasping in our *SptssaT51I/+* mouse model.

1480. Intra-Articular Delivery of an AAV-Anti-TNF-α Vector Alleviates the Progress of Arthritis in a RA Mouse Model

Shuang Luo[1], Qing Xie[1,2], Qingwei Li[1], Qiang Zheng[1,2]

[1]Chengdu Origen Biotechnology Co., Ltd, Chengdu, China,[2]Sichuan Provincial Key Laboratory of Innovative Biomedicine, Chengdu, China

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by erosive arthritis, which occurs at any age, with a peak incidence between 30 and 50 years of age. The pathological feature includes chronic inflammation in the synovial membrane, formation of vascular opacities and destruction of cartilage and bone in the joints, leading to joint deformity and loss of function eventually. Anti-TNF-α such as Adalimumab, Etanercept and Golimumab are commonly used medication drugs, which are administrated by intra-articular injection directly into the articular cavity, the operation is normally conducted at a frequency of 1 to 4 doses per month depending on the severity of the symptom, causing great inconvenience to the patients and compromised compliance. Therefore, a non-invasive, low-frequency medication for treating rheumatoid arthritis is urgently needed. AAV-mediated gene therapy is considered a treatment strategy involving a single administration with long-term effectiveness, holding immense potential in the treatment of RA. To develop a gene therapy approach, an AAV8 vector harbouring a transgene expressing the anti-TNF-α (KH656) was created in this study. The potency of this vector for the

treatment of RA in a type II collagen-induced RA mouse model was explored. In this study, the KH656 was injected into the mice's ankle joint at a dose of 1E11vg/joint; Animals injected with Adalimumab (8 mg/kg, bi-week) were used as the benchmark. The disease progress/severity is determined by the following indicators: (1) the paw thickness and articular damage clinical scores; (2) cartilage damage; (3) inflammatory cytokines and the infiltration of M1/M2 macrophages; (4) chondrocyte damage, osteoclast damage, osteoprotegerin and osteoclast differentiation factors.The paw thickness and the clinical score of the animals were measured first. The disease animals showed swollen paws, with an average thickness of 3.42±0.42 mm, and a clinical score of 5.21±1.58 at day-48. In the benchmark group, the treatment of Adalimumab alleviated the symptoms of animals by reducing the paw thickness and clinical score to 3.11±0.44 mm, 4.00±1.17 respectively. Notably, the KH656 alleviated the paw swollen with a thickness of 2.92±0.1 mm and a clinical score of 2.43±1.24 on average, which is superior to the Adalimumab group in terms of therapeutic efficacy. The biomarkers of the therapeutic effect were detected after the treatment. The M1 macrophage infiltration and the secretion of the inflammatory factors including IL-1β, IL-6 and TNF-α were inhibited in all treated animals' joint fluid, and the KH656 group showed a stronger inhibitory effect than the Adalimumb group. The animals' joint tissues were subjected to immunohistochemical analysis as well. Compared with the Adalimumab group, KH656-treated animals had a less inflammatory response, slighter chondrocyte and osteoclast damage, lower M1/M2 macrophages ratio, lower levels of osteoprotegerin, osteoclast differentiation factor and fewer apoptotic cells.In summary, Intra-articular delivery of KH656 was found to be capable of protecting articular cartilage, inhibiting synovial inflammation, alleviating arthrophlogosis and restoring normal joint functions in mice. In light of these promising preclinical observations, future NHP studies and clinical trials using the anti-TNF-α-based AAV gene therapy are expected to show substantial benefits to human RA patients.

1481. Utilizing Recombinant AAV and Liposome-Liquid Droplet Form for Improving Osteoarthritis Treatment

Hyeonjee Seo, Kye Il Joo

Ewha Womans University, Seoul, Korea, Republic of

Osteoarthritis(OA) is a common joint disease, but it is still difficult to treat due to a lack of disease-modifying therapy. The impaired articular cartilage possesses limited inherent healing capabilities, necessitating alternative approaches like cartilage reconstruction. This research aims to utilize recombinant AAV (rAAV), genetically engineered with cloned proteins, as a therapeutic intervention. AAV is a comparatively safe viral vector, and its modified form, tailored for gene therapy, transforms into a tool for delivering specific genes to the body to address diseases. This rAAV is anticipated to be introduced into the joint to facilitate the differentiation of resident stem cells, thereby aiding in tissue repair. To this end, we propose a novel liquid droplet-based biomaterial platform to enable precise spatiotemporal administration of rAAV for the efficient treatment of osteoarthritis. Lipo-coa, incorporating liposomes into the existing coacervate, demonstrates an enhanced capacity to adeptly navigate and adhere to narrow crevices. Additionally, it exhibits effective lubrication along the shear reaction boundaries, fostering anticipation for more efficacious treatment and drug delivery.

1482. Transduction Efficiency of Nine Myotropic AAV Variants in a Human Skeletal Muscle Xenograft Model

Matthew James Burke[1], Jin Han[1], Xiufang Pan[1], Yongping Yue[1], Keqing Zhang[1], Lei Jin[2,3,4], Jun Li[2,3,4], Shi-Jie Chen[2,3,4], Dongsheng Duan[1,5,6,7]

[1]Department of Molecular Microbiology and Immunology, University of Missouri, Columbia, MO,[2]Department of Physics, University of Missouri, Columbia, MO,[3]Department of Biochemistry, University of Missouri, Columbia, MO,[4]Institute for Data Science and Informatics, University of Missouri, Columbia, MO,[5]Department of Biomedical, Biological & Chemical Engineering, University of Missouri, Columbia, MO,[6]Department of Neurology, University of Missouri, Columbia, MO,[7]Department of Biomedical Sciences, University of Missouri, Columbia, MO

Myotropic AAV variants hold promise to treat inherited neuromuscular diseases such as Duchenne muscular dystrophy (DMD). Several bioengineered AAV capsid variants recently demonstrated superior muscle transduction in mice and nonhuman primates. Many of these capsids contain a surface-exposed RGD motif. Despite promising results, their performance in human skeletal muscle is unknown. To address this, we used a human muscle xenograft model to compare six RGD-motif capsids with three AAV capsids that are currently in use in clinical trials (AAV8, AAV9, and AAVrh74). Fully regenerated muscle grafts were established in immune-deficient NSG mice using skeletal muscle from adult human donors. To evaluate the efficiency of gene delivery and gene expression in the same animal, we utilized a novel exonic barcode system. This system allows for detection of each barcoded vector genome and mRNA transcript using conventional qPCR techniques. Barcoded but otherwise identical reporter AAV genomes expressing EGFP driven by the ubiquitous CAG promoter were packaged in each of the nine capsids. Viral titers of each vector were determined by barcode-specific qPCR. A cocktail containing equal ratios of each AAV variant was delivered to host mice by intravenous injection at two doses (3E+12 vg/kg/AAV, n=6 and 1E+13 vg/kg/AAV, n=5). At one-month post-injection, we collected tissues and evaluated graft regeneration by immunofluorescence staining and morphometric analysis. We then used TaqMan qPCR assays unique to the DNA and mRNA barcodes to evaluate vector genome and transcript copy numbers in the human muscle graft, mouse tibialis anterior muscle, and mouse liver. Our preliminary results indicate differential transduction and transgene expression between AAV variants in human muscle and mouse tissues. The full data set will be presented at the Annual Meeting.

1483. Microdystrophin Configuration Optimization for Duchenne Muscular Dystrophy Gene Therapy

Xiufang Pan[1], Nalinda B. Wasala[1], Lakmini P. Wasala[1], Chady H. Hakim[1], Yongping Yue[1], Keqing Zhang[1], Matthew J Burke[1], Dongsheng Duan[1,2,3,4]

[1]Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO,[2]Department of Biomedical Sciences, Department of Biomedical Sciences, College of Veterinary Medicine, University of Missouri, Columbia, MO,[3]Department of Neurology, School of Medicine, University of Missouri, Columbia, MO,[4]Department of Chemical and Biomedical Engineering, College of Engineering, University of Missouri, Columbia, MO

Duchenne muscular dystrophy (DMD) is a lethal muscle-wasting disease caused by the null mutations in the *DMD* gene. This results in the loss of the sarcolemmal stabilizing protein dystrophin. Dystrophin is one of the largest proteins. The dystrophin coding sequence exceeds the packaging capacity of the adeno-associated virus vector (AAV), the only viral vector capable of efficient bodywide muscle gene delivery. Systemic microdystrophin gene therapy aims to replace the missing dystrophin protein with a highly abbreviated form of dystrophin. Several microdystrophin constructs are currently in clinical trials, and one has received accelerated approval from the FDA to treat 4 to 5-year-old patients. The full-length dystrophin protein has four hinges in the rod domain. Two hinges flank the rod domain, and the other two are in the middle of the rod domain. Some clinical trial microdystrophins contain a centrally located hinge while others do not. To determine the functional implication of the central hinge, we engineered two microdystrophin constructs; one contains a centrally located hinge, while the other does not. The microdystrophin cassettes were packaged in AAV9 and delivered to 2.5-m-old mdx mice via the tail vein at 4e14 vg/kg. Microdystrophin expression, muscle histology, grip force, and muscle force were examined when mice were 6 -7 months old. Both constructs yielded saturated expression in skeletal muscle and the heart. Both constructs resulted in similar histological improvements and equivalent grip strength and muscle-specific force. However, the construct without the central hinge was more effective in protecting against eccentric contraction-induced force drop. Our data suggest that fine-tuning microdystrophin configuration is a promising strategy to improve DMD gene replacement therapy (Supported by NINDS, NIH).

1484. A Proof-of-Concept AAV Gene Replacement Therapy for GNE Myopathy in a Knock-in Mouse Model

Katherine E. Koczwara[1], Petcharat Leoyklang[2], Angela Lek[3], Alec DeSimone[1], Shushu Huang[1], Vincent Ho[1], Qi Zhang[1], Justin Cohen[1], Kaiyue Ma[1], Stella Mitrani-Rosenbaum[4], Marjan Huizing[2], Monkol Lek[1]

[1]Yale University, New Haven, CT,[2]NHGRI, NIH, Bethesda, MD,[3]MDA, Chicago, IL,[4]Hadassah-Hebrew University of Jerusalem, Jerusalem, Israel

Rationale: GNE myopathy is a rare, autosomal recessive disease, leading to progressive skeletal muscle atrophy and weakness in the limbs. Myopathy occurs due to pathogenic mutations in the *GNE* gene, resulting in reduced enzymatic activity of the GNE protein, a bifunctional enzyme involved in the sialic acid biosynthesis pathway. To date, over 250 pathogenic mutations in *GNE* have been reported, making mutation correction a poor therapeutic approach. While oral supplementation of N-acetyl-D-mannosamine (ManNAc), the substance produced by GNE within the sialic acid biosynthesis pathway, may be a viable therapeutic strategy, its efficacy in treating GNE myopathy patients is still being evaluated (NCT04231266). Alternatively, delivery of a fully functional *GNE* gene to muscle tissue should restore proper GNE function, regardless of the particular pathogenic mutation. AAV-based gene delivery has proven successful for the treatment of other genetic neuromuscular diseases, and the relatively small size of the *GNE* coding sequence (<2.2kb) make this approach an attractive treatment strategy for GNE myopathy. Study Design: In this study we designed, optimized, and evaluated the efficacy of a gene replacement therapy for GNE myopathy. A library of AAV vectors was developed and evaluated *in vitro* for GNE expression due to: (1) the promoter used; (2) the inclusion of an intron; (3) the GNE isoform; and (4) the position and type of protein tag. Following selection of an optimal vector, two high expressing constructs were packaged into AAV9 particles, and a dose and biodistribution study was performed in C57BL/6 wildtype mice to determine the safety and efficiency of GNE expression across a variety of tissues. Lastly, the efficacy of gene replacement therapy was evaluated in a knock-in mouse model of GNE myopathy. In brief, both AAV9 and AAVmyo2 serotypes were utilized to treat 6-8 week-old mice with constructs that express the *GNE* coding sequence under control of muscle-specific promoters. At 12 weeks post-injection, several outcome measures were determined, including (1) the effects of gene replacement therapy on muscle hyposialylation, (2) the biodistribution of viral genomes depending on AAV serotype used, and (3) GNE protein expression across a variety of tissues. Results/Conclusions: *In vitro* optimization demonstrated that the CK8e-SV40-*hGNE1*-V5 and MCK-SV40-*hGNE1* constructs resulted in high *GNE* transcript and GNE protein expression in myoblasts. The AAV9 dose and biodistribution study in C57BL/6 mice indicated robust, dose-dependent transcript and GNE protein expression across a variety of muscle tissues at 8 weeks post-injection. While the effects of GNE gene replacement therapy on muscle hyposialylation in a mouse model of GNE myopathy are still being investigated, preliminary results of this study indicate a high dose (3e14 vg/kg) of AAVmyo2-MCK-SV40-*hGNE1* led to strong exogenous GNE protein expression in skeletal muscle. Additionally, the use of the AAVmyo2 serotype resulted in significantly decreased liver targeting of the viral vector compared to AAV9, while exhibiting increased skeletal muscle targeting. This proof-of-concept study provides key insights into the optimal muscle-specific promoter, dose, and AAV serotype to be used within the context of GNE myopathy. In-progress and future work investigating the effects of AAV therapy on muscle hyposialylation and other disease-relevant phenotypes will be important in determining the efficacy and minimum required dose for a successful gene replacement therapy.

1485. Expression of Large and Highly Functional Dystrophins Using Split Inteins and Myotropic AAV Vectors

Hichem Tasfaout[1], Christine L. Halbert[1], James M. Allen[2], McMillen Tim[1], Stephen D. Hauschka[1], Dirk Grimm[3], Michael Regnier[1], Jeffrey S. Chamberlain[1]

[1]University of Washington, Seattle, WA,[2]University of Washington School of Medicine, Seattle, WA,[3]Heidelberg University Hospital, Heidelberg, Germany

Gene replacement using Adeno-associated viral (AAV) vectors is a promising therapeutic approach for many diseases. However, this therapeutic modality is challenged by the packaging capacity of AAVs (~4.7 kb), limiting its application for disorders involving large coding sequences, such as Duchenne muscular dystrophy, with a 14 kb mRNA. Here, we developed a novel method for expressing large dystrophins by utilizing the protein *trans*-splicing mechanism mediated by split inteins. We identified several split intein pairs that efficiently join two or three fragments to generate a large midi-dystrophin or the full-length protein. We show that delivery of two or three AAVs into dystrophic mice results in robust expression of large dystrophins and significant physiological improvements compared with micro-dystrophins. Moreover, using the potent myotropic AAVMYO and strong muscle-specific expression cassette CK8e, we demonstrate that low total doses ($2x10^{13}$ vg/kg) are sufficient to express large dystrophins in striated muscles bodywide with significant physiological corrections in young or very old *mdx*^{4cv} dystrophic mice. Our data support the feasibility of this approach *in vivo* and show a clear functional superiority of large dystrophins over micro-dystrophins that are being tested in clinical trials. This novel method could benefit many patients with Duchenne or Becker muscular dystrophy regardless of genotype and, if not complicated by immune responses to intein polypeptides, could be adapted to numerous other disorders caused by mutations in large genes exceeding the carrying capacity of AAV.

1486. Control of Cardiotoxicity Related to AAV Gene Transfer

Isabelle Richard, Ariane Biquand, Evelyne Gicquel, Jérôme Poupiot, Anthony Brureau

GENETHON, Evry, France

Gene delivery technologies based on adeno-associated virus vectors (AAV) have yielded promising results for the treatment of monogenic diseases, leading in recent cases to marketing authorization in Europe and US. However, preclinical studies and clinical trials have highlighted that infusing high vector doses can induce cytotoxicity in a number of organs, leading in a few cases to the death of the patient. We investigated the consequences on the heart of administration of high doses of AAV vectors expressing 3 different transgenes corresponding to muscular dystrophies: a form of microdystrophin for Duchenne Muscular Dystrophy, the human full-length SGCG cDNA for γ-sarcoglycanopathies and the human full-length FKRP cDNA for FKRP deficiencies. We observed in all these cases the possibility of cardiotoxicity with high doses injection whereas no abnormality was present in the skeletal muscle. Dissection of the mechanisms at stake revealed activation of different molecular cascades due to supraphysiological transgene expression in the heart and leading to cardiomyocyte death. Preventing transgene expression in heart through the use of a cardiac specific microRNA or a heart detargeting promoter was shown to prevent the appearance of such cardiotoxicity. Overall, our data highlighted a specific sensitivity of the heart to transgene expression, in particular compared to skeletal muscle, suggesting the importance to regulate finely the expression of transgene in this organ in any gene therapy approach. The manuscript presenting this work is ready for submission.

1487. U7snRNA-Mediated Exon 17 Skipping in Duchenne Muscular Dystrophy

Liubov V. Gushchina[1], Gabrielle Dufresne[2], Ezgi Saylam[2], Adrienne Bradley[2], Natalie Rohan[2], Allison Lowery[2], Hui Lin[2], Nicolas Wein[2], Kevin M. Flanigan[2]

[1]Center for Gene Therapy, The Ohio State University and Nationwide Children's Hospital RI, Columbus, OH,[2]Center for Gene Therapy, Nationwide Children's Hospital RI, Columbus, OH

Duchenne muscular dystrophy (DMD) is a severe genetic disease affecting approximately 1 in 5,200 live male births and characterized by progressive muscle weakness, leading to premature death due to cardiac and respiratory failure. DMD is caused by mutations that disturb the open reading frame (ORF) of the dystrophin gene leading to loss of the dystrophin-glycoprotein complex. Currently approved and experimental gene therapies have resulted in expression of multiple internally deleted, partially functional dystrophin isoforms, the efficacy and durability of which are still limited. An alternative and most effective strategy to induce exon skipping is to incorporate antisense oligonucleotides (ASOs) into U7 small nuclear RNA (U7snRNA) and deliver them using adeno-associated virus (AAV). This approach has already demonstrated long-term potential to restore either full-length dystrophin or a highly functional N-terminal truncated protein in Dup2 mice and patients with exon 2 duplication mutations (NCT04240314). Here, we examined the therapeutic potential of rAAV.U7snRNA-mediated therapy to correct disrupted ORF in DMD patients carrying variable exon 17 skip-amenable deletions. We designed several AAV vectors and tested them *in vitro* and *in vivo*. Two DMD patient-derived cell lines—Immortalized tet-inducible-MyoD fibroblasts (FM cells)—carrying exon 17 amenable deletions were treated with rAAV.U7snRNA vectors at four different doses and then differentiated them for 8-14 days. Both cell lines revealed robust exon 17 skipping in a dose-dependent manner. Semi-quantitative analysis of RT-PCR gel images also confirmed the presence of In-frame (IF) dystrophin mRNA transcripts in both cell lines. To confirm exon 17 exclusion *in vivo*, all three vectors were tested using a novel DMD mouse model carrying an out-of-frame (OOF) deletion of exons 18-41. Del18-41 mice were intramuscularly injected into both the left and right sides of the tibialis anterior (TA) and gastrocnemius (Gast) muscles. Animals were euthanized 4 weeks after vectors administration, and tissues were collected for analysis. RT-PCR analysis revealed exclusion of exon 17 for two of the three target sequences in both TA and Gast muscles, resulting in dystrophin protein levels of 0.9-5.2% detected by Western blotting. Overall, we clearly demonstrate that rAAV.U7snRNA-mediated therapy is a powerful therapeutic tool that can benefit nearly 5.5% of all DMD patients carrying amenable mutations (duplication and deletion) flanking exon 17.

1488. AAV9 Mediated Dars2 Gene Therapy Prevents Neurodegeneration in Two Mouse Models of LBSL

Ines L. Garofolo[1], Yajie Liang[2], Miroslaw Janowski[2], Piotr Walczak[2], Ali Fatemi[1,3], Christina L Nemeth[1,3]

[1]Moser Center for Leukodystrophies at Kennedy Krieger, Kennedy Krieger Institute, Baltimore, MD,[2]Department of Diagnostic Radiology and Nuclear Medicine, University of Maryland, Baltimore, Baltimore, MD,[3]Department of Neurology, Johns Hopkins University School of Medicine, Baltimore, MD

Leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation (LBSL) is a rare white matter disease caused by mutations in the mitochondrial aspartyl-tRNA synthetase (mt-AspRS, or *DARS2*) gene, which is important for the synthesis of proteins in mitochondria. Abnormalities within cerebral white matter, brainstem, and spinal cord are observed in all patients' MRI; however, a large phenotypic spectrum exists. Previously, we have shown that *Dars2* deletion from CamkII alpha excitatory neurons results in a severe and slowly progressive phenotype emerging at 22 weeks old, consisting of significant cortical atrophy and ventricular enlargement (Nemeth et al., 2019). More recently, we have generated mice with *Dars2* deletions in Advillin-expressing cells, or sensory neurons of the dorsal root ganglia. *Dars2*/Advillin deletion mice display severe ataxic behavior starting at as young as p14, eventually leading to death by 6 weeks. As LBSL patients primarily experience symptoms associated with ataxia, disordered gait, and white matter abnormalities, these models provide a representation of disease phenotype for the further study of disease mechanism, cell-type specific effects, and therapeutic efficacy. Our current focus in therapeutic testing within these mouse models is via treatment with an Adeno-Associated Viral (AAV) vector, serotype 9, containing a human *DARS2* plasmid. Our hope is to improve the biochemical and phenotypic deficiencies in our models by way of intracerebroventricular (ICV) or intrathecal (IT) injection of the AAV9 vector. At 8 weeks of age, and prior to onset of symptoms, *Dars2*/CamKIIα mice were treated with a single *DARS2*-AAV9 via ICV injection. Open field analysis was performed at baseline and every 2 weeks following treatment until mice were sacrificed at 32 weeks. Behavior data indicated a positive effect of the therapy to decrease excitatory behavior and prevent cortical atrophy (figure 1). *In situ* hybridization detected presence of human *DARS2* in neuronal cells, suggesting that cell uptake of human *DARS2* has therapeutic effects in mice. On or around p6, *DARS2*-AAV9 was administered via IT injection to *Dars2*/Advillin mice. On p35, gait was assessed in wild-type, *Dars2*/Advillin knock-out mice, and AAV9 treated knock-out mice. Preliminary results indicate improvements to spasticity through a normalization of base of support measures, a significant reduction of weight loss, and improved life span. Together, these data suggest that gene delivery via AAV9 reduces the phenotypic burden of *Dars2* deletion in two different mouse models. Ongoing studies are currently addressing biodistribution and toxicity of the *DARS2* vector.

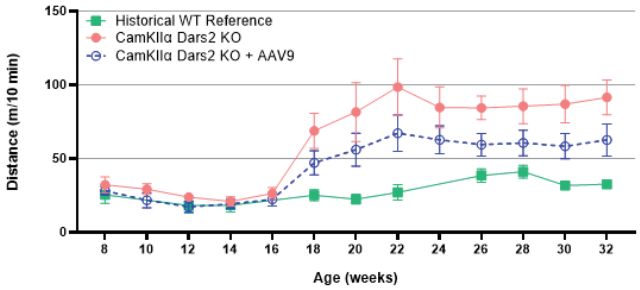


Figure 1. Intracerebroventricular injection of AAV9 decreased excitatory behavior in *Dars2*/CamKIIα KO mice.

1489. Highly Effective Expression of Full-Length Dysferlin Protein in Bla/J Mouse Muscle After Systemic MyoAAV Administration

Danielle Velez, Chady H. Hakim, Ana Maria Manso, Graham Salmun, Stephanie Landeros, Mark Fielden, Sharif Tabebordbar

Kate Therapeutics, San Diego, CA

Dysferlinopathy (LGMD2B/R2) is a genetic muscle disease caused by mutations in the *DYSF* gene resulting in the absence of dysferlin, a membrane protein involved in muscle membrane repair (membrane patch). Dysferlin deficiency compromises sarcolemmal integrity, leading to progressive muscle degeneration. Gene replacement therapy is a promising therapeutic strategy for dysferlinopathy. However, the dysferlin coding sequence (~6.2 kb) exceeds the packing capacity of a single AAV. Dual AAV strategies have been proposed as a strategy to overcome this hurdle and allow expression of full-length dysferlin. However, production of full-length dysferlin with conventional dual AAV strategies is often inefficient, because it requires delivery of vector genomes from both AAVs into the same nucleus in muscle fibers, and because the efficiency of homologous recombination in post-mitotic cells is low. To address this, we used an alternative dual AAV strategy utilizing split protein inteins to express full-length dysferlin. In this strategy, two segments of the dysferlin protein are expressed individually and the full length protein is generated by protein splicing in the muscle fiber cytoplasm. We tested multiple split inteins *in vitro*, and identified a lead dual AAV candidate containing a split intein that results in high efficiency of protein splicing and full length dysferlin protein production in myotubes. Systemic delivery of dual MyoAAVs for the lead candidate in Bla/J mice at low dose resulted in physiological or supraphysiological full-length dysferlin expression in multiple skeletal muscles. Immunofluorescence analysis showed widespread dysferlin expression across most muscle fibers, which provided protection against strain injury in Bla/J muscles.Our results provide evidence for the feasibility of a dual AAV strategy utilizing split protein inteins for effective expression of full-length dysferlin protein in dystrophic muscle following low-dose systemic MyoAAV administration.

1490. Cell-Type Specific Enhancer AAVs for the Treatment of Motor Deficits in Parkinson's Disease

Ananya Chowdhury, Alex Fraser, Max Departee, Alex C. Hughes, John K. Mich, Yemeserach Bishaw, Bryan B. Gore, Emily A. Kussick, Naz Taskin, Victoria Omstead, Nathaly Munoz, Ximena Opitz-Araya, Refugio Martinez, Kathryn Gudsnuk, Boaz P. Levi, Ed S. Lein, Jonathan T. Ting, Tanya L. Daigle

Human Cell Types, Allen Institute for Brain Science, Seattle, WA

Gene therapy has the potential to provide long-term clinical benefits for debilitating human disorders of the central nervous system (CNS) and the body. Among others, human clinical trials are in progress to test gene therapies for the treatment of Parkinson's disease (PD), a debilitating neurodegenerative disease impacting the brain's dopaminergic system. These trials showed promising results using AAV2 to drive the expression of human aromatic l-amino acid decarboxylase (hAADC)—a rate-limiting enzyme in dopamine synthesis—under the control of a ubiquitous promoter (CMV). However, this required a bilateral, intraoperative MRI-guided putaminal infusion, which is expensive as well as highly invasive, and prone to technical issues. The ability to deliver the therapeutic cargo via a non-invasive route of administration like the intravenous (IV) method would be a huge step forward in the field of gene therapy. To achieve that, we need to ensure they cross the blood-brain barrier (BBB) and show strong and widespread transduction in the brain while minimizing off-target effects in non-diseased cells of the brain, sensory neurons, and peripheral organs to avoid unwanted side effects and mortality in PD patients. To that effect, we have created a powerful toolkit of enhancer-based AAVs with commercially available PHP.eB capsid, which enables precise delivery of genes of interest to diverse cell types within the CNS with inherently low off-target expression. Our enhancer-AAVs showed strong and specific drive hAADC expression in striatal medium spiny neurons (pan-MSNs), indirect or direct MSNs, striatal cholinergic neurons, and midbrain dopamine neurons following systemic delivery in mice. The lead pan-MSN vector was further evaluated in non-human primates (NHP) following intraparenchymal and intracerebroventricular delivery, demonstrating cross-species expression and translational capability. We tested the efficacy of our lead pan-MSN vector in a 6-OHDA toxin model and two genetic PD models in mice, Mitopark and MCI, using multiple behavioral paradigms like accelerating rotarod, open field, contralateral paw touches, and gait analysis. Our work showed that expressing hAADC in all MSNs by systemic (IV) delivery ameliorated specific motor-related deficits in these models. A single administration of a lower dose of L-Dopa further improved their motor behaviors, which could better the quality of life by delaying the onset and possibly diminishing the detrimental side effects of L-Dopa observed in the patients. Furthermore, our lead pan-MSN vector showed more robust behavioral rescue when compared to ubiquitous CMV-promoter AAV when administered locally to the dorsal striatum of mice, suggesting the superiority of a cell-type specific intervention. In summary, our work provides strong proof-of-concept data in support of enhancer-AAVs as potential circuit-selective therapies for treating PD. Cell-type specific expression of hAADC via a non-invasive route of administration has the potential to improve motor functions in PD patients alone or alongside a lower dose of L-DOPA, bypassing invasive brain surgery to achieve a similar therapeutic effect.

1491. Biodistribution of AAV6.2-mCherry in C57BL/6J Mouse Dosed via Nose-Only Inhalation Route

Mayuri Prasad[1], Justine Damiano[2], Phillip Gouldthorpe[2], Ambre Brink[1], Lori Bedient[2], Emily Resseguie[2], Sheri Vesperman[1], Sarah Wills[1], Simon Moore[3], Brian E. Mcintosh[1]

[1]Labcorp, Madison, WI,[2]Labcorp, Somerset, NJ,[3]Labcorp UK Limited, Huntingdon, United Kingdom

In the past 7 years the United States Food and Drug Administration (USFDA) has approved 5 Adeno associated virus (AAV) based gene therapy products treating a range of disorders and deadly monogenic diseases. In order to meet demands of the evolving AAV gene therapy product landscape, successful AAV vector delivery technique is necessary to evaluate appropriate cell targeting and sometimes even lab personnel safety. Monogenic pulmonary diseases such as cystic fibrosis and α-1-antitrypsin (AAT) deficiency, require targeted delivery of AAV gene therapy aerosolized droplets to respiratory tract airway epithelium cells via an inhaled route. In this study, we demonstrate and establish a rodent, nose-only AAV inhalation model for assessing the efficiency of administration, biodistribution, toxicity and the persistence of an AAV6.2 expressing mCherry. The study design was reviewed and approved by the Institutional Animal Care and Use Committee at Labcorp, Madison, WI. Male C57BL/6J mice were administered AAV6.2-mCherry aerosols at concentrations of 3.61×10^9 viral genome copies/L for 6 or 60 minutes to achieve 1 log fold different doses. The target delivered dose of animals administered AAV6.2-mCherry aerosols for 6 minutes was 2.3×10^{10} GC/kg and for animals administered AAV6.2-mCherry aerosols for 60 minutes was 2.3×10^{11} GC/kg. Biodistribution, toxicity and persistence of the AAV6.2 was evaluated after 2-and 4-weeks. The AAV aerosol was generated using an Aeroneb Solo nebulizer into a nose-only exposure system. qPCR was used to evaluate the aerosol concentration and particle size of aerosol samples collected from the exposure system. The dosing procedure was performed within a biosafety cabinet in an ABSL2 designated area in the facility. In addition, various mitigation strategies were employed to ensure that personnel and laboratory exposure was minimized. The persistence of the viral genome and tissue-specific transduction efficiency was evaluated by qPCR, Immunohistochemistry for the mCherry protein and Qualitative Whole Body Fluorescence Assay in respiratory and other major organ system tissues. No AAV6.2-mCherry-related adverse clinical observations or mortality were noted during the study, and expression of mCherry was limited to respiratory system tissues. Mass median aerodynamic diameter (MMAD) of the AAV6.2-mCherry was towards the upper limits of respirable droplet size, yet contained viral aerosol that could reach the pulmonary parenchyma. A dose dependent increase in transduction efficiency was seen in selected respiratory tissues via qPCR analysis. Minimal or minimal to slight expression of mCherry protein was seen in the trachea and tracheal bifurcation, respectively, in animals administered $\geq 2.3 \times 10^{10}$ GC/kg AAV6.2-mCherry and was limited to the mucosal epithelial cells. In the lung, minimal to slight expression of mCherry protein was

seen in mucosal epithelial cells of the bronchi in animals administered ≥2.3 x 10^{10} GC/kg AAV6.2-mCherry. Minimal to moderate expression of mCherry protein was observed in the respiratory epithelium of the nasal turbinates in animals administered ≥2.3 x 10^{10} GC/kg AAV6.2-mCherry. Our data demonstrates the feasibility of using AAV nose-only murine inhalation model in AAV based toxicology and biodistribution studies aimed at monogenic pulmonary diseases. We were able to safely and efficiently administer AAV6.2-mCherry droplets simultaneously in multiple animals and minimize viral contamination in the facility by adopting contamination mitigation strategies. The development of this AAV inhalation murine model is a promising path forward in the safety assessment of inhalation gene therapy studies focused on pulmonary diseases such as Cystic fibrosis and α-1-antitrypsin (AAT) deficiency.

1492. Investigating the Mechanisms of AAV-Mediated Dorsal Root Ganglia (DRG) Toxicity by Single Nucleus RNA Sequencing in C57BL6 Mice

Sam Hana[1], Dan Ferretti[2], Benjamin Doyle[2], Zachary Hawley[2], Patrick Cullen[2], Thomas Carlile[2], Christelle Lasbleiz[3], Maria Zavodszky[2], Nadine Mastre Frances[3], Fergal Casey[2], Shih-Ching Lo[2]

[1]Biogen Inc | PSL-EPHE, Montpellier, France,[2]Biogen Inc, Cambridge, MA,[3]PSL-EPHE, Montpellier, France

Somatosensory neurons play a critical role in the sensation and the responses to external stimuli. Sensory neurons have shown to be susceptible to toxicities induced by high doses of adeno-associated virus (AAV) and AAV gene products. This pathology is generally characterized by neuronal and axonal degeneration in NHPs, piglets, rats and others. In this report, mice were shown to exhibit molecular and transcriptomic alterations consistent with findings in prior studies. Wild-type C57BL6 mice were intrathecally dosed with AAV9-duplex-amiR vector (PackGene Biotech) at various doses, animals were then sacrificed for lumbar DRGs used in single-nucleus RNA sequencing. At 3 weeks post-injection, serum neurofilament light (NfL) levels surge in response to the AAV9 vector. A notable reduction of neuronal (>50%) and Schwann nuclei (~8%) were detected in mice with high serum NfL levels. A new cluster labelled repair/activated satellite glial emerges along with an increase in T cells and macrophage cells. Key developmental and repair associated (axonal guidance) genes were upregulated in repair/activated satellite glia. Innate and adaptive immune genes were upregulated in macrophages and T cells. This study provides valuable cell type specific mechanistic insights in response to AAV-induced DRG toxicity in mice. These findings may aid in designing of immunosuppressant-based mitigation approach to alleviate AAV-related DRG toxicity for improved use of gene therapies in treating CNS diseases.

Friday Posters: AAV Vectors - Product Development Manufacturing and Approval Considerations

1493. Intact Mass Analysis of Adeno-Associated Virus (AAV) Capsid Proteins Using a LC-QTOF Mass Spectrometer

Xiaomeng Xia, Evelyn Wang, Yoshiyuki Okamura, Tairo Ogura

Shimadzu Scientific Instruments, Columbia, MD

Over the last three decades, the field of gene therapy has witnessed remarkable progress, evolving from laboratory experiments to the approval of products by the Food and Drug Administration (FDA). Adeno-associated viruses (AAVs) have emerged as widely utilized vectors in gene therapy due to their high transduction rates, wide tissue specificity, low immunogenicity, and minimal genotoxicity. AAVs comprise two primary components: a viral vector protein capsid and encapsulated DNA transgene. The AAV capsid consists of three highly homologous viral proteins (VPs) known as VP1, VP2, and VP3. The comprehensive characterization of these VPs, encompassing their sequence, stoichiometry, and post-translational modifications (PTMs), is crucial due to their pivotal role in viral infectivity and vector potency. LC-MS is known to be a powerful analytical technique by offering a robust method to directly measure the protein molecular weight, thus providing both specificity and sensitivity for rapid confirmation of AAV capsid serotypes. However, several challenges remain to be addressed for the analysis of AAVs compared with conventional recombinant proteins. AAV samples exhibit greater structural complexity and are typically present at very low concentrations. Consequently, the LC-MS platform chosen for characterizing AAVs must possess exceptional sensitivity for detecting these rare viral proteins and ensure precise measurement of their intact mass. In this study, we present the characterization of AAV capsid proteins using liquid chromatography and mass spectrometry (LC-MS) based assays, specifically intact mass and PTMs. AAV8 was chosen as a specific case study. The titer of the AAV8 sample received was 4.691E12 vp/mL. The AAV8 sample underwent treatment with 10% (v/v) acetic acid for 15 minutes, followed by centrifugation at 12,000 rpm for 5 minutes. The dissociated AAV8 proteins were analyzed using LC/MS-QTOF, employing a Shim-pack Scepter C4 column on a Shimadzu LCMS-9030. The C4 column was pre-conditioned with 0.1% difluoroacetic acid to increase the separation of the viral proteins. Mobile phases were 0.1% formic acid in water and acetonitrile without any ion-pairing reagent, which minimized the mass spectrometer fouling. The chromatographical separation of three viral proteins of AAV8 capsid was achieved in a gradient of 30 minutes. The intact mass workflow in Protein Metrics (PMI) was employed for the deconvolution of intact protein spectra. The masses for peaks at 5.59, 6.02, and 6.84 min were calculated to be 81,747.5, 66,598.2, and 59,804.5 Da upon MS charge deconvolution using PMI. The peak eluting at 8.15 min was found to have a mass of 50,593.0 Da, matching the mass of a VP3 fragment resulting from hydrolysis. The observed masses were agreeable with the theoretical masses of VP1, VP2, VP3 and VP3 clip, suggesting that the developed method can measure intact VP masses. The accurate mass measurement also provides a means to deduce the

potential PTMs on the VPs including acetylation and phosphorylation. For example, VP3 and VP3 clip show a mass difference of 42 Da between the measured mass and the corresponding theoretical mass, suggesting the addition of N-terminal acetylation as the potential molecular form, whereas the peaks showing 80 Da mass shift on VP2 were attributed to the phosphorylated proteoforms. VP1 was observed with both acetylation and phosphorylation PTMs from the mass shift. In conclusion, a LC-MS based method was developed to enhance the thorough characterization of AAV capsid proteins. This contributes to improved analyses of AAV capsid proteins, enhancing understanding and providing guidance for the development and manufacturing of new AAV therapeutics.

1494. Breakthrough Technology for AAV Mass Production -One-Step Producer Cell Lines Establishment with Originally Designed Novel Plasmid-

Yusuke Mori, Taichi Muraguchi, Katsuko Sato
Fujifilm Corporation, Kaisei-machi, Kanagawa, Japan

[Purpose] Recombinant adeno-associated virus (rAAV) vectors are popular emerging modalities for gene therapy. Current manufacturing processes using plasmid-polymer complex transfection are expected to be insufficient to meet the demand for the estimated patient numbers, due to difficulties in controlling the plasmid-polymer complex during manufacturing . The plasmid-polymer complex size changes with time and impurities, creating difficulties in handling at production scale. The high cost of the plasmid itself is another limiting factor. To solve these problems, we have developed "AAV producer cell lines" in which the genes necessary for AAV production were incorporated into the chromosomes of the production cells, and established a method for high production of AAV vectors using these cell lines. [Methods] A unique design was constructed in which the genes required for AAV production, REP/CAP/Helper/GOI, were carried in a single plasmid and a drug-inducible switch was incorporated to avoid cellular damage caused by AAV gene expression. Plasmids were integrated into the HEK293 cell chromosomes, and cell lines with all genes incorporated were selected within 2 months. [Results] The established AAV producer cell lines showed proliferation comparable to the parental HEK293 cells without inducers. Subsequently, AAV production was confirmed by the addition of inducers. The culture was expanded in the bioreactor and reached a cell density of 30 Mcells/ml, and then >$1x10^{12}$ vg/ml AAV vectors were produced by adding the inducer. Furthermore, the cell culture medium was withdrawn from the perfusion reactor every 24 hours for 7 consecutive days, and AAV production was induced with the withdrawn cell culture medium, allowing the continuation of high production. In the near future, this technology will be able to meet various needs such as mass production and scale-up/down of vector manufacturing.

1495. Small Molecule Screen Identifies Targets That Increase AAV9 Production in Suspension HEK293 Cells

Francesca Ispaso[1], Kaylin Fisher[1], Francis Grafton[1], Markus Hörer[2], Chris A. Reid[1], Mo Mandegar[1]
[1]Ascend Gene and Cell Therapies, Alameda, CA,[2]Ascend Gene and Cell Therapies, Munich, Germany

The use of recombinant AAV as a vector for gene delivery is widespread, with over 900 pre-clinical and clinical programs underway. However, inefficient manufacturing methods result in high costs, limiting the availability of gene therapies. In this study we describe a high-throughput small molecule screening strategy to identify compounds that increase the capacity of cells to produce AAV9. Previously we had reported identification of a novel small molecule (SM-016) from a screen of 3,000 bioactive molecules that we validated in shake flasks and Ambr15 bioreactors. For our current study we wanted to further expand on the diversity of biological targets screened. In this study, we first developed a miniaturized suspension adapted high throughput screening strategy: ATLAS (Arrayed Targeted Library for AAV Screening). We performed optimization studies to show translatable, reproducible, and comparable AAV9 yields from 96 well to 125mL shake flask format. Next, we performed a screen using a curated compound library of over 700 small molecules. Targets identified include epigenetic modulators, DNA damage response, GPCR and transmembrane transporters, cell cycle modulators, anti-infection, and metabolic targets. Evaluation of top hits are currently underway.

1496. Advancing Serological AAV Assays with Human Chimeric Antibody Standards

Dana Holzinger[1], Silja Syrbe[1], Lisa Mutz[1], Christina Querfurth[1], Katharina Hauser[1], Klaus-Ingmar Pfrepper[1], Morten Seirup[2], Trish Hoang[2], Marjeta Urh[2], Caroline Odenwald[1], Maik Lander[1]
[1]PROGEN, Heidelberg, Germany,[2]Promega Corporation, Fitchburg, WI

Recombinant adeno-associated virus (AAV) vectors are leading tools for viral gene therapy. However, many humans in the general population have developed antibodies against AAV as a result of naturally acquired infections, which might affect efficiency and safety of the gene transfer using AAV vectors. Therefore, testing for pre-existing AAV antibodies in patient sera is an indispensable step for the selection of patients for AAV gene therapy clinical trials. The use of suitable positive controls for the development of reliable and reproducible serological AAV assays is crucial. Therefore, we developed recombinant human chimeric AAV antibodies based on our exclusive portfolio of neutralizing anti-AAV mouse monoclonal antibodies (see antibody clones in Tab. 1). The antibodies retain the identical variable region compared to the mouse monoclonal AAV antibodies while containing a human Fc region. This allows the detection with an anti-human secondary antibody and ensures comparability with human serum antibodies. Here, we show characterization data of our new human chimeric antibodies, including binding affinity, cross-reactivity and their application in serological assays.

1497. Unraveling AAV Bioprocessing: Advanced Analytics for In-Depth Insight and Process Optimization

Qiuge Zhang, Morgan Ludwicki, Jacob Calhoun, Masa Nakamura, Mauhamad Baarine
3M HealthCare, Saint Paul, MN

In Adeno-Associated Virus (AAV) manufacturing, the lack of comprehensive analysis on AAV quality and host cell DNA removal at each unit operation through the entire bioprocess is impeding our ability to identify the key steps for further optimization. This study tried to bridge the knowledge gaps by developing advanced analytics capabilities to characterize both AAV quality and impurities from upstream to downstream. In a case study, we varied nuclease concentrations during cells lysis, clarified the lysates using the charge-based 3M™ Harvest RC Chromatographic Clarifer, and processed the AAV-containing fluids through AAVX affinity chromatography. We employed qPCR for residual host cell DNA/plasmid DNA quantification and droplet digital PCR (ddPCR) to profile residual host cell DNA size distribution after each unit operation. Additionally, ELISA (Enzyme-Linked Immunosorbent Assay), ddPCR, Stunner, and SEC-MALS (Size-Exclusion Chromatography with Multi-Angle Light Scattering) were utilized to assess AAV quantity and quality at different steps in the purification process. This comprehensive analytical study throughout the entire bioprocess has helped us develop an understanding of the DNA impurity removal dynamics. The investigation also enabled us to pinpoint the key clarification step to simultaneously simplify the AAV purification process bioprocess and improve the purity of the resultant AAV.

1498. A Versatile Suspension-Based Platform for the Manufacturing of AAVs

Ahmed Youssef, Melanie Langhauser, Andreas Schulze, Alexandra Schoberth, Florian Sonntag, Markus Hoerer, Karl Heller
Ascend, Planegg-Martinsried, Germany

Recombinant adeno-associated virus (**rAAV**) is among the most widely used viral vectors for gene therapy. However, **vector manufacturing** remains a challenge as the demand for high quality AAV vectors is far greater than the available capacity, hence manufacturers tend to scale up the process which impacts the costs as well as the quality of the AAV produced. Additionally, manufacturing processes vary from one AAV serotype to another and show dependency on vector length and molecular configuration, increasing the time for process development, scale-up and having AAV ready for clinical supply. We have invested in developing a **robust modular suspension platform** process that minimizes batch variability and builds quality into the product by design. Using high-throughput scale-down models this process decreases complexity, increases the flexibility for scale-up, and thereby decreases the cost of goods (COGs). Our platform is an **end-to-end process solution**, composed of multiple unit operations that are differentiated from the industry. This process platform was optimized for vector yield and potency at best possible quality, to align with the stringent, continuously evolving regulatory constraints, while allowing for flexibility and adaptability to specific customer program needs. We present here **yield and product-related impurity data** for the most frequently clinically applied AAV serotypes at different bioreactors scales using our proprietary split two plasmid platform. We are continuously expanding our data to show universal applicability of our modular platform over a wide range of AAV serotypes. The modularity of our platform and our continuous investment in understanding process dependencies that require specific solutions, offers the necessary flexibility to quickly adapt the manufacturing process to specific product needs. This streamlines overall process development and scale-up and reduces overall development costs. We continue to **innovate AAV gene therapy** with a major focus on CMC to support the field in making AAV gene therapy accessible to patients suffering from more common diseases with higher risk benefit profiles and accordingly high regulatory bars. Our commitment is to develop the safest possible products for our clients and their patients.

1499. Characterization of Recombinant Adeno-Associated Virus 2 rAAV2 Capsid Content Variation for Enhanced Gene Therapy Efficacy

Michael J. Born
Process Analytical Development-Viral Vectors, MilliporeSigma - Carlsbad, Carlsbad, CA

rAAV have revolutionized the field of gene therapy, exhibiting immense potential in treating a diverse range of diseases. Understanding and characterizing the AAV capsid content variation is crucial for optimizing their efficacy. Here, we will present AAV2 capsid characterization data from upstream and downstream vector processing. Our study aims to comprehensively characterize the critical quality attributes of AAV2, including empty/full ratios, partially filled capsids, and various biophysical properties. By leveraging advanced analytical techniques, we elucidate the impact of these factors on the process performance of AAV2 in gene therapy applications. The investigation of AAV2 capsid content variation is vital for unraveling the complexities associated with vector production and optimizing therapeutic outcomes. The findings presented shed light on important considerations for the design and development of AAV-based gene therapies.

1500. Advancing AAV Bioprocessing: Cost-Effective Clarification with a Charge-Based Chromatographic Clarifier

Morgan Ludwicki, Qiuge Zhang, Jacob Calhoun, Mauhamad Baarine, Matthew Peters, Joris Van de Velde
3M Healthcare, St. Paul, MN

AAV (Adeno-Associated Virus) bioprocessing poses a unique challenge in that the cells often must be lysed in order to extract and harvest the AAV particles from the host cell culture. The lysis of the cells concurrently releases host cell DNA which must then be removed during the purification process. Typically, expensive nucleases are deployed to degrade the host cell DNA prior to clarification. Our solution addresses this challenge by employing the charge-based 3M™ Harvest RC Chromatographic Clarifier to remove host cell DNA and substantially reduce the amount of nuclease utilized in the purification process. Through experiments with varied nuclease concentrations during lysis, we demonstrate that Harvest RC can effectively remove

host cell DNA while achieving high AAV recovery yield and substantial turbidity reduction. A case study further demonstrated the seamless processing of clarified fluid on AAVX affinity chromatography, achieving equivalent AAV quality and purity. The Harvest RC-based clarification process has been effective with multiple serotypes, is scalable across device sizes, and is compatible with upstream and downstream unit operations. This versatility positions Harvest RC as an adoptable solution for AAV bioprocessing, streamlining the workflow by enabling high AAV quality and reduced costs.

1501. AAV Vectors Produced by the AAVone Single-Plasmid AAV Production System Show Low Levels of rcAAV

Rongze Yang[1], Zhen Shi[1], Yu Liu[1], Yuyan Wang[1], Yuling Dai[1], Leming Wang[1], Cuiping Chen[1], Haniya Zaidi[1], Jiarui Liang[1], Ngoc Tam Tran[2,3], Daozhan Yu[1], Phillip WL Tai[2,3], Qizhao Wang[1]

[1]AAVnerGene Inc., Rockville, MD,[2]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[3]Department of Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA

We previously reported the development of an all-in-one, single-plasmid AAV production system, namely AAVone, in which the Ad helper genes (*E2A*, *E4orf6*, and *VA RNA*), AAV helper genes (*rep* and *cap*), and the AAV vector genome are consolidated into a single compact plasmid (pAAVone). This design has a 13-kb 'backbone' (*i.e.*, obligate plasmid sequences not packaged into vectors), which is significantly larger than AAV's packaging capacity. In this system, an AAV vector can be efficiently generated by simply transfecting the corresponding pAAVone plasmid into host cells. The system shows a two- to four-fold increase in AAV productivity, compared to traditional triple-plasmid transfection production system for the same transgene. One major concern for the AAVone system is the potential for generating replication-competent (rc) AAV, since *rep*, *cap*, and the inverted terminal repeat (ITR) sequences are present in the same plasmid. To test the rate of rcAAV in AAVone system, we employed three different methods: (1) Using qPCR primers targeting both ITR and *rep*, comparable levels of ITR-*rep* hybrids, which represent a potential for rcAAV contamination, were observed at relatively low percentages, in both the AAVone and triple plasmid systems. (2) For a comprehensive analysis of recombination events, we employed single molecule, real-time (SMRT) sequencing to examine AAV-like genomes. The analysis revealed that the probability of ITR-rep-cap triple-chimeras was 0.046% (90 out of 196,753) in the AAVone system and 0.021% (27 out of 131,989) in the triple-plasmid system, and none of these sequences exhibited an intact wtAAV-like, typical rcAAV structure (ITR-rep-cap-ITR). (3) Moreover, a cell-based assay(Conducted by WiXi Apptec) indicated that rcAAV level from the AAVone-produced vector negative at 10^9 vector genome level. These results indicate that the AAVone system can effectively produce AAV vectors with low risk of rcAAV. AAVone represents a revolutionary AAV production system to make AAV gene therapy more accessible and affordable.

1502. Scalable, High-Titer Production of Clinical Grade Adeno Associated Virus in the Gibco™ CTS™ AAV-MAX Helper-Free AAV Production System

Chao Yan Liu[1], Kenneth W. Thompson[1], Paula Decaria[2], Kyle Williston[1], Collin Rasmussen[1], Mariah Weinkauf[1], Natalie Miller[1], Emily Jackson-Holmes[3], Jonathan Zmuda[1]

[1]Thermo Fisher Scientific, Frederick, MD,[2]Thermo Fisher Scientific, Logan, UT,[3]Thermo Fisher Scientific, Carlsbad, CA

Production of high-titer AAV at clinical scales remains a significant challenge for the gene therapy industry. Developers need to understand how to optimize, scale, and qualify their manufacturing process for AAV production under the tightest of timelines. Due to high demand in the gene therapy space, effective solutions to address these challenges must be developed and implemented. Until recently, AAV production took place primarily in adherent cultures using 293T cells in the presence of fetal bovine serum; however, such adherent systems suffer from a number of significant drawbacks including difficulty in scaling up, the presence of the SV40 large T antigen in the producer cell line as well as cost, consistency and regulatory considerations stemming from the use of animal sera. To address these shortcomings, we developed the Gibco™ CTS™ AAV-MAX Helper-Free AAV Production System, a chemically defined, suspension based AAV production system that allows for scalable, high titer production of AAV viral vectors in a non-293T cell lineage. The AAV-MAX system comprises all of the components required for scalable AAV production in mammalian cells including: (1) a clonally-documented, high-titer 293F-derived producer cell line (VPC2.0), (2) a chemically defined growth and expression medium, (3) a production enhancer, (4) a cationic lipid-based transfection reagent and booster, (5) a protein-free complexation buffer, and (6) a Polysorbate 20-based lysis buffer. The AAV-MAX system delivers AAV titers at ≥5x10^{10} viral genomes per mL (vg/mL) across multiple AAV serotypes with excellent scalability from multi-well plates to stirred tank bioreactors making the AAV-MAX system an ideal production platform for generating clinical grade AAV. Here, we provide characteristics around the key components of the system to assist in the development of robust AAV production processes from bench to clinical/commercial scales.

1503. Evaluation of Media Additive for Enhancing rAAV Productivity in Triple Transfection Platforms

Harini Dhamodaran Sampath, Misha Chittoda, Swetha Kannan Iyer, Lisa Stukenborg, Shawn Bourque, Aditya Utturkar, Samantha Desmarais, Neil Shah, Pete Clarner, Junghae Suh, Davide Gianni

Gene Therapy Accelerator Unit, Biogen, Cambridge, MA

Over the last decade, there has been significant and progressive growth in the number of AAV (Adeno Associated Virus) gene therapy programs moving into clinical trials, supported by the recent approval of multiple rAAV-based gene therapies. This translated into an increased demand for clinical and commercial grade rAAV vectors, thus exposing weaknesses and limitations of the current rAAV

manufacturing paradigms. Hence, extensive interest has been focused on the refinement of existing processes to improve the capability to generate high-quality material in a scalable fashion while abating manufacturing costs. The use of media additives has been historically adopted in biologics production processes as a key approach to address this problem. Here, we demonstrate that supplementation of compound X in the production medium at the pre-transfection phase can increase rAAV productivity in adherent triple-transfected human embryonic kidney (HEK293T) cells up to six-fold. The media additive was assessed at different concentrations showing a dose-response effect. Genome titer improvement was confirmed to be scalable across two different scales (T150 flask and CellSTACK®-10 chamber) as well as transgene-independent. Critical quality attributes such as potency and viral genome integrity are currently being assessed. Furthermore, the optimal time for addition, and identification of the molecular target(s) associated with the observed effects, will be investigated. These findings confirm the value of chemical additives to overcome some of the challenges associated with rAAV manufacturing and highlight the impact of an improved understanding of the cellular pathways regulating rAAV production on the development of focused screening of new compounds.

1504. Novel Dual-Plasmid System: Next-Generation AAV Production Strategy with High Productivity and High Quality

Weixiao Huang[1], Danqing Xiao[1], Longxiang Sheng[1], Xueer Yao[1], Ye Bu[1], Paul Li[2], Liying Yang[1], Xin Swanson[1]

[1]PackGene Biotech INC, Houston, TX,[2]PackGene Biotech, Houston, TX

The traditional triple-plasmid transfection system has been widely employed in industrial AAV manufacture due to its stable scalability and flexibility. Approximately two decades ago, a dual-plasmid system, consolidating RepCap and adenovirus helper genes into a single plasmid, was developed to reduce the cost of GMP-level plasmid manufacture and simplify the process. However, challenges such as relatively low productivity, quality issues, and the risk of replication-competent AAV (rcAAV) persist in the dual-plasmid system. In this report, we present a novel dual-plasmid system featuring a uniquely designed TP vector with optimized sequences and arrangement of vector elements. Our findings indicate that this novel system demonstrates higher AAV productivity and quality compared to the traditional triple-plasmid system, with minimal risk of rcAAV contamination. For process development, we screened seven different transfection reagents and identified a low-cost option suitable for high-titer production in the dual-plasmid system. Transfection process parameters have also been optimized to enhance productivity and reduce process-related impurities. To assess scalability, we tested the system with various therapeutic Gene of Interest (GOI) plasmids on both Ambr 250 bioreactor and 3L stirred bioreactor, demonstrating either equal or higher yields and lower process-related impurities for the dual-plasmid system. In conclusion, we have developed a next-generation dual-plasmid AAV production system that establishes a new manufacturing process platform with high productivity, quality, and cost-effectiveness.

1505. A Genome-Wide High-Throughput Gain-of-Function Screen Identifies Novel Targets for Improved AAV9 Production

Chad Wang[1], Muskan Sheth[1], Francesca Ispaso[1], Isha Ukani[1], Markus Horer[2], Mo Mandegar[1], Chris A. Reid[1]

[1]Ascend Gene and Cell Therapies, Alameda, CA,[2]Ascend Gene and Cell Therapies, Planegg, Germany

Recombinant AAV, a widely used vector for in vivo gene delivery, is being utilized in ongoing pre-clinical and clinical programs. Current manufacturing inefficiencies lead to high costs, poor yields and compromised quality in gene therapy products. The high costs restrict broader application, while quality issues affect the vector's potency, immunogenicity and overall safety, necessitating higher dosages and increasing the risk of severe adverse events following dosing. Our study utilized the ATLAS (Arrayed Targeted Library for AAV Screening) miniaturized screening platform to systematically over-express approximately 18,000 ORFs, aiming to enhance AAV9 production. Our screen identified transcription factors, epigenetic regulators, DNA replication factors, RNA regulators, protein ubiquitination, and metabolic targets as primary enhancers of AAV9 production. Confirmatory studies indicated a dose-dependent increase in AAV9 production, up to 3-fold from baseline. Moreover, we confirmed that these factors do not negatively impact the potency of AAV. Next, we confirmed these findings in other AAV serotypes including AAV2, 5, and 8. Further work is in progress to identify lead hits which next will be validated in our current manufacturing platform at Ambr15 scale towards yield, potency and key quality parameters focusing on product-related impurities.

1506. High-Throughput Purification Suite Enabling Quality Determination in AAV Bioreactor Development

Matthew G. Petroff[1], Lavoisier Ramos-Espiritu[2], Mi Jin[3], Jonathan Palmer[1], Conor Leahy[1]

[1]Technology Development, Spark Therapeutics, Philadelphia, PA,[2]Technology Development, Spark Therapeutics, Monroe Township, NJ,[3]Spark Therapeutics, Philadelphia, PA

The upstream (USP) production of AAVs generates multiple product variants, such as fragments, empty capsids, or capsids with incorrect transgene. Purification is typically required prior to assessing these variants in a cell culture, bottlenecking optimization of quality in bioreactor processes. We describe a high-throughput (HT) workflow to enable quality assessments of bioreactor cultures. The workflow combines automated miniature chromatography to support affinity purification of small-scale cultures, and a HT testing strategy to facilitate rapid turnaround of key product attributes, including empty and partial concentrations and viral protein ratio. In the first case study, this workflow enabled the understanding of product quality from the triple-transfection industry standard. The HT affinity product was subsequently purified through HT polishing chromatography, revealing key bioreactor parameters that led to product variants not removed by the platform downstream process. In a second case study, the workflow supports understanding quality during development of

cell culture process intensification. Overall these tools enable improved understanding of upstream processes for improved drug substance quality and manufacturing robustness.

1507. Preparation and Administration of Low Dose AAV Drug Products via Systemic, CNS and Ophthalmic Delivery Routes: Complexities and Solutions

Smrithi Padmakumar, Leslie Lam, Andrew Jones, Mi Jin
Drug Product Development, Spark Therapeutics, Philadelphia, PA

Ensuring patient safety and efficacy during preparation and administration of low dose AAV drug products can be significantly challenging due to concerns such as adsorption losses, impact on Critical Quality Attributes, particle leaching, microbial contamination etc. The complexity of available specialized administration devices (used particularly for CNS and ocular delivery), filters, infusion components and closed system transfer devices (CSTD) with multiple contact parts and large surface areas may exacerbate the problem. Probable risks include loss in titer and potency imparted by AAV adsorption to product contact surfaces, as well as sub-visible particle (SVP) formation compromising the vector quality. In addition, complex surgical procedures may require extended hold or operation time bringing risks to both product stability and patient safety. This also emphasizes the requirement to develop stable formulations particularly compatible with the intended route of administration and simpler approaches to make the administration process error free and less complex. Herein, we present a few examples from case studies for IV, CNS and ocular administration of low dose AAV drug products and the associated risk mitigation strategies.

1508. Development of Off-the-Shelf pRepCap and Customizable pGOI Plasmids for AAV Production

Ruigong Wang[1], Siddhartha Paul[1], Olga Nikiforova[1], N'Dea Wheeler[1], Johann Umali[1], Kavita Sapru[2], Bhargavi Kondragunta[1]
[1]Internal R&D and Product Development, Catalent Pharma Solutions, Baltimore, MD,[2]IPT - Process Development, Catalent Pharma Solutions, Baltimore, MD

Recombinant adeno-associated viral (AAV) vectors have emerged as one of the powerful tools in the field of human gene therapy, based on their excellent safety profile and ability to achieve long-term efficacy both in pre-clinical and clinical models. AAV-based gene transfer vector manufacturing usually involves transient co-transfection of human embryonic kidney (HEK) 293 cells with three vector plasmids. The quality and titer of AAV product has been correlated with improved vector design. Standardized plasmids could greatly speed-up AAV production platform development along with reducing the variability of these critical raw materials. At Catalent, we have now developed off-the-shelf (OTS) RepCap vectors, along with a novel customizable ITR (Inverted terminal repeat)-plasmid backbone to add to our repertoire of vectors. We recently developed multiple pRepCap plasmids for serotype such as AAV1, AAV3, AAV8, and AAVrh10, which are uniquely offered by Catalent as OTS plasmids. We designed these plasmids by assembly of IP-free elements to achieve maximum productivity, stability, and safety. The pRepCap plasmids offered at Catalent resulted in high vector genome titer, above 1E11 vg/mL by digital droplet PCR, along with low host cell DNA (hcDNA), ≤ 200ng normalized hcDNA per 1E13 vector genome, and percent full capsid between 50- 80 % by AUC (analytical ultra-centrifugation) following one-step affinity purification. These vectors have been integrated with Catalent's UpTempo^{SM}AAV Platform Process. We recently also developed a customizable pGOI plasmid, including customizable features for clients, such as enhancer, promoter, and regulatory elements, along with multiple cloning sites.We have evaluated our pGOI plasmids with GFP (Green Fluorescent Protein), and pGalacorin® gene sequences in conjunction with our proprietary clonal HEK-293 cell line. Each of these plasmids has been transformed into E. coli strains, and high producing clones with highest plasmid titer and quality were selected for generating Research Cell Banks (RCBs). Currently R&D grade plasmid and GMP plasmid are ready to be offered to clients to support clinical manufacturing of AAV gene therapy products.

1509. Process Development to Achieve a Novel, Scalable and Highly Cost-Effective Method of AAV Manufacturing Using Whole Plants as Bioreactors

Kevin Wong, Pranav Dinesh Mathur, Qingqing Xie, Terri Grider, Finley Burns, Katarina Dumar, Matthew Schafer, Jake Connors, Daniel Gibbs
Cirsium Biosciences, San Diego, CA

Introduction: Advancements in the development of plant expression vectors and glycoengineering have positioned plants as a superior option for biologic production. Manufacturing costs, scalability, and production capacity heavily influence product market availability, affordability, and profitability. Gene therapy manufacturing faces challenges in these aspects. Plant-based systems offer advantages in reducing the production cost of recombinant biologics and simplifying scale-up by bypassing the necessity for significant capital investments in sophisticated cell culture facilities and expensive culture media for biomass generation. Therefore, we have developed a novel AAV-manufacturing platform using transient gene expression in whole plants to produce high quality AAV vectors that are significantly cheaper and faster to manufacture. Methods: Plant expression plasmids containing each of the required components (Capsid, Rep, GOI and helper genes) for producing AAV were transformed into Agrobacterium bacteria. Cultures of these bacteria were used to infiltrate Nicotiana Benthamiana (N. Bentha) plants grown in an environmentally controlled grow room via hydroponics. We used vacuum infiltration to infiltrate the N. Bentha plants with the Agrobacterium containing the necessary plasmids, wherein the plant leaves are submerged into infiltration media and then subjected to a negative atmospheric pressure. Post infiltration, plants were grown for another ~1 week after which the leaves were homogenized to obtain the crude lysate. Post clarification, we concentrated the cleared lysate using Tangential Flow Filtration, purified by FPLC affinity chromatography and enriched for full capsids using Iodixanol gradient purification. We conducted several quality assays on the AAVs produced by this method. Results: Agrobaterium containing our recombinant plasmids were able to successfully infiltrate the N. Bentha plants and assemble

the AAV vectors in the leaf cells. We demonstrated yields of greater than 1e14 viral genomes from 1 kilogram of plant biomass, equating to roughly 10-15 plants. We employed identical downstream purification methods typically utilized for purifying AAVs from mammalian cell cultures and achieved comparable recovery percentages. Quality testing showed that purified AAVs had 90% full particles, contained significantly below the FDA recommended endotoxin, and were free of any bacteria, fungi or mycoplasma. Plant produced AAV was infectious to the cells in vitro and ex vivo and in vivo. Conclusion: We were able to successfully use transient expression methods in whole plants to produce high-quality and potent AAVs carrying the GFP gene. Using this AAV manufacturing method reduces the upstream cost of AAV production by over 10-fold. This method does not require excessive amounts of high-quality plasmids or scaling up of cell cultures and therefore drastically reduces the scale up time. The conventional mammalian-based system encounters reliability challenges during the scale-up process. Conversely, in the plant-based manufacturing method, individual plants function as modular and predictable bioreactors, rendering this approach highly scalable with exceptional reliability and speed, with potentially unlimited capacity. In summary, this innovative method of AAV manufacturing holds significant promise. This innovative approach holds promise for revolutionizing AAV manufacturing and enhancing global access to gene therapies.

1510. Vial Containment Systems for Gene Therapies

Margaret Craig, Page McAndrew

Scientific Communications, West Pharmaceutical Services, Inc., Exton, PA

Background Viral vector based gene therapies typically are stored and distributed at ultra-low temperature. There are several requirements of the vial containment system (i.e., vial, elastomer stopper, seal): - maintain container closure integrity (CCI) performance - present no adverse interaction with gene therapy - provide adequate recovery and functionality of vector Understanding performance against these requirements is essential for a manufacturer to decide if a vial containment system is suitable. The purpose of the present study was a multi-year characterization of the performance of vial containment systems comprising cyclic olefin polymer (COP) vials, bromobutyl elastomer stoppers with ethene tetrafluoroethene (ETFE) laminate film facing drug product] and aluminum seals. **Results** CCI performance (laser-based headspace analysis with both oxygen and carbon dioxide) evaluation was made of vial containment systems stored at -80°C for up to two years. Example data are shown in the Figure. No ingress of oxygen was observed over 1 year and only a small amount (5%) was observed over 2 years (likely through permeation of COP vial). There was no system breach. Potential interaction with drug product was evaluated in two ways. The first was by exposure of model proteins to COP vials, glass vials, and various stoppers under agitated conditions and measuring the levels of particles formed and protein loss - both indicators of unwanted interaction that may be extrapolated to virus capsids. Example data are shown in Table 1 - stoppers with ETFE gave enhanced performance as indicated by a lower level of particle formation and better protein recovery. Virus recovery and functionality were evaluated after storage at -80°C and thaw for selected adeno-associated viruses, adenoviruses, and lentiviruses. Example data for the adenovirus Ad5.F35-CMV-eGFP are shown in Table 2. The system comprising COP vials performed equivalent, within error limits, to the standard polypropylene screwcap vial system. **Conclusion** All factors considered (CCI performance, reducing risk of potential interaction, virus viability), vial containment systems comprising COP vials, bromobutyl elastomer stoppers with ETFE laminates, and aluminum seals, offer very good performance for the storage of virus-based gene therapies versus systems comprising glass or polypropylene. Table 1. Particle Formation and Protein Loss upon Exposure of Select Protein to Elastomer Stoppers with and without ETFE Laminate Film

Stopper	Particles per ml (1-10 µm) after 6 hrs agitation	Recovery at 24 hrs (%)
with ETFE	44	98
without ETFE	290	95

Table 2. Vitality of Select Adenovirus after Storage at -80°C in COP and Polypropylene Containment Systems

Ad5.F35-CMV-eGFP **14 days at -80°C**	**Recovery** (viral particles per ml)	**Functionality** (fluorescence absorption units)
COP System	2.3×10^{11}	4.3×10^{5}
Polypropylene System	2.6×10^{11}	4.3×10^{5}

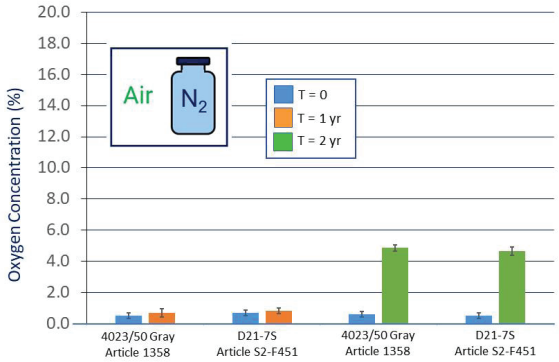


Figure. CCI Performance after Storage at -80°C for One and Two Years

1511. A High Throughput AAV Production Method Development to Support Preclinical Studies

Nannan Jia, Shiliang Hu, Yoni Bason, Nicholas J. DiGioia, Yinxing Chen, June Deng, Janvi Shah, Noel Walsh, William Lee, Lauren Drouin, Matthias Hebben, Jing Liao

Alexion Genomic Medicine, AstraZeneca Rare Disease, Lexington, MA

The cost of adeno-associated virus (AAVs) manufacturing remains a significant challenge in genomic medicine. Preclinical studies, including in vitro and animal studies, demand less total vector genome (vg) but higher throughput for testing multiple AAV vector candidates concurrently. Traditional production methods, encompassing both upstream and downstream purification, limit high throughput production. To address this, we developed mAAVRx, a novel 2-plasmid design that achieves high-yield, high-quality, and potent AAV vectors in scalable suspension HEK293 cells. This optimized upstream process enables high throughput production when combined with an efficient downstream purification. Traditional purification methods, such as

chromatography with CsCl ultracentrifuge, are time-consuming and pose constraints. To address this, we developed a high throughput purification method to enhance efficiency. Various purification methods were tested, and vector titer and recovery rates were measured. To validate potency, luciferase was used as a reporter gene, and vectors purified by different methods were transduced into Huh7 cells. The results indicated that vectors purified by traditional methods exhibited a wide signal range linearly related to dose, which signal range could represent by one of new developed high throughput purification method, while other methods showed a narrow range, reaching a signal plateau easily. To further validate in vivo, vectors with different full capsid percentages were used by spiking empty capsid into a full capsid vector prep, and the experimental results demonstrated similar readouts for 100%, 50%, and 20% full capsid vectors. In contrast, 5% full capsid showed about a 2-fold decrease of in-vivo potency. This suggests that plateau effects with empty capsids are more dramatic in vitro than in vivo. In summary, the high throughput purification method, combined with mAAVRx, streamlines vector production for preclinical studies. Vectors purified by the high throughput methods meet the needs of small-scale in-vivo studies, providing reliable data. However, challenges remain in removing empty capsids and addressing impurities for in vitro studies, necessitating further refinement.

1512. Development of an Assay to Perform High-Throughput Drug Screen in HEK293 Cells to Improve AAV Vector Production

Santosh Panthi, Alan Baer, Nirjal Bhattarai

Office of Therapeutic Products, US Food and Drug Administration, Silver Spring, MD

Adeno-associated vector (AAV)-based gene therapy holds great promise for treating a range of human diseases. Despite the significant potential of AAV vectors in gene therapy, challenges persist in producing high-quality vectors on a large scale. Therefore, innovative strategies are needed to improve the AAV vector manufacturing process. Currently, AAV vectors are commonly produced using HEK293 cells, but during production, the activation of various cellular responses has been observed. The impact of these cellular responses on vector production and quality remains unknown. To explore this, we sought to perform a high-throughput drug screen in HEK293 cells, utilizing commercially available drug libraries that target antiviral, inflammatory, cell signaling, and checkpoint pathways to identify restriction factors that affect AAV quality and yield. To perform such a drug screen, first, we needed a high-throughput screening assay. Here, we report the development of a high-throughput drug screen assay that can be employed to identify small molecule compounds to improve AAV vector production in HEK293 cells. This 96-well plate assay uses an AAV-luciferase vector in adherent HEK293 cells, and the assay is reproducible, robust, and exhibits a high signal-to-noise ratio. Current studies are underway to identify compounds that improve AAV production using this high-throughput assay. Once chemical compounds are identified and their cellular targets in HEK293 are validated, we aim to develop a novel HEK293 producer cell line with knockouts of inhibitory cellular factors using CRISPR/Cas9. This novel HEK293 producer cell line may help to improve AAV vector quality and yield during manufacture.

1513. Development of an Analytical Method for AAV Capsid Proteins for Use in Stability Studies

Aitor Navarro Nieto

Viralgen Vector Core, San Sebastian, Spain

Gene therapy based on adeno-associated virus (AAV) vectors is now a reality, with several products already approved by the FDA and EMA (HEMGENIX®, LUXTURNA®, ROCTAVIAN® ZOLGENSMA®,) and some more to come in the following years. In parallel to this evolution, the requirement for fast and reliable characterization of the Critical Quality Attributes (CQAs) to ensure AAV product quality is also increasing. Among those CQAs, the viral capsid proteins (VP) charge distribution is important for the tropism and potency of the different serotypes. The capsid protein's charge heterogeneity can account for possible post-translational modifications (PTM) that can happen during different steps of AAV vector manufacturing or storage; some of these modifications can hinder the development of gene therapy products. To monitor these modifications, we have developed a method based on imaged capillary isoelectric focusing (icIEF). Since different AAV products can require different formulation buffers which are not always compatible with icIEF analysis, we have developed a procedure that can be theoretically used with any product and then adjusted to obtain the best results in terms of reproducibility. It was previously shown that deamidation of amino acids on the surface of the AAV capsids correlates with charge heterogeneity modifications of the capsid proteins and can have an impact on vector potency. Using icIEF, we show here that the deamidation of AAV9 capsid proteins over time is correlated with the reduction of vector potency. We also checked if this reduced potency was correlated with capsid disassembly. We believe such a method could be beneficial for the development of improved formulation buffers.

1514. Addressing Downstream Purification Challenges: Solving AAV2 Aggregation Issue and Developing Innovative Approaches for Empty Capsid Removal

Ting Yang[1], Jeffrey Kan[1], Chinmay Patkar[2], Gina Zhang[2], Yiling Fang[2], Robert Lu[3]

[1]Process Development, Frontera Therapeutics, Bedford, MA,[2]Analytical Development, Frontera Therapeutics, Bedford, MA,[3]Technical Operation, Frontera Therapeutics, Bedford, MA

Two distinct case studies are highlighted in this presentation. The first delves into the challenges posed by the propensity of AAV2 capsid to aggregate, particularly under low ionic strength buffer conditions. These aggregation issues have significantly complicated downstream purification process development. The narrative unfolds with the successful resolution of aggregation. Additionally, insights are shared on addressing aggregation occurring during the final UFDF formulation step.The second case study explores an innovative method targeting empty capsid removal, Frontera's purification platform showcases its effectiveness in reducing empty capsids to less than 1% in the final drug substance. This breakthrough underscores the platform's capability in enhancing the purity of the drug substance and mitigating potential immunogenicity concerns.

1515. Forced Degradation Study on the AAV Empty Capsid

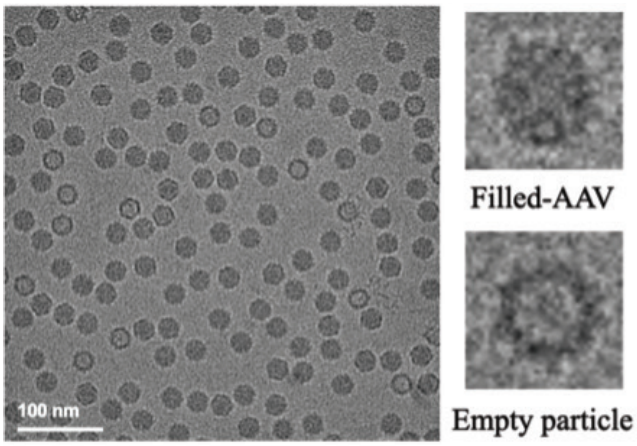
Alesha Arshad[1], Jack Eifert[1], Alexander Yarawsky[1], Dana Holzinger[2], Lisa Mutz[2], Lake Paul[1], Angela Mohs[1]
[1]BioAnalysis LLC, Philadelphia, PA,[2]PROGEN, Heidelberg, Germany

Recombinant adeno-associated viruses (AAVs) have become widely used in gene therapies. The AAV viral vector consists of a protein capsid packaging the single-stranded DNA encoding for the gene of interest. To date, over 13 different naturally occurring serotypes have been discovered which have tropism for different tissues. All serotypes consist of a total of 60 molecules of a mixture of three viral proteins VP1, VP2 and VP3 in the estimated ratio of 1:1:10. Stress studies play an important role in identifying potential degradation pathways which might affect the AAV transduction efficiency. Little is currently known about the degradation pathway of different AAV serotypes. In this study we focus on two serotypes, AAV8 and AAV9, that are commonly used in the development of gene therapy products. A forced degradation study is performed which compares the effect of oxidative stress, low pH, high pH, and freeze-thaw stress on the empty AAV protein capsid. Size exclusion chromatography, dynamic light scattering, sedimentation velocity analytical ultracentrifugation and mass spectroscopy are used to identify the degradation mechanism for the two serotypes.

1516. Development of Image Analysis Methods to Characterize the Wider Adeno-Associated Viral Products Using 100-keV Cryo-Electron Microscopy

Haruka Nishiumi[1], Kiichi Hirohata[1], Mitsuko Fukuhara[1,2], Aoba Matsushita[1], Yasuo Tsunaka[1], Mark Allen Vergara Rocafort[1], Takahiro Maruno[1], Tetsuo Torisu[1], Susumu Uchiyama[1,2]
[1]Department of Biotechnology, Osaka University, Suita, Osaka, Japan,[2]U-Medico Inc., Suita, Osaka, Japan

Adeno-associated viral (AAV) vectors have been the leading platform for gene delivery, especially for *in vivo* gene therapy. AAV-related drugs are safer and more effective than other virus-mediated drugs owing to the non-pathogenicity and specific tropism of AAVs. Meanwhile, one of the significant challenges regarding the AAV vector is the excessive production of empty particles (EPs). EP is a gene-lacking AAV capsid and is generated at the same time as fully genome-containing AAV particles (FPs) during manufacturing. EPs can inevitably be contained in AAV products, potentially leading to reduced transduction efficiency and increased immunogenicity. Therefore, removing EPs and determining the FP to EP ratio (F/E ratio) of AAV products are recommended. To date, several methods to evaluate the F/E ratio and eliminate the EPs in AAV products have been proposed such as analytical ultra-centrifugation (AUC). These methods achieved the high resolution to separate the FPs and EPs; however, most approaches have still challenges in evaluating the exiguous EPs contained in highly purified AAV products. Hence, in this study, we developed image analysis methods to extend the range of F/E ratios using cryo-electron microscopy (cryo-EM). Cryo-EM can visualize the FPs and EPs while showing different morphology that reflects the encapsulated genome owing to their different electron densities inside the capsid; therefore, FPs and EPs can be discriminated through image analysis. Using our developed image analysis methods, we successfully calculated the F/E ratios of various AAV products while showing excellent correlation with theoretical F/E ratios determined using band sedimentation AUC. Subsequently, we assessed the performance of our image analysis methods in evaluating the F/E ratio of highly purified AAV products (purity > 95%). As a result, our methods provided the F/E ratios of highly purified AAV products containing less than 10% of EPs. Furthermore, we demonstrated that our methods revealed the differences in the F/E ratios arising from the different numbers of density gradient ultracentrifugation implementations. These results indicate that our image analysis methods can support the robust characterization of AAV products containing a wide population of EPs with high accuracy, leading to potential improvement of the safety of AAV medication in clinical trials.



1517. Identification of an AAV Affinity Chromatography Elution Buffer That Maximizes Product Recovery and Minimizes Product Degradation

Sierra VanSuch, Ryan DeGroot, Ashley Sacramo, Benjamin Wright
Process Development, Solid Biosciences, Boston, MA

Recombinant adeno-associated virus (rAAV) continues to be a promising vector for delivery of gene therapies due to its proven success in clinical trials and high potential for treatment of diseases with unmet medical needs. To meet the clinical and commercial demands while ensuring an effective product, manufacturing sufficient rAAV quantities while maintaining superior product quality is of paramount importance. Chromatography-based purification methods using AAV Affinity resin are commonly used during manufacture of rAAV due to their ability to effectively remove process and product-related impurities. However, to efficiently elute rAAV vector from these resins at acceptable recoveries, harsh buffer conditions, such as low pH, are typically employed that often result in a rapid negative impact to product quality, such as increased product aggregation or decreased product activity. Here, we discuss development work that was performed on an rAAV Affinity chromatography step that identified modifications to the elution buffer pH, elution buffer components,

and eluate collection method/neutralization that resulted in a two-fold improvement in product recovery, reduced impact to product, and improved manufacturability.

1518. A High Quality *Minicircle* Production Scale-Up Success Story: AEX-HPLC In-Process Controls to Guide Upstream Process Modification

Ram Shankar, Nina Schäffer, Christa Krüsemann, Marco Schmeer, Martin Schleef

PlasmidFactory GmbH, Bielefeld, Germany

Minicircles enhance the quality of AAV vectors in the context of the packaged genomes thanks to the elimination of false encapsidation of plasmid backbone sequences. *Minicircle* DNA production starts with the propagation of a parental plasmid (PP) molecule within a bacterial host strain. At the end of the cultivation phase, an *in vivo* recombination is triggered that allows the *minicircle* (MC) region containing the Gene of Interest (GOI) to be separated as an independent supercoiled construct. The resulting mix of DNA molecules is called a recombination product (RP) which is taken through further proprietary purification stages to obtain the pure MC free of side products such as miniplasmid (MP) which contains the plasmid backbone. Capillary Gel Electrophoresis (CGE) is a routine analytical tool to characterise the final purified MC product. Although CGE can be used to analyse RP, its applicability as an in-process control is limited to cases where the MC and MP have different sizes. When MC and MP are of similar sizes, one had to rely on agarose gel electrophoresis for the differentiation between the two molecules, wherein again a meaningful quantification based on band intensity is not possible. We employed Anion Exchange High Performance Liquid Chromatography as a second method to analyse one-step purified RP samples from our upstream department. AEX-HPLC could beautifully complement the data from CGE and was able to resolve the MP and MC of sizes 4.6 kb and 4.5 kb. Measuring the MC content in fermentation samples gave us unprecedented insight into RP composition and allowed us to adjust the upstream process with the aim of maximising DNA yield per batch. Interestingly, we found that parameters such as induction timepoint and specific growth rate at induction notwithstanding, the proportion of MC in the RP was quite stable whereas the levels of PP and MP balanced each other out. Since only the total amount of these two contaminating molecules mattered in the downstream purification and not the individual amounts, variations in these two molecules could be followed without any consequence. The CGE data for proportion of PP correlated over the entirety of the production project with that from the HPLC data. The final purified MC DNA amount matched with the prediction from in-process controls (27% of RP). We achieved an overall scale up of MC production in *High Quality* grade of over 30X. To allow for possible losses in amount during purification, a large amount of starting material as RP was produced as a safety net. Nevertheless, the final MC product overshot the required amount by 3X. Scaling up MC production is required to cover the increasing demands of starting material for large scale AAV vector production while ensuring vector safety and quality according to regulatory guidelines. On the other hand, large scale production of *minicircles* in *High Quality* grade is also attractive for non-viral vector applications which are making a big comeback.

1519. Matrix Approach for Potency Assay Development of Cell and Gene Therapy Products - A Case Study for Adeno-Associated Virus (AAV) Vectors

Karen Doucette, Sascha Karassek, Marina Alber, Ulrike Herbrand

Biologics Testing, Charles River Laboratories, Erkrath, Germany

Objective: Develop a phase-appropriate potency assay matrix for an AAV gene therapy product. Introduction: Potency assays for cell and gene therapy (CGT) products are more complex than other molecules since these products have several mechanisms of action (MoA), higher variability in the manufacturing process, and limited material for testing. The MoA of an AAV gene therapy product includes a multi-step process of infection, transcription, translation, and function. This complexity presents a challenge to developers to identify a single in vitro assay that reliably and accurately measures potency which is also correlated to in vivo activity. Authorities therefore recommend a matrixed approach for potency assay development for CGT products. This case study illustrates a phase-appropriate potency assay matrix approach reflecting the complex MoA of an AAV gene therapy product. Description: Three separate analytical methods were developed to reflect the MoA of an AAV product: RNA expression of exogenous transgene by RT-qPCR, protein expression by flow cytometry, and activity by functional reporter gene assay. Data is presented from two AAV serotypes and targets. Each method involved transduction of AAV reference material in HEK293T cells to demonstrate dose response over a range of MOI and stability indicating properties when exposed to varying stressed conditions. For functional activity, a test and reference material were evaluated in parallel in a cell-based reporter gene assay for determination of relative potency. Luminescence, as measure of dose dependent activation of that reporter system, was quantified with a plate reader, and the data was plotted and analyzed with a four-parameter logistic fit using PLA 3.0. Conclusions: All developed cell-based assays were specific for the intended AAV product and demonstrated stability indicating properties. These methods can serve as building blocks to develop a potency assay matrix for AAV products with varying capsid serotypes and therapeutic transgenes. It is important to plan sufficient time for thorough assay development and understanding of the capabilities and limitations of each method. Optimal conditions might vary from lot to lot, even with a well-controlled production process; therefore, system suitability criteria need to be set thoughtfully after thorough product characterization. Meetings and contact with regulatory authorities is important to support long term strategic planning for the potency assay matrix approach.

1520. Streamlined Process for Large Scale cGMP Plasmids Manufacturing at Low-Cost Utilizing Continuous Flow Alkaline Lysis, Depth Filters for Clarification, and Two-Step Chromatography

Yujun Wang[1], Xianxiao Chen[1], Guoqiang Li[1], Yanping Yan[2], Hua Zhang[1], Ming Zhong[1], Xiulian Sun[1]

[1]uBriGene Biosciences International Co., Suzhou, China,[2]uBriGene Biosciences International Co., Jinan, China

High-quality plasmids are critical starting materials for packaging viral vectors for gene therapy (e.g. Lentiviruses and rAAVs) using the multiple transient transfection method. Linearized plasmids also serve as templates for *in-vitro* transcription of RNA vaccines and drugs. CGMP-compliant production of large quantities of plasmids at a low cost has emerged as a major demand within the CGT industry. As a one-stop CDMO service provider in the GCT industry, here we present a streamlined process for plasmids manufacturing. It features optimized induction strategies to boost plasmid amplification in *E. coli* culture to as high as 0.5 grams per liter. Bacterial cells are harvested by concentration and exchanged into a resuspension buffer with tangential flow filtration (TFF). Alkaline lysis is carried out via an enclosed contentious flow system, comprising single-use tubing and a mix with no requirement for complex and pricey equipment. Divalent salts are added to the neutralization stage of cell lysis to promote precipitation and removal of host cell RNAs. Clarification of lysis is achieved by depth filtration, which compared to centrifugation is fully enclosed and more scaling-friendly. Anion exchange chromatography (AEX) and hydrophobic interaction chromatography (HIC) are used respectively to capture plasmid from clarified lysis and to enrich supercoiled plasmid species. Notably, AEX with membraneous matrix is utilized, so a higher flow rate can be supported compared to traditional columns packed with resins. Because of this high flow rate of loading, pre-chromatographic TFF concentration can be omitted to save material cost and operation time. The downstream recovery rate has been proven to be stable between 40% to 50% and typical contamination levels are competitive in the industry. In summary, we have streamlined the process for cGMP manufacturing of plasmids at a scale as large as tens of grams at low cost for applications in viral and non-viral vector production.

1521. A Reliable and Robust Methodology for AAV Genome Quantification Using Droplet Digital PCR

Jennifer Srnak, Yicheng Zhao, Andrew Charles Hedman, Shou Liu, Hung Do, Lin Liu

M6P Therapeutics, St. Louis, MO

Adeno-associated virus (AAV) serves as a prevalent gene therapy vector, yet inconsistencies exist in determining its viral concentration within the field. Labs employ an array of techniques—like qPCR, silver stains, and ELISA—to assess correctly packaged viral particles. The need to identify and develop a dependable and standardized method to measure complete viral genome packaging is at the forefront for advancing the field. Drawing from available literature, we conducted a comprehensive survey of existing standard operating procedures for viral genome packaging using the droplet digital PCR (ddPCR), uncovering discrepancies in previously established methodologies. Notably, gene of interest measurements often deviated from adjacent sequence measurements such as promoters or inverted terminal repeats (ITRs). This problem might stem from two potential sources: either the viral genome was truncated, or the protocol required further optimization. Through modification of multiple protocol parameters—such as DNase I digestion, capsid lysis methods, dilution schemes, and restriction enzyme digestion—we carefully refined our approach to improve reliability and consistency. During this progress, a crucial aspect was rectified: the ratio between the ITRs and other genomic components (the gene of interest and regulatory sequence elements) began aligning at the anticipated 2:1 ratio. This alignment correlated with a fully packaged AAV genome, underscoring the unique single-stranded genome structure of AAV with ITRs at both ends that is crucial for stability and packaging efficiency. In addition, our adjustments yielded more highly reproducible results and aligned more closely with measurements from capsid-specific assays such as silver stains. Here, we describe this reliable and robust methodology for AAV genome quantification using the ddPCR system and AAVs produced from a variety of vendors.

1522. Development of an Inducible AAV Rep and Cap Gene-Integrated Production Cell Line for Efficient AAV Manufacturing

Tianqi Jiang[1], Xiaojing Sheng[2], Yixiong Chen[2], Qing Lin[2]

[1]Dept. of Discovery, Lingyi Biotech Co., Ltd., Shanghai, China,[2]Lingyi Biotech Co., Ltd., Shanghai, China

The production of adeno-associated virus (AAV) predominantly utilizes transient triple-plasmid transfection in HEK293-based cells, a method underpinning five of the seven EMA/FDA-approved AAV gene therapy products due to its high product quality assurance. However, the substantial costs of transfection reagents and plasmid production, combined with the limited stability of the transfection complex and challenges in achieving efficient mixing in bioreactors, restrict the scalability of this method, particularly beyond 500-liter production volumes. These limitations highlight the urgent need for an advanced AAV production cell line that integrates all necessary components for efficient AAV production. Addressing this need, our study introduces a novel AAV production cell line, termed 293RSS1.0, through the integration of inducible expression cassettes for AAV Rep and Cap proteins, driven by proprietary inducible promoters and regulatory elements. This strategic integration facilitates the conditional expression of key viral proteins crucial for AAV packaging. The resulting cell line, 293RSS1.0, exhibits exceptional stability across over 20 passages, minimal leakage of Rep and Cap proteins, and a substantial increase in expression upon inducer addition. Significantly, the 293RSS1.0 cell line demonstrates versatility in AAV production, compatible with transient transfection using a Helper plasmid and an AAV gene of interest (GOI) plasmid alongside inducer supplementation. This adaptability positions the 293RSS1.0 cell line as a promising foundation for the development of a comprehensive, cost-effective, and scalable AAV production platform.

1523. An Integrated Process Characterization Approach to Enable Accelerated Commercialization Timeline of an Adeno-Associated Virus Based Treatment for Sanfilippo Syndrome

Jack Ren

Pharmaceutical Development, Ultragenyx Inc, Woburn, MA

Mucopolysaccharidosis type III (MPS III), also known as Sanfilippo syndrome, is a rare disorder that primarily affects the brain and spinal cord (central nervous system). To treat this syndrome, Ultragenyx

developed a recombinant adeno-associated virus (rAAV) based gene therapy product, named UX111, which is currently in its late-stage development. Process characterization (PC) and process performance qualification (PPQ) are essential life-cycle events for late-stage therapeutic product development and required BLA-enabling activities. Usually, the PC activities require a lengthy duration (year or even years) to acquire extensive experimental data to define the optimal process operation space. Here, we present an integrated risk-based PC approach to enable an accelerated pathway for biologics license application (BLA). Specifically, UX111 historical manufacturing data, UX111-specific PC data, and rAAV platform PC data are leveraged to generate the process and analytical control strategy (PACS) and to define PPQ ranges. The integrated PC approach not only provided a solid foundation to design a robust and reliable process, but also may enable the timely delivery of drug product to Sanfilippo syndrome patients in need.

1524. Early Planning and Regulatory Interactions to Facilitate the Development of an AAV Gene Therapy for PaVe-GT

Richa Madan Lomash[1], Jean Dehdashti[2], Oleg Shchelochkov[3], Randy J. Chandler[3], Lina Li[3], Irini Manoli[3], Pramod Terse[1], Xin Xu[1], Rodica Stan[1], Dimah N. Saade[4], Philip J. Brooks[2], Donald C. Lo[1], Carsten G. Bonnemann[4], Charles P. Venditti[3], Anne Pariser[2], Elizabeth Ottinger[1]

[1]Therapeutic Development Branch, NCATS/ NIH, Rockville, MD,[2]DRDRI, NCATS/ NIH, Bethesda, MD,[3]NHGRI/ NIH, Bethesda, MD,[4]NINDS/NIH, Bethesda, MD

Gene therapy development presents multiple challenges, and early planning is vital in the implementation and success of such programs. The Platform Vector Gene Therapy (PaVe-GT) program is an NIH initiative developing adeno-associated virus (AAV) gene therapies for four rare diseases of low prevalence: two organic acidemias and two congenital myasthenic syndromes. Utilizing the platform-based approach, the program aims to incorporate efficiencies throughout the pre-clinical and clinical development process and publicly disseminate scientific and regulatory learnings and experience. As we advance the development of these gene therapies, we share the value of establishing a Target Product Profile (TPP) early in the drug development lifecycle to better inform the development steps and guide the desired clinical outcomes for any investigational product. Paving the way for PaVe-GT's first gene therapy lead candidate, AAV9-hPCCA, we also sought early feedback from FDA in an INitial Targeted Engagement for Regulatory Advice on CBER ProducTs (INTERACT) meeting. The primary objective of this meeting was to receive preliminary FDA feedback on our AAV9-hPCCA proof-of-concept animal studies in supporting prospect of direct benefit (PDB) for the proposed patient population, and the manufacturing and desired critical quality attributes (CQAs) for the gene therapy product. Additionally, in planning for the target population, route of administration, and projected clinical dose, we sought FDA feedback on our proposed clinical trial design. The productive discussions and outcomes from the INTERACT meeting have been applied in the further development of AAV9-hPCCA. Of broader relevance to the field, the FDA tentatively agreed that we could leverage capsid related biodistribution data as we develop the next PaVe-GT AAV gene therapy products using the same capsid. Additionally, FDA requested that we take into consideration the route of administration and transgene expression in tissues positive for vector presence in the case of these subsequent products. This can eliminate the need for some of the duplicative animal experiments and create efficiencies in the process. Overall, early implementation of a regulatory strategy, by establishing a TPP, helps to align study objectives across multidisciplinary teams, with the goal of reducing drug development delays. Here, we detail the elements of a TPP and how its utilization can inform a preclinical development program. We also share the regulatory strategy of AAV9-hPCCA with a focus on INTERACT package preparation, and lessons learned from the meeting with the FDA. This communication aims to provide stakeholders resources that can be applied to rare disease drug development in establishing a viable regulatory path, to clinical trial initiation.

1525. Investigating Impact of Two Detergents on Affinity Chromatography in the Purification of Recombinant Adeno-Associated Virus

Hui Zhang, Lijun Wang

CoJourney Bio Ltd., Lower Gwynedd, PA

Efficient release of adeno-associated virus (AAV) from host cells is a pivotal stage in the purification process. Triton X-100, a commonly used nonionic surfactant and detergent for cell lysis, has traditionally shown satisfactory overall recovery in the purification of viral vectors. However, in response to regulatory changes, Triton X-100 has been banned by the European Union in 2021, and in 2024, the CDE has recommended alternative lysis reagents fit for purpose. This study presents a comparative analysis between Triton X-100 and Tween-20 as lysis agents, assessing their effectiveness in cell lysis, dynamic binding capacity (DBC), and yield during affinity chromatography. The results indicate that both agents exhibit similar efficiency in cell lysis, as determined by viral genome (vg) titer. Furthermore, the product quality after affinity chromatography remains comparable. Notably, Triton X-100 displays a non-specific binding interaction with the POROS CaptureSelect AAVX affinity resin, unlike Tween-20. This unique interaction hinders the resin's binding sites, resulting in a reduction of dynamic binding capacity and overall yield compared to Tween-20.

1526. Prequalification of Analytical Test Methods for Determining the Potency of an Adeno-Associated Virus-Based Gene Therapy Product

Amanda Bries, Lakmini Wasala, Adam Slechta, Vickie Dowling, Wayne Yount

Analytical Development, Kriya Therapeutics, Durham, NC

For recombinant AAV based gene therapy products, several analytical tools are required to fully characterize the potency of these complex therapeutics. Factors such as infectivity, empty/full capsid ratio, transgene expression efficiency and protein function should be considered when developing and establishing potency methods. Here we demonstrate a framework for evaluating potency-related method performance early in product development. A clinical candidate AAV vector was concentrated and then used to generate six different

vector genome (vg) concentrations spanning from above to below the specification range. Each test article was subjected to method performance parameters: range, linearity, accuracy, precision, and specificity. HEK293 cells were transduced by the vector at 3 different multiplicity of infection (MOI) targets. Efficiency of capsid uptake into the cell was analyzed by flow cytometry following product specific staining, whereas transgene expression was determined by quantifying protein expression. Each transduction plate contained a reference standard comparator and results were reported as the average relative potency across 3 MOI doses. The impact of varying ratios of empty capsids on AAV potency was also assessed. Linearity was detected across the range of vg concentrations (16-172% of nominal) when compared against the reference standard at 3 MOI doses. Accuracy was achieved for protein expression at both high and low vector doses (83% and 115%, respectively) when compared against the nominal target. Additionally, a high correlation ($R^2 = 0.9962$) between MOI dose and protein expression was observed in the transduced cells. Methods for both capsid infectivity and protein expression demonstrated specificity for the protein of interest, as well as precision at three different levels including repeatability, reproducibility, and intermediate precision. Notably, when evaluating the impact of empty capsids on potency, results indicated that a greater empty to full ratio attenuated protein expression when dosed at a constant vg/cell target. These data demonstrate that analytical potency methods can meet prequalification standards during early phase development. This work supports future method qualifications and long-term strategies for potency characterization.

1527. Lessons Learned in a Pre-IND Meeting for an AAV-Based Gene Therapy for a Rare Disease

Rodica Stan[1], Richa Madan Lomash[2], Oleg Shchelochkov[3], Irini Manoli[4], Jennifer L. Sloan[5], Randy J. Chandler[3], Venkata RM Mangalampalli[6], Pramod Terse[1], Jean Dehdashti[7], Xin Xu[1], Carsten G. Bonnemann[8], Charles P. Venditti[4], Philip J. Brooks[7], Elizabeth A. Ottinger[1]

[1]NIH/NCATS, Rockville, MD,[2]NCATS, Rockville, MD,[3]National Institutes of Health, Bethesda, MD,[4]NIH, Bethesda, MD,[5]National Human Genome Research Institute, Bethesda, MD,[6]NIH - National Center for Advancing Translational Science (NCATS), Rockville, MD,[7]NIH/NCATS, Bethesda, MD,[8]NINDS/NIH, Bethesda, MD

The Platform Vector Gene Therapy (PaVe-GT) program is an initiative started by a multi-disciplinary group of collaborators to develop adeno-associated virus (AAV) gene therapies for four rare diseases. Specifically, PaVe-GT seeks to increase the efficiency of clinical trial startup by using the same gene delivery system and manufacturing methods for multiple rare disease gene therapies. The regulatory pathway used for the development of the first PaVe-GT platform product can be applied to the next products, and moreover, the process efficiencies and regulatory lessons learned with one product will most likely reduce the time to Investigational New Drug (IND) submission for the remaining PaVe-GT products in development. Public dissemination of the regulatory lessons acquired overall with the platform may also benefit other sponsors in the development of AAV gene therapies for rare diseases. The first PaVe-GT investigational gene therapy product, AAV9-hPCCA, is being developed to treat propionic acidemia resulting from a deficiency of propionyl-CoA carboxylase, alpha subunit (PCCA). As part of a Type B pre-IND meeting with the Food and Drug Administration (FDA), the Agency reviewed our regulatory package and provided valuable feed-back for the proof-of-concept studies in a propionic acidemia mouse model, manufacturing process and analytical testing of AAV9-hPCCA, as well as pivotal efficacy study, GLP toxicology study, and clinical protocol synopsis. The FDA concurred with critical aspects of our manufacturing, pre-clinical and clinical development program and provided detailed recommendations for potency assay development, compatibility protocol design, and clinical trial cohort selection. This input will help us complete the IND-enabling studies and prepare the IND submission. In conclusion, early planning and regulatory interactions with the FDA can help design a better drug development plan and reduce failure mode. The knowledge gained by advancing PaVe-GT program's first product through pre-IND stage will be applied to the other investigational products under development and is being disseminated to the public to aid sponsors of other AAV gene therapy efforts for rare diseases. This research was supported in part by the Intramural research program of the NCATS, NIH.

1528. Forced Degradation of KRIYA-893 Vectors to Demonstrate Product Stability with Stability-Indicating Assays

Catherine Dial, Robert Damitz, Victoria Dowling, Wayne Yount

Kriya Therapeutics, Morrisville, NC

Full understanding of AAV vector degradation pathways and degradation products is limited, which poses a challenge for the development of investigational products. Because recombinant AAV products are comprised of protein and DNA, there are many possible degradation pathways that must be considered during vector development, manufacture, and storage. KRIYA-893 is an investigational gene therapy comprising two AAV1 vectors that contain expression cassettes for insulin and glucokinase for the treatment of type I diabetes. These AAV1 vectors have shown excellent stability at -80°C and after freeze-thaw cycling in the clinical formulation. Forced degradation studies were performed on KRIYA-893 vectors to understand what stresses cause instability and what analytical methods can be used to assess product stability. By comparing degradation profiles, differences in stability of the two component vectors were examined. To understand degradation pathways, the component vectors were subjected to physical and chemical stresses which represented extreme conditions that may be encountered during product manufacture, storage, and handling including: exposure to acidic (pH 2, 4), alkaline (pH 8, 10), thermal (40°C), photooxidative (~2000 lux), and chemical oxidative (1 - 30000 ppm H_2O_2) conditions for durations ranging from 4 hours to 14 days. Product attributes evaluated included appearance, vector genome titer (ddPCR), capsid titer (Gyros), capsid purity (CE-SDS), aggregation (SEC), capsid charge distribution (AEX), genome integrity (CE), and relative potency by protein expression (cell-based assay). These degradation conditions generally resulted in changes to one or more product attributes that were readily detected by the analytical methods used. Highly acidic

conditions for extended durations caused viral genome degradation detectable by CE and ddPCR and accompanying decreases in vector potency. Thermal stress caused a positive shift in capsid charge distribution and decreased potency. Highly alkaline conditions caused a negative shift in capsid charge distribution, but no impact to vector potency was observed. High concentrations of hydrogen peroxide resulted in an almost complete loss of potency at very short durations with no other detectable product attribute changes. Longer duration exposure to oxidants caused decreases in capsid and genome titer and chemical modifications to capsids. The same degradation pathways were observed for both vectors, however the magnitudes of soluble aggregates formed, genome and capsid titer decreases, and vector genome degradation differed between insulin and glucokinase vectors. Conditions encountered during AAV manufacturing and handling have the potential to cause product degradation. The changes identified in this study can inform limits for manufacturing and supply chain control strategies. While most of the degradation pathways identified were common for AAV1 component vectors, some results suggest there may be differences in stability of two vectors of a single serotype that arise from inherent differences, supporting that the vector genome can contribute some modes of instability. A broad analytical panel is needed to detect these product quality changes to ensure gene therapy products have optimal and consistent quality attributes important for product safety and efficacy.

1529. An Improved Affinity Chromatography Process for Adeno-Associated Virus, Serotype 6 (AAV6)

Laura E. Pickrell, Kelley Kearns, Thomas C. Scanlon
Repligen, Lebanon, NH

Improved methods for purifying recombinant adeno-associated virus (rAAV) vectors can greatly facilitate manufacturing scale-up, while reducing costs. The present work focuses on improving recovery of AAV6 capsids from the affinity capture step and demonstration of resin reuse using sodium hydroxide. Capsid recovery at pH 3 can be improved using AVIPure® AAV6 resin, compared to other affinity resins. High-throughput screening and optimization of elution conditions can also enable similarly high capsid recoveries at a softer pH elution condition to alleviate potential concerns regarding viral stability. The alkaline stability and process robustness using AVIPure AAV6 are demonstrated, along with the effects of elution pH, conductivity, salt type, and additives on overall yield and purity.

1530. Utilization of Tenaya's AAV Productivity Boosting Small Molecule (SMB) for Intelligent Design of HEK293 Cell Line to Improve AAV Productivity

Jackson Leong, Beatriz Lim, Farshad Farshidfar, Brooke Rathie, XiaoShan Ke, Joe Woods, Sushanthi Ramesh, Bill Prince, Frank Jing, Kee-Hong Kim
Tenaya Therapeutics, South San Francisco, CA

Adeno-associated virus (AAV) gene therapy is quickly becoming a safe and effective therapeutic modality for the delivery of potentially curative treatments for genetic diseases. One of the major challenges associated with AAV GT is to cost effectively produce AAV Drug Product (DP) with adequate levels of critical quality criteria (safety, identity, strength, purity, potency, and quality). Triple transient transfection using HEK293 process is widely used in both clinical and commercial scale manufacturing. The main limitation of HEK293 is scalability and viral productivity. Tenaya has developed a proprietary Small Molecule Booster (SMB) that can significantly improve AAV productivity in HEK293 transient transfection process using different HEK293 cell lines, processes, and cell culture medium for a wide range of AAV serotypes. Using RNA-Seq, Tenaya has identified multiple genes that are influenced by the addition of SMB. Using genome editing, Tenaya has generated multiple proprietary HEK293 cell lines with improved AAV productivity, demonstrating the unique advantage of HEK293 cell line engineering guided by small molecule booster vs. conventional genome-wide screening strategies.

1531. Development of a Scalable High Yield HEK293 Expression Platform for AAV Manufacturing

Charles Feathers[1], XiaoShan Ke[1], Brooke Rathie[1], Jackson Leong[2], Joseph Woods[1], Frank Jing[2], Bill Prince[1], Kee-Hong Kim[1]
[1]Tenaya, South San Franscisco, CA,[2]Tenaya Therapeutics, South San Francisco, CA

Adeno-associated virus (AAV) gene therapy is quickly becoming a safe and effective therapeutic modality for the delivery of potentially curative treatments for genetic diseases. One of the major challenges associated with AAV GT is to cost effectively produce AAV Drug Product (DP) with adequate levels of critical quality criteria (safety, identity, strength, purity, potency, and quality). Triple transient transfection using HEK293 process is widely used in both clinical and commercial scale manufacturing. The main limitation of HEK293 is scalability and viral productivity. A number of novel expression technologies have been developed in recent years to improve AAV productivity in HEK293 suspension system, but have yet to prove scalability beyond bench-scale. At Tenaya, we have developed a proprietary transfection-based HEK293 expression platform technologies resulting in a scalable process and significantly increase yields, with 7 to 10 times the upstream cell culture titer of conventional plasmid systems and 2 to 3 times yield of novel plasmid systems. Characterization of the AAV products made by Tenaya's proprietary HEK293 platform have been demonstrated to be comparable to traditional plasmid subunit stoichiometry, % full capsids and in vitro expression.

1532. Engineering and Banking of Differentiated iPSC Cells from Wildtype and Isogenic Homozygous Null iPSC Cells to Support Potency Assay Development

Xiaoshan Ke, Jane Yang, Isaac Peria-Gil, Alex Aycinena, Tisho Tefera, Jun Liu, Charles Feathers, Frank Jing, Bill Prince, Kee-hong Kim
Tenaya Therapeutics, South San Francisco, CA

Potency assessment development for AAV Gene Therapy is crucial to support adeno-associated virus (AAV) vector release and is required

for future marketing authorization. Potency assay development is especially challenging for AAV GT used in cardiovascular indications as the products are structural proteins instead of enzymes. One of the key requirements for development of functional potency assay is having a readily available biologically relevant cell line. To meet this need, Tenaya has developed a robust and scalable differentiation process for Induced Pluripotent Stem Cell Derived Cardiomyocytes (iPSC-CMs) suitable for continued potency assay development from early phase potency assay qualification to late phase validation to support routine product characterization and release purposes. This process has demonstrated efficiency of differentiation and maintenance of both wildtype and homozygous null cell lines which exhibit disease phenotypes. Additionally, the iPSCM-CM cell line generated using Tenaya's process is comparable to commercial sources in terms of quality, purity, and performance.

1533. Development of Highly Productive, Rhabdovirus-Free SF9 Insect Cell Line for Large-Scale AAV Production for Cardiovascular Gene Therapies

Jackson Leong, Charles Feathers, Tae Won Chung, Xiaoshan Ke, Sushanthi Ramesh, Frank Jing, William Prince, Kee-Hong Kim

Tenaya Therapeutics, South San Francisco, CA

Adeno-associated virus (AAV) has become a safe and effective modality for in-vivo delivery of gene therapy (GT) into patients suffering genetic diseases. Recent approvals of AAV-based gene therapies, with dozens more in the late-stage development, highlight the increasing demand for efficient and cost-effective large-scale AAV manufacturing with high productivity and consistent quality across scales. Sf9/recombinant baculovirus expression vector system (BEVS) remains as one of well-established production platforms for AAV manufacturing with several advantages over HEK293 platform using the triple transient transfection. While the HEK293 platform can rapidly supply AAV vectors for research and early-stage clinical trials, Sf9/BEVS boasts high packaging efficiency (vector genome yield) and reliable scalability that is suitable for large-scale AAV manufacturing to deliver therapies to larger patient population. Since the identification of a novel invertebrate Rhabdovirus (SfRV) in ATCC Sf9 cell line by the FDA in 2014, demonstrating adequate clearance of rhabdovirus from Sf9/BEVS has been challenging and costly, especially for early stage AAV GT products. In this work, we report the successful development of the new generation of Sf9 cell line that is SfRV-free at Tenaya Therapeutics, which can critically improve the safety and purity of AAV produced in Sf9/BEVS system. Furthermore, Tenaya's SfRV-free cell line also demonstrates excellent scalability, producing AAV at significantly higher productivity compared to SfRV containing Sf9 cells across shake flask, benchtop bioreactor (3 L), and pilot-scale bioreactor (200 L) with comparable product quality and potency. Additionally, the SfRV-free cell line outperforms other commercially available SfRV-free Sf9 cell lines in both serum-free and chemically defined cell culture medium.

1534. Development and Scale Up of a Robust End-to-End Manufacturing Process for High Quality AAV Production

Azam Hassaninasab

Process Development, Elevate Bio, Waltham, MA

Adeno-associated virus (AAV)-based gene therapies have demonstrated substantial therapeutic benefit for the treatment of a variety of disorders and have been commercially approved. With promising clinical data and continued success in pre-clinical and clinic studies, AAV-based gene therapies have emerged as a new class of molecular medicines. Yet, there is limited AAV GMP manufacturing capacity and robust processes readily available for the development of novel AAV therapeutics. Here we describe an end-to-end AAV manufacturing process that was optimized for AAV5 vectors attaining high genome titers and a significantly improved downstream processing yield. During upstream process development, several DOE studies were performed to fine tune critical process inputs. As a result of the work, there is a significant improvement in productivity as well as 60% increase in full capsid from upstream crude harvest. The downstream process was also fully developed based on a two-column chromatography strategy. Several DOE screens were conducted to improve the affinity capture and the IEX purification steps. The recovery of AAV5 affinity capture increased from 45% to >80%. Importantly, the step recovery of IEX was greatly improved with full capsid enrichment >70%, which was confirmed by analytical ultracentrifugation (AUC) data. In addition, a significant reduction in the impurity levels was achieved using this purification scheme, including more than 7 logs reduction in host cell protein (HCP) levels bringing those levels below the detectable limit in final AAV product. Moreover, data also demonstrated multiple logs of DNA impurities reduction in downstream process. The process was successfully scaled up from 2 L to 50L in order to generate representative material for Non-Human Primate (NHP) studies.

1535. Purification of Adeno-Associated Virus Serotype 9 from HEK293 Cell Lysate via the Flow-Through Affinity Adsorbents

Wenning Chu, Stefano Menegatti

Department of Chemical and Biomolecular Engineering, North Carolina State University, Raleigh, NC

Adeno-associated viruses (AAVs) are the vector of choice for delivering gene therapies that can cure inherited and acquired diseases. Clinical research on various AAV serotypes significantly increased in recent years alongside regulatory approvals of AAV-based therapies. The current AAV purification platform relies on protein ligands—typically camelid antibodies—that provide high binding capacity and selectivity but suffer from high cost and harsh elution conditions (pH < 3) that can harm the transduction activity of recovered AAVs. Addressing this challenge, we introduced the flow-through affinity chromatography that target the HCPs in Human Embryonic Kidney (HEK) 293 cell lysate and enable AAVs flow through directly with low retaining. This study describes the mixture of an adsorbent A & adsorbent B, and the mixture of an adsorbent A & adsorbent C, at a volume ratio of 0:1, 1:4, 1:3, 1:2, 1:1, respectively. With the continuing injection of 20 CVs of AAV9 HEK293 Lysate at 1-min residence time, both the mixture of an adsorbent A & adsorbent B, and the mixture of an adsorbent

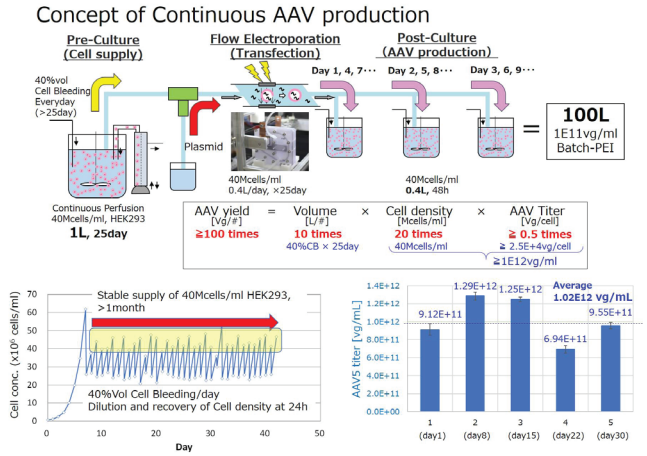
A & adsorbent C show the ability to remove medium components (conductivity dropped from~15 mS/cm to ~10 mS/cm) and HEK HCPs (UV_{280nm} dropped from~900 mAU to ~100 mAU) with the increase of volume ratio of adsorbent A&B or A&C from 0:1 to 1:1. Meanwhile, the logarithmic removal values of HEK HCPs were increased first until reaching to a volume ratio of adsorbent A &B or A&C at 1:3 with a cumulative LRV up to 1.6, and corresponding cumulative AAV9 yield above 78%, then kept drop after that. Because of its flow-through mode, the downstream purification process can be utilized to a broad range of the other AAV serotypes with minimal intervene of transduction efficiency.

1536. Scalable Flow through Electroporation for High Yield Continuous AAV Production

Takashi Yakushiji, Yoichi Nagai, Michiko Edo, Atsushi Inada, Masafumi Nishino, Nozomu Tonoike, Yuka Kobayashi, Yusuke Mori

FUJIFILM Corporation, Ashigarakami-gun, Japan

In recent years, several recombinant-AAV (rAAV) - based gene therapies have gained regulatory approval, and the number of rAAV therapies in development pipelines continues to increase. It is expected that as rAAV based therapies begin to be applied to more common conditions and are systemically administered, the need for large scale supply of rAAV will increase. Increasing productivity of conventional PEI Triple Transfection based rAAV production by increasing the cell concentration and/ or using larger volume bioreactors are problematic due to the difficulty controlling the complicated reaction between the PEI-Plasmid complex and cells. To accelerate rAAV clinical research and meet the potentially enormous future demand, high-yielding breakthrough rAAV production technologies are needed. We have developed a PEI-Free Continuous rAAV production system that combines high density HEK293 perfusion cell culture with a Scalable Flow Electroporation device with very high Electrode durability. HEK293 cells were cultivated in a 1L bioreactor at a concentration of 40Mcells/ml using an ATF based perfusion system. Cells were continuously withdrawn as a cell bleed at a rate of 40% of the bioreactor volume each day for one month. The withdrawn cells were continuously electroporated using a novel flow electroporation device connected aseptically to the bioreactor. The electroporation device was used without replacement for one month, and a cumulative total of over 10,000 pulses were applied to the cell suspension via the electrodes. On 5 days (day1, 8, 15, 22, and 30), the cell suspension was continuously mixed with rAAV plasmids (pGFP, pRC5, and pHelper) before electroporation and the transfected cells fed into a recovery bioreactor. An average of 1E12vg/ml rAAV titer was achieved 48h post electroporation on each occasion. These results demonstrate supply of high-concentration HEK293 cells continuously for one month, good electrode durability in the Flow electroporation device across 10,000 pulses which is sufficient to continuously operate for 1 month, and consistent transfection and AAV production across this period. It is expected that if plasmids were continuously supplied for the 30 day duration a total of 1E16vg of rAAV would be produced for a 1L bioreactor. This yield corresponds to a 100-fold increase in output compared to using a bioreactor of the same volume in batch mode with PEI transfection. We have demonstrated proof of concept of an innovative continuous rAAV production platform and are continuing to further improve efficiency and scale up to support larger bioreactor volumes. This scalable platform may enable mass production from modestly sized bioreactors and contribute to the acceleration of clinical development and commercialization of rAAV.



1537. Advanced Approach to the Purification and Characterization of AAV Vectors Based on Equilibrium Density Gradient Ultracentrifugation

Kiichi Hirohata, Yuki Yamaguchi, Takahiro Maruno, Takayuki Onishi, Tetsuo Torisu, Susumu Uchiyama

Department of Biotechnology, Graduate School of Engineering, Osaka University, Suita, Japan

For the development of gene therapy products using AAV vectors, one of the challenges is the removal of product-related impurities including empty particles. The presence of empty particles may be a cause of reducing therapeutic efficacy and increasing the risk of immunogenicity. Hence, a number of methodologies have been reported for both the purification of AAV vector in the downstream process and the evaluation of the ratio of full to empty particles, regarded as a critical quality attribute (CQA). In addition, the viral protein (VP) stoichiometry of the capsid has been recognized as a CQA and quality control (QC) because several studies suggested a correlation between the VP stoichiometry and transduction efficiency. To meet this increased demand of the quality of AAV vectors, there is a need to develop a further approach that can assess the particle heterogeneity contained in AAV vectors in detail. Equilibrium density gradient ultracentrifugation (DGE-UC) is a conventional but effective approach to the purification and characterization of AAV vectors. Under a cesium chloride (CsCl) density gradient, particles are separated based on the differences in buoyant density at equilibrium. Due to its high density resolution and sensitivity, DGE-UC is now a powerful method that can evaluate even full particles with different VP stoichiometry. We demonstrate the utility of DGE-UC in achieving the separation and fractionation of full particles with different VP stoichiometry. Quantitative analysis can also be performed using analytical ultracentrifugation (AUC) instrument equipped with optical detection system. We are in the process of evaluating various AAV vectors to validate the practical applications of AUC utilized optimization of DGE-UC.

1538. Quantitative Analysis Reveals the Impact of the Empty to Full Capsid Ratio on AAV Vector Transduction *In Vivo*

Shiyong Wu[1], Kentaro Yamada[1], Lohra M. Miller[2], Xin Li[1], Miguel Gonzalez[1], Sreevani Arisa[1], Jiahe Tian[3], Martin F. Jarrold[4], Roland W. Herzog[1], Weidong Xiao[1], Benjamin E. Draper[2], Junping Zhang[1]

[1]Herman B Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis, IN,[2]Megadalton Solutions Inc., Bloomington, IN,[3]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY,[4]Chemistry Department, Indiana University, Bloomington, IN

Monitoring the empty/partial to full capsid ratio is crucial throughout AAV-based gene therapy development for product safety and efficacy. Particle effects on transduction may be linked to their abundance, but existing technology poses challenges for the exact ratio analysis of each AAV vector preparation. Charge detection mass spectrometry (CD-MS) emerges as a robust tool, enabling accurate quantification of these ratios at different scales. Here we employed CD-MS to quantitatively analyze various empty-to-full capsid ratios and their impact on AAV vector transduction in vivo. The two pAAV-ApoE-hAAT-hFIX (Padua) and AAV8-ApoE-hAAT-CohFVIII vectors were produced in suspension HEK293 cells by the triple transfection system. The vectors were purified by iodixanol gradient ultracentrifugation. To get various ratios of empty vs full capsid particles, the vectors from the first-round purification were subjected to a second-round gradient ultracentrifugation, and then different fractions were separately harvested. All the vector preparations were characterized by CD-MS and then injected into hemophilia A (HA) or hemophilia B (HB) mice for transgene expression and immunoassays. For AAV-ApoE-hAAT-hFIX (Padua) vectors, the empty to full capsid ratio for 1st round purification was around 90:10. The ratios of three fractions (Fraction 1-3) from 2nd round purification were 99:1, 80:20, 10:90 from the top to bottom three layers, respectively. Injecting equal amounts of AAV capsid particles from both purification rounds showed a significant difference in hFIX expression at mRNA level, consistent with their full particle abundance. Nonetheless, no significant changes in anti-AAV8 IgG levels were observed. Further experiments used AAV-ApoE-hAAT-hFIX particles with equivalent gene copies from both rounds, in which the bottom fraction 3 yielded highest hFIX expression but showed no changes in anti-AAV8 IgG levels among various groups. The same procedure was applied to AAV8-ApoE-hAAT-CohFVIII vectors. The three vector fractions from the second-round purification along with the first-round AAV vector, was respectively administered to HA mice with equal gene copies. Their ratios of empty to full capsids were around 100, 47:53, 6:94, and 92:8, respectively. Notably, Fraction 3 from the bottom layer, which contained the lowest ratio of empty particles, exhibited the highest transgene expression, while Fraction 1, with the highest ratio of empty particles, showed the most pronounced immunogenicity by enhanced α-TNF, α-IL1, and ISG-15 expression. Additionally, Fraction 1 also exhibited the highest anti-AAV8 IgG response compared to the other groups. Fraction 2 and Fraction 1 had lower anti-AAV8 IgG than that of the first-round vector preparation. These findings suggest that a high concentration of empty AAV particles may adversely affect transgene expression and potentiate the host's immune response. Lowering empty particles in AAV vectors are essentially for gene therapy efficiency. Especially, for research grade AAV vectors, monitoring a lot-to-lot variation in the ratio of empty to full capsids is required to ensure experiment consistency.

1539. AE1-BHK Novel Cell Line for rAAV Production of Multiple Serotypes for Gene Therapy Applications

Amber Chevalier Plambeck, Grace Loegering, Andrzej K. Noyszewski, Santhalingam Elamurugan, Alex Deiulio, Erica Kelly Venberg, Jeremy Houser, John Ballantyne, Michael Chambers, James Brown, Jagadish Loganathan

Agathos Biologics, Fargo, ND

Adeno-associated virus (AAV) vector-based gene therapies have attracted increasing interest for the treatment of monogenic disorders and as a valuable research tool to study gene function. Currently, the HEK293 Ad5E1 packaging cell line is the most popular platform for producing AAV of different serotypes due in part to the simple triple transfection production process. In addition to increasing the yield and scalability of HEK293, developers are looking to new cell lines for improving rAAV production and to address ethical concerns with cell lines derived from aborted fetal tissue. We have previously shown the mammalian platform AE1-BHK (formerly BHK-[wt E1]) stably expresses the E1 proteins with high cell growth and was able to produce rAAV2 using the same triple transfection process as used with HEK293. In the current work, we present data for other serotypes scaled up in cell stacks and purified and we show infectivity data for the rAAV produced in AE1-BHK. These data show AE1-BHK is a flexible platform to produce rAAV of different serotypes by triple transfection.

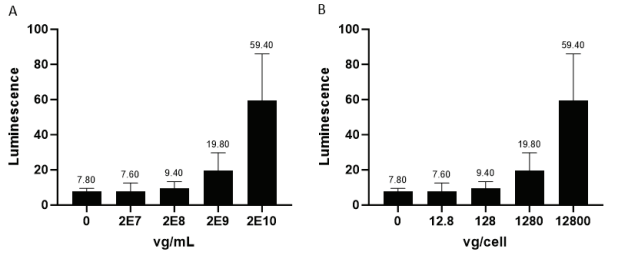


FIGURE 1. Luciferase activity from HepG2 cell line infected with rAAV8-luciferase. (A) HepG2 cells were infected with rAAV8- luciferase at different vg/mL (B) same data as (A) expressed as vg/cell. The data shown here are the mean ± SD from 5 technical replicates of one experiment.

1540. Establishment of Serum Free Suspension Adapted AE1-BHK-S Novel Cell Line for the Manufacturing of rAAV

Santhalingam Elamurugan, Amber Chevalier Plambeck, Grace Loegering, Andrzej K. Noyszewski, Alex Deiulio, Erica Kelly Venberg, John Ballantyne, Michael Chambers, James Brown, Jagadish Loganathan

Agathos Biologics, Fargo, ND

Adeno-associated virus (AAV) vectors are a promising biological product for gene therapy due to their non-pathogenic nature, ability to infect both dividing and non-dividing cells, and robust transgene expression. Adherent HEK293 is a well-established cell line for producing rAAV and has been successfully adapted to grow

in suspension in animal component-free chemically defined media and is commonly used for rAAV production at large scale. Here, we show a novel AE1-BHK cell line that stably expresses the Ad5 E1 proteins which can produce rAAV of different serotypes by the triple transfection method. To scale-up the production we adapted the adherent AE1-BHK cell line to a suspension AE1-BHK-S cell line using serum-free chemically defined media. We will present data on efforts to select clones based on robust growth and high cell density and evaluation of the clones for suspension virus production.

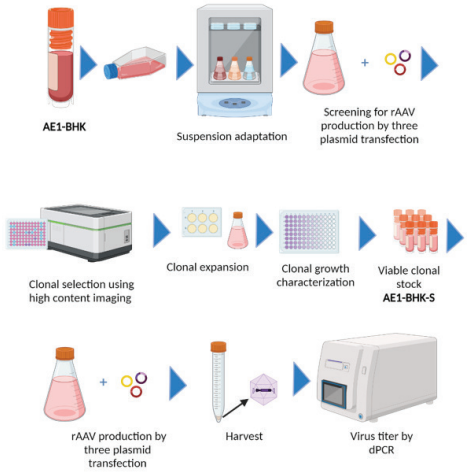


FIGURE 1 Workflow for AE1-BHK serum free suspension adaptation and clonal selection to create AE1-BHK-S for rAAV production.

1541. Optimization of AAV Production Yields through Dual Transfection

Sarath C. Mandava, Adam S. Cockrell, Lauren Peters, Margaret Moran, Neil Templeton, Ben Wright, Jen C. Gifford

Solid Biosciences, Charlestown, MA

Adeno-associated virus (AAV) vectors have emerged as versatile tools for gene therapy applications, necessitating the continual improvement of production methodologies to enhance yields and minimize undesired effects related to safety. This study investigates a dual transfection approach for AAV production, comparing its efficiency to the conventional triple transfection process currently in use. Our results demonstrate a consistent 2-3-fold increase in AAV production yields when employing the dual transfection approach across various capsid and gene of interest (GOI) combinations tested, highlighting this production method as a potential alternative to supply large amount of virus for in vivo gene therapies with high needs at reduced costs. Moreover, the dual transfection approach exhibits similar product quality attributes when compared to similar triple transfection generated AAV. Through a comprehensive series of in vitro assays, we analyzed various parameters including but not limited to vector purity, titers, transduction efficiency, and capsid integrity. Our findings not only underscore the enhanced productivity achieved with dual transfection but also emphasize its potential to streamline AAV production processes and reduce cost of goods, while mitigating concerns related to mispackaging, product quality and replication competent (rc)_AAV. This optimized approach holds promise for advancing the scalability and cost-effectiveness of AAV vector manufacturing, thereby contributing to the ongoing development and accessibility of gene therapy applications.

1542. Integrated Development of a Scalable, High Productivity, and High Yield Manufacturing Process to Enable Supply of an Investigational rAAV Gene Therapy to Treat Duchenne's Muscular Dystrophy

Dennis P. Chen, Wei Xue, Logan K. Nichols, Jian Lin, Mike Dullen, Carli D'Antonio, Amy Medeiros, Greg Mantenuto, Chao Huang, Jan Thomas Panteli, Patrick Hossler, Matt Marrichi, James Warren

Pharmaceutical Development, Ultragenyx Pharmaceutical Inc., Woburn, MA

Systemic delivery of recombinant adeno-associated virus (rAAV) gene therapies to treat neuromuscular genetic disorders such as Duchenne muscular dystrophy (DMD), require high doses (e.g., ≥ 1E+14 GC/kg of body weight). However, low batch productivity leads to high manufacturing costs and challenges to clinical supply. There is an urgent need to improve existing rAAV manufacturing platforms to achieve robust, high-yielding, scalable and cost-effective processes to meet high dosing requirements and reduce the overall cost of treatment. We employed a cost-effective and scalable rAAV production process using our Pinnacle producer cell-line/adenovirus helper platform that reduces the high cost of goods sold (COGS) associated with transfection-based processes. Through optimization of our intensified upstream process, we've increased the productivity of our rAAV expression platform and designed a robust downstream process capable of handling the challenging feed stream of a high-cell density production, while consistently achieving the desired product quality. Herein, we highlight the key results from our harvest clarification development, emphasizing the interdependence between upstream and downstream processing. We demonstrated improvement over our legacy platform batch process by increasing upstream volumetric productivity and process yield 5-fold to achieve approximately 1E+12 GC/mL; and developed a robust approach to the removal and inactivation of the adenovirus helper virus. Furthermore, our COGS analysis shows that our process intensified platform can reduce the number of cGMP batches required to initiate clinical studies compared to our legacy process at significantly reduced cost while meeting high dosing requirements for the treatment of DMD.

1543. High-Throughput Workflows to Accelerate Development of Chromatographic Purification of rAAV Viral Vectors

Ryan H. DeGroot, Sierra VanSuch, Ashley Sacramo, Ben Wright

Solid Biosciences, Charlestown, MA

Establishing a robust and scalable chromatographic full capsid enrichment step for adeno-associated virus (AAV) gene therapies presents an evolving challenge as novel combinations of capsids and genes of interest are continually developed. Traditional chromatographic development systems often require large quantities of feed-stream material, costly consumables, and a significant personnel commitment

to plan, setup, and execute these experiments. Thus, high-throughput screening workflows at small-scale that provide directionally relevant performance data can be instrumental to the rapid, efficient, and successful development of a chromatographic process step. Here, we discuss development and application of a high-throughput workflow to screen and optimize an anion exchange (AEX) chromatography step for enriching full AAV capsids using an automated liquid handling system and micro-scale columns that delivers comparable process performance in terms of chromatographic profiles, step yield, and product quality as traditional bench-scale columns.

1544. Case Study on Insect Cell-Produced AAV6 Gene Therapy Vector in a Rhabdovirus-Free Sf9 Cell Line

Thera Mulvania[1], Brooks C. Hayes[1], Scott Walker[1], Buel D. Rodgers[2], Joseph Rininger[3]

[1]Expression Systems, LLC, Davis, CA,[2]AAVogen Inc., Rockville, MD,[3]Latham Biopharm Group, Elkridge, MD

Developing AAV gene therapeutics for systemic delivery presents unique manufacturing challenges that are potentially addressed using inherently safe and scalable insect cell production systems. This presentation provides a case study of production and activity of AVGN7, an AAV6-based gene therapeutic for treating rare neuromuscular diseases, using this system. The upstream manufacturing process employs a newly introduced expression system comprised of a rhabdovirus negative clonal Sf9 RV-Free cell line derived from a single cell printing method, as well as a co-developed chemically defined insect cell medium. Upstream process development employs intracellular capsid detection in concert with GFP expression from the ITR-containing vector. This novel approach allows for screening of expression conditions that lead to successful co-expression of the required components for AAV production. Preliminary studies demonstrate the ability to produce high levels of AAV expression using a high cell density, high MOI (multiplicity of infection) methodology. Downstream processes for purification will be discussed along with assessments of bioactivity and post-translational processing of capsid proteins.

1545. Assessment of Host Cell DNA in AAV Gene Therapy Product Using PacBio Sequencing

Hsin-I Jen[1], Wei Zhang[2], Xiaohui Lu[3]

[1]Analytical Development, Ultragenyx, Woburn, MA,[2]Ultragenyx Pharmaceutical, Acton, MA,[3]Ultragenyx Gene Therapy, Woburn, MA

The presence of residual host cell DNA in gene therapy products is a critical concern for patient safety, given the associated uncertainties and potential risk of oncogenicity. To understand the characteristics of encapsidated host cell DNA, PacBio sequencing (Sequel II) was employed to determine the size and the transcription potential of the residual host cell DNA fragments.This study first assesses the accuracy of PacBio sequencing in detecting fragments of different sizes. Different proportions of inverted terminal repeats (ITR)-containing fragments of different sizes were deliberately spiked into the viral DNA before undergoing PacBio library preparation and sequencing. ITR sequences in viral genomes were known to be crucial for second-strand synthesis and transcription. It is also notoriously hard to sequence by traditional Illumina sequencer. Subsequent analysis quantified the percentage recovery of spiked-in fragments, demonstrating the detectability and accuracy of PacBio sequencing across various fragment sizes.Next, various viral products were sequenced using PacBio and the results revealed that the majority of host cell DNA does not contain ITR. Additionally, among all the analyzed host cell DNA, no complete coding sequences were observed. The absence of a complete coding sequence and ITRs in the identified host cell DNA indicates a low likelihood of safety risk associated with unintended host cell protein expression.

1546. A Commercially Producible Non-Lethal Virus Insect Cell Line

Shixian Zhang, Yan Wu, Yujing Liu, Yangyang Sheng, Xin'an Lu

Beijing Cygenta BioTechnology Co., Ltd, Beijing, China

The baculovirus expression system stands out as a robust tool for producing recombinant proteins, vaccines, and AAV vectors. In 2014, the FDA reported contamination of commercially produced baculovirus cell lines sf21 and sf9 cells with rhabdovirus, emphasizing the need for rhabdovirus-free insect cells to ensure the safety of biological products. In this study, we successfully established a rhabdovirus-free Sf9 (RVF-Sf9) insect cell line using a monoclonal screening method with antiviral reagents. The RVF-Sf9 cell line, cultured for 44 passages, exhibited no rhabdovirus detection, maintaining stable doubling time and cell viability across passages. Baculovirus titers at different passages showed no significant differences. Genomic copies of Adeno-Associated Virus Vectors (AAV2-VEGF) produced in RVF-Sf9 and SF9 (with rhabdovirus) cells were comparable. Further production of AAV2-VEGF in 200-liter batches demonstrated a higher crude harvest yield of 5X10^14 vg/L using RVF-Sf9 cells compared to 3X10^14 vg/L with SF9, with no discernible differences in purity or product characteristics. Our results showcase the successful creation of a stable rhabdovirus-free cell line, providing a safer platform for commercial baculovirus system production.

1547. Development of Orthogonal Cell-Based Potency Assay to Demonstrate the Biological Effect of AAV Vector-Based Gene Therapy Products

Sree Kattala, Kalpana Rajanala, Subechhya Neupane, Dinesh Kumar Singh, Pushpendra Singh, Arun Upadhyay

Research and Development, Ocugen Inc, Malvern, PA

Background: Gene therapies show great potential in treating inherited diseases and have been increasingly proven successful across a broad spectrum of conditions, ranging from systemic diseases to ocular disorders. However, the manufacturing of gene therapy is a complex and resource-intensive process that requires rigorous quality control measures across various production batches. In order to ensure the consistency of each batch of gene therapy and determine the effectiveness of the product over time under diverse storage conditions, an in vitro cell-based potency assay is essential. This assay is critical

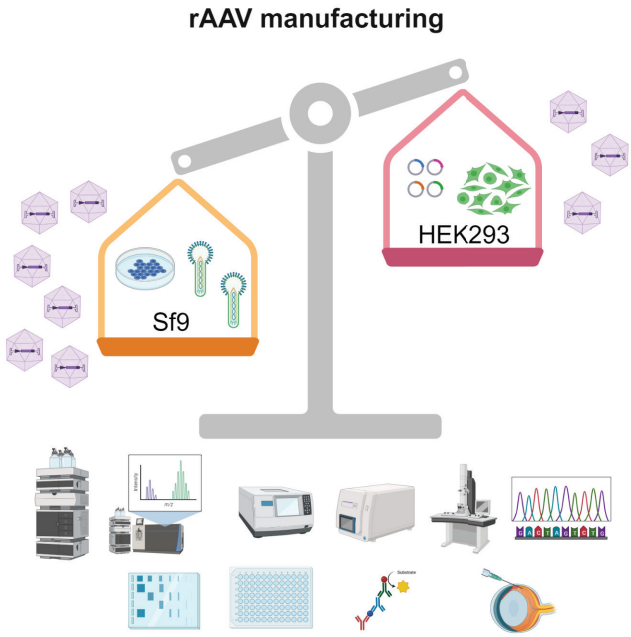
in establishing comparability across different manufacturing batches, both in clinical trials and for eventual commercial use. **Methodology:** In this study, we developed a matrix-based approach for *in vitro* cell potency assay that allows to measure the transduction, expression, and biological effect of a therapeutic transgene. For our ocular gene therapy product, multiple retinal cell lines were screened to determine the transduction efficiency; for this, AAV-GFP was used with a GFP-based imaging approach. Thereafter, AAV containing the transgene was used to assess the transduction efficiency and RNA level expression using the qPCR method. Subsequently, the biological effect of the transgene was evaluated using a luminescence-based approach. These assays were qualified for different parameters such as reproducibility, specificity, selectivity, and dose-dependence linearity according to the gene therapy potency assay regulatory guidance. These potency assays are vital in assessing the product quality and were used as release and stability indicating methods and meet the regulatory expectations for gene therapy products. **Results:** Ocugen developed a cell-based system that can reproducibly quantify the functional efficacy of different AAV-based gene therapy products. These cell-based potency assays are orthogonal and show a strong cross-correlation to one another with a dose-dependent effect of gene therapy products. We demonstrate that this potency assay showed acceptable assay performance and was deemed comparable across manufacturing batches. Further, this approach would help in evaluating the variability and stability of this gene therapy product during the product life cycle. These assays are easy to implement and can be adapted to other AAV gene therapy products and used to reliably assess the molecular function of other products.

1548. Comprehensive Head-to-Head Comparisons Demonstrated Key Advantages of The Sf9 System Over HEK293 in rAAV Manufacturing

Shengjiang Shawn Liu[1], Jinzhong Li[1], Sameera Peraramelli[1], Ningguang Li[1], Alan Chen[1], Minghua Dai[1], Fang Liu[2], Yanbao Yu[3], Ying Li[1], Eduardo Santillan[1], Kevin Lin[1], Derrick Huynh[1], Shuyi Li[1], Li Ou[1]

[1]Avirmax, Inc., Hayward, CA,[2]Stanford University, Stanford, CA,[3]University of Delaware, Newark, DE

Recombinant adeno-associated (rAAV) vectors could be manufactured by plasmid transfection into human HEK293 cells or baculovirus infection of *Spodoptera frugiperda* (Sf9) insect cells. However, systematic comparisons between these two systems using large-scale, high-quality AAV vectors are lacking. rAAV from Sf9 cells (Sf9-rAAV) at 2-50 L and HEK293 cells (HEK-rAAV) at 2-200 L scales were manufactured and systematically characterized. SDS-PAGE identified non-GOI impurities and differences in the VP1:2:3 ratio, while agarose gel electrophoresis observed multiple bands that indicate truncated genomes. Sf9-rAAV had up to 40-fold higher yields than HEK-rAAV across various scales, measured by ddPCR. Electron microscopy observed a lower full/empty capsid ratio in HEK-rAAV (70.8%) than Sf9-rAAV (93.2%), while dynamic light scattering (DLS) and HPLC analysis showed that HEK-rAAV had more aggregation. NGS with PacBio and Illumina showed that HEK-rAAV had ~10-fold more host cell DNA and more chimeric events with host cell DNA. LC-MS/MS identified different post-translational modifications (PTM) profiles, with more phosphorylation, acetylation, methylation, ubiquitination, and glycosylation, but less deamination in Sf9-rAAV. Furthermore, Sf9-rAAV had a higher $TCID_{50}$/vg than HEK-rAAV, indicating better infectivity. Additionally, Sf9-rAAV achieved higher *in vitro* transgene expression of Aflibercept, measured by ELISA. Finally, after intravitreal dosing into a mouse laser choroidal neovascularization model, Sf9-rAAV and HEK-rAAV achieved similar efficacy in reducing lesions. Overall, this study detected notable differences in the physiochemical characteristics of HEK-rAAV and Sf9-rAAV. However, the *in vitro* and *in vivo* biological functions of the rAAV from these systems were highly comparable. Sf9-rAAV may be preferred over HEK293-rAAV for advantages in yields, full/empty ratio, scalability, and cost.



1549. A Robust Upstream Process for AAV8 Production Scaled up from 15 Liters to 1000 Liters

Shixiong Dong

OBiO Technology (Shanghai) Corp., Ltd., Shanghai, China

Adeno-Associated Virus (AAV) has emerged as a pivotal tool in gene therapy, distinguished by its low immunogenicity, minimal toxicity, and non-pathogenic characteristics. While various methods exist for AAV production, the transient transfection of suspension HEK293 cells with three plasmids remains the predominant approach for large-scale production. Challenges arise in large-scale AAV production during HEK293 transfection exceeding a 1000-liter working volume, including (a) large-scale HEK293 suspension cell culture, (b) rapid mixing of DNA-PEIpro complexation in a large volume, and (c) rapid transfer of large volume transfection complexes into the bioreactor. Leveraging insights from prior small-scale bioreactor experiences, we optimized and developed the production process in a 2000-liter single-use bioreactor, culminating in a successful run. We present key data as follows: • Upstream harvest virus titer: 1.97E+11 vg/ml (ddPCR) • Host cell DNA residue: 3.4 ng/1E+12 vg • Host cell protein residue: 2 ng/ml • Full virus percentage: 84.2% (AUC) • Downstream total

harvest ratio: 34% Significantly, virus yield and impurity levels closely match the 200-liter data, demonstrating successful scale-up from a 15-liter reactor to a 2000-liter reactor. This achievement represents a groundbreaking advancement in overcoming the challenges associated with large-volume HEK293 transient transfection processes.

1550. The AAV Analytical Toolbox - A Comparison of Multiple Methods, Instruments and Data

Agnieszka Lass-Napiorkowska, Henry George
MilliporeSigma, St. Louis, MO

AAV (Adeno-Associated Virus) analytics is a key element in the development of robust AAV production, purification and polishing platform. Only reliable, accurate and reproducible analytical techniques enable optimization of AAV upstream and downstream processes. This poster focuses on comprehensive comparison of analytical methods used for AAV2, AAV5 and AAV6 titer assessment (Elisa, ddPCR), % full comparison (ddPCR/Elisa vs SamuxMP mass photometer vs CDMS vs AUC), content of AAV packaging (Illumina NGS, ssDNA extraction vs CGE), aggregation (DLS, AUC), purity (SDS-PAGE, CGE) and biological activity (HeLa-based infectivity assay).

Friday Posters: AAV Vectors - Immune Modulation

1551. Engineering Human CNS Receptor-Targeted Capsids for Improved Antibody Evasion

Simon Pacouret, William Donahoe, John W. Harvey, Pamela P. Brauer, Qingxia Zheng, Chin-Yen Lin, Binhui Zhao, Gabrielle Clouse, Qin Huang, Jencilin Johnson, Casey Keyes, Catherine P. Pirtle, Jarrett Rios, Albert T. Chen, Fatma Elzahraa Eid, Andrew J. Barry, Yujia A. Chan, Ken Y. Chan, Benjamin E. Deverman
Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA

Adeno-associated virus (AAV) capsids have been successfully engineered via surface loop modifications to target different organs such as the central nervous system (CNS), muscle, retina, and liver following systemic delivery. However, the population of patients that can benefit from AAV-based gene therapies is limited by pre-existing neutralizing antibodies (NAbs) to AAV capsids. NAbs not only activate the complement system but can hinder AAV transduction and thus the gene therapy's efficacy. Therefore there is a need for capsids with reduced sensitivity to antibodies that are compatible with surface loop modifications that confer enhanced in vivo tropisms. In this work, we aimed to reduce the NAb sensitivity of novel capsids developed by our group that cross the blood-brain barrier (BBB) via interactions with human CNS receptors such as human Transferrin Receptor (TfR1). Through sequential steps, we generated a library of TfR1-targeting capsids diversified at five distributed surface locations. We then subjected the library to high-throughput neutralization assays, in the presence of increasing concentrations of human intravenous immunoglobulin (IVIG), with transduction of human brain endothelial cells as a readout. Based on library screening data, we identified several capsid variants that (1) exhibited decreased neutralization by human IVIG, (2) retained their ability to transduce human cells via interactions with TfR1, and (3) possessed production fitness scores that were comparable to AAV9. We are now evaluating these capsids individually for their manufacturability, TfR1-dependent transduction, BBB crossing in vitro and in vivo, and maintained transduction efficiency in the presence of human sera. Ultimately, this work should yield novel antibody-evading capsids that can be used in combination with other capsid modifications that confer enhanced in vivo functionalities.

1552. CpG Content of Adeno-Associated Viral Vectors Can Be Manipulated to Control Neuroanatomical and Synaptic Impacts

Connor E. Dunn[1], Brigett V. Carvajal[2], Jie Ma[1,2], Caroline Jung[2], Ai Vy Nguyen[2], Christos Suriano[1,2,3], Lisa M. Boulanger[1,2]
[1]Department of Molecular Biology, Princeton University, Princeton, NJ,[2]Princeton Neuroscience Institute, Princeton University, Princeton, NJ,[3]Department of Biology and Sokol Institute for Pharmaceutical Life Sciences, Montclair State University, Montclair, NJ

Recombinant adeno-associated viruses (rAAVs) are used extensively as gene delivery vectors for basic research and are the basis of multiple FDA-approved gene therapies. Despite AAV's overall strong safety profile, the host immune response can constrain transgene expression and cause tissue-specific forms of toxicity. To realize the potential of AAV-mediated gene delivery, it is important to manage harmful and efficacy-limiting immune responses. In the rodent nervous system, AAV can unexpectedly disrupt neuronal morphology and synaptic communication, changes that are prevented by blocking the innate immunoreceptor Toll-like receptor 9 (TLR9). The AAV genome contains unmethylated cytosine-phosphate-guanine (CpG) motifs that are ligands for TLR9, and lower CpG content has been correlated with reduced immune responses and enhanced transgene expression in preclinical and clinical studies. We hypothesized that depleting CpG motifs from the AAV genome would protect neurons from TLR9-mediated structural and functional dysregulation. To directly test the impact of CpG depletion on responses to AAV in the brain, we reduced the number of CpG dinucleotides in the AAV1-*pCAG-FLEX-EGFP* genome by ~60%. To do this, we removed all CpG dinucleotides from the intronic regions and from the transgene, where we made codon-optimized synonymous mutations. These substitutions did not affect expression of the encoded transgene (EGFP), which was detected at comparable levels in the brains of mice that received intraparenchymal injections of either intact or CpG-depleted AAV. However, CpG depletion of the AAV genome fully prevented the loss of dendritic complexity seen with intact AAV1-*pCAG-FLEX-EGFP*. CpG depletion also prevented impacts on synaptic transmission that occur with intact AAV1-*pCAG-FLEX-EGFP*. Taken together, these findings suggest that CpG depletion can protect neurons from harmful TLR9-mediated impacts of AAV. To facilitate the design and adoption of CpG-minimized AAVs, we created a web-facing application that allows users to (1) quantify the CpG content of ssDNA sequences using multiple metrics, including RF3, a measure created by J. Fraser

Wright that differently weights known TLR9 -activating and -inhibiting motifs and aims to estimate TLR9 activation potential; (2) interactively explore the CpG content of a library of over 130 commonly-used AAV components, including ITRs, promoters, and transgenes; and (3) deplete CpG dinucleotides from noncoding regions and use codon-optimized synonymous mutations to deplete CpGs from coding regions. To our knowledge, this study provides the first evidence that CpG depletion can combat immune-mediated impacts of AAV on neural circuits. It also provides a community tool to streamline the use of CpG minimization or depletion as a non-invasive, cost-effective strategy to improve the safety and efficacy of AAV-mediated gene delivery.

1553. Benchmarking Natural and Synthetic AAV Capsids for Neutralizing Antibody Evasion

William Donahoe, Simon Pacouret, Pamela P. Brauer, John W. Harvey, Catherine P. Pirtle, Ken Y. Chan, Yujia A. Chan, Benjamin E. Deverman

Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard, Cambridge, MA

Adeno-associated viruses (AAVs) exhibit significant promise as delivery vehicles for gene therapy but their broad applicability is hindered by pre-existing immunity in patients. Efforts have been made to circumvent neutralizing antibodies (NAbs) by employing various capsid engineering strategies such as capsid shuffling, mutagenesis of monoclonal antibody (MAb) footprints, and modifying naturally occurring serotypes with relatively low NAb binding. However, data is lacking on how well different capsids can evade NAbs relative to each other. To bridge this knowledge gap and set a baseline for our internal efforts to develop capsids with improved antibody evasion, we assessed the relative neutralization effectiveness of two pooled human IgG batches against natural serotypes (AAV1, AAV4, AAV8, and AAV9), loop VIII modified capsids (AAV-BI28 and Dyno bCap1), MAb footprint mutagenesis capsids (CAM130 and AAVhum.8), and capsid shuffling/hybrid variants (AAV-DJ and AAVrh32.33). Specifically, we determined the concentration of each pooled human IgG sample required to trigger a 50% reduction in AAV transduction efficiency (IC50) for each capsid. We used this control dataset to evaluate our progress in engineering an antibody evasion phenotype in AAV-BI-hTFR1, a blood-brain barrier (BBB)-penetrating capsid that targets the human Transferrin Receptor (TfR1). Among the naturally occurring serotypes, AAV1 and AAV4 had the lowest and highest IC50, respectively. Across all capsids tested, AAVrh32.33 had the highest IC50. Altering loop VIII alone had no impact on AAV9 resistance to pooled human IgG in the specific cases of AAV-BI28, Dyno bCap1, and AAV-BI-hTFR1. CAM130, AAVhum.8, and AAV-DJ were all more resistant to pooled human IgG than their parental capsids, with 1.2-1.6, 1.9, and 1.8-2 fold higher IC50s, respectively. Lastly, modification of multiple sites on the AAV-BI-hTFR1 capsid yielded novel variants with IC50s up to 3.58-fold greater than that of the parental capsid, AAV9. This comparative analysis will help guide future engineering endeavors focused on developing novel AAV capsids with enhanced resistance to neutralizing antibodies.

1554. Rapamycin is an Important Component of an Immunosuppression Regimen to Inhibit Capsid-Specific Humoral Immune Responses in High-Dose AAV Gene Therapy in Cynomolgus Macaques

Deniz Erturk-Hasdemir[1], Weasel Scott[1], Leah N. Cardwell[1], Justin Serio[1], Sanket Bhagat[1], Lina Song[1], Lorraine Sullivan[1], Stephanie M. Morin[1], Brian E. McIntosh[2], K. Reed Clark[1], Lorelei Stoica[1], Barbara A. Sullivan[1]

[1]Ultragenyx Pharmaceutical Inc., Novato, CA,[2]Labcorp Drug Development, Madison, WI

Host immune responses against recombinant adeno associated viruses (rAAVs) pose a significant challenge for safe and effective gene therapy. Some patients receiving high systemic doses of rAAVs in different clinical trials have experienced serious adverse events such as thrombotic microangiopathy (TMA) with hemolytic anemia, thrombocytopenia, and atypical hemolytic uremic syndrome (aHUS) leading to kidney damage due to complement activation. A proposed mechanism for complement activation is the binding of IgM and IgG anti-capsid antibodies to rAAV vectors in circulation to form rAAV-antibody immune complexes that trigger the classical complement pathway. We hypothesize that prophylactic immunosuppression (IS) to inhibit de novo anti-capsid IgM and IgG responses within the first two weeks of dosing may be useful in mitigating complement-mediated adverse events in high-dose rAVV gene therapy. We evaluated IS with prednisolone, rapamycin, and rituximab-pvvr for its ability to decrease the development of anti-capsid IgM and IgG in two non-human primate (NHP) studies. Our previous work showed that prednisolone/rapamycin decreased IgG but not IgM and that a triple IS regimen is necessary to inhibit both IgG and IgM. Here, in a follow-up study we tested whether prednisolone/rituximab-pvvr would be sufficient to inhibit anti-capsid humoral immune responses. An investigational AAVhu37 gene therapy vector expressing human microdystrophin (μDys) for the treatment of Duchenne muscular dystrophy was administered at 2e14 VG/kg in male cynomolgus macaques. Seronegative animals for both anti-AAVhu37 neutralizing antibodies (NAb) and for AAVhu37 anti-capsid IgGs were included. Animals received either AAVhu37 vector or vector along with one of the following IS regimens: prednisolone/rituximab-pvvr or prednisolone/rapamycin/rituximab-pvvr. Plasma, serum, and whole blood were assessed at different time points to evaluate immune endpoints such as anti-capsid IgM and anti-capsid IgG levels, platelet numbers, and complement protein levels. Whole blood was monitored throughout the study for B cell numbers and rapamycin levels. Rapamycin levels were above the target level of 12 ng/mL for the duration of the study. Peripheral B cells were depleted (75-100%) by rituximab-pvvr. The AAVhu37-μDys gene therapy was well tolerated with no adverse effects noted during this study. A transient elevation in liver enzymes (ALT, AST) was observed in all dose groups within the first week following vector dosing. The combination of prednisolone/rapamycin/rituximab-pvvr demonstrated a significant decrease in the anti-capsid IgM and IgG levels compared to the no IS group, consistent with our previous work. Prednisolone/rituximab-pvvr did not have a significant effect on mean IgM or IgG levels compared to the no IS

group. The extent of peripheral B-cell depletion correlated with the anti-capsid antibody levels. Four days after vector administration, the prednisolone/rapamycin/rituximab-pvvr group exhibited significantly (~1000-fold) higher vector genome DNA levels in the serum compared to the no IS group. A transient increase in complement proteins and a transient decrease in platelet numbers were observed in only a subset of animals from all dosed groups within the first week. The combination immunosuppression regimen of prednisolone/rapamycin/rituximab-pvvr was well tolerated, reduced anti-capsid IgM and IgG, and increased VG levels in the serum following high-dose rAAV gene therapy with AAVhu37. These results suggest that rapamycin is an important component of the IS regimen to reduce anti-capsid humoral immune responses.

1555. Elucidating the Impact of AAV Immune Complexes on Macrophage Uptake and Chemokine Induction

Robert J. Jimenez, Blake Williams, Michael R. Emami, Melissa J. Spencer

Department of Neurology, David Geffen School of Medicine at UCLA, Los Angeles, CA

High systemic doses of AAV have been associated with excessive complement activation, which can lead to thrombotic microangiopathy and organ damage. Previous human studies have indicated that AAV-dosing activates classical complement, which is dependent on the presence of AAV-antibody complexes (Salabarria et al, 2023). In murine models that emulate antibody-dependent complement activation it has been shown that certain myeloid-derived chemokines (CXCL10, MCP-1, and MIP-1β) are also dependent on the presence of antibody-AAV immune complexes (Emami et al, 2023). To better understand how classical complement influences induction of myeloid-derived chemokines, we used C1qa knockout mice (Jackson Labs *B6(Cg) C1qatm1d*(EUCOMM)Wtsi/TennJ) as C1q is the first complement protein to recognize antibody opsonized immune complexes. C1q homozygous knockout mice and C57Bl6/J wildtype mice were given an initial dose of empty AAV9 vector to prime adaptive immunity and given a second dose of AAV9_CMV_mCherry (1.16 E14 vg/kg) four weeks later. Plasma was collected before dosing, and then five hours and two weeks after each dose. Chemokines were assessed and observed to be elevated in an antibody dependent manner in both genotypes. Despite equal antibody levels in both genotypes, C1q knockout mice showed inflated chemokine responses. To further elucidate if the elevated chemokine response was antibody or complement mediated, we used an *in-vitro* system to test in parallel the chemokine activation signatures between complement or antibody-AAV immune complexes. After exposure to antibody-AAV immune complexes, THP-1 induced macrophages showed an antibody-dependence on both AAV uptake and transgene expression, leading to the concomitant induction of myeloid derived chemokines. This chemokine induction was partially blunted by blocking FcR1 and eliminated by blocking both FcR1 and FcR3 simultaneously. C3b opsonization of AAV particles did not increase AAV uptake nor chemokine induction of THP-1 macrophages. However when C3b was converted to iC3b, induction of chemokines was observed. These data suggest that AAV entry to macrophages likely occurs through an Fc receptor-dependent mechanism, and once internalized, activates macrophages. The study also suggests that there may be redundancy between Fc receptors on macrophages. Finally, our data also show that C3b and iC3b do not promote AAV entry, but that C3b processing to iC3b can induce chemokines in the absence of AAV internalization. These data provide insight into how antibody- and complement opsonization of AAV influence macrophage uptake and chemokine production.

1556. A Strategy to Select Minimally Immunogenic Candidate AAV Vectors to Treat GNE Myopathy

Bradley A. Hamilton[1], Rachana Patil[1], Benjamin Ruland[2], John Day[3], J. Fraser Wright[1]

[1]Pediatrics, Stanford School of Medicine, Stanford, CA,[2]Computer Science, Stanford University, Stanford, CA,[3]Neurology, Stanford School of Medicine, Stanford, CA

Innate immune responses to AAV vectors have emerged as a significant safety concern, especially after high dose systemic administration. Toll-like receptor 9 (TLR9)innate immune receptor activation has been indicted as a potentiator of serious adverse events. CpG dinucleotides in AAV genomes are TLR9 ligands, but their removal can produce unanticipated effects. GNE myopathy is characterized by progressive skeletal muscle weakness caused by mutations in the enzyme responsible for producing sialic acid. Strategies to realize the benefit of AAV based gene therapy for GNE myopathy and other neuromuscular diseases must include efforts to reduce the inherent innate and adaptive immunogenicity of AAV vectors through better design and manufacture, reduction of dose coupled with increased expression efficiency, and predicting and modulating immune responses pre- and post-vector administration. Here, we provide a vector development strategy centered on selection of minimally immunogenic open reading frames (ORFs) achieved through CpG reduction and measured by our novel assay, which opsonizes vectors with engineered antibodies prior to peripheral blood mononuclear cell (PBMC) exposure, revealing detectable interferon alpha 1 (IFNa1) secretion from PBMCs to measure TLR9activation by proxy. Using our previously reported algorithm accounting for CpG frequency, methylation status, and the TLR9 stimulatory or inhibitory potential of CpGs intetranucleotide contexts, we generated six candidate CpG reduced ORFs encoding GNE kinase with low normalized risk factors. We then cloned these sequences into expression plasmids with identical distal genetic elements. Next, we transfected HEK293T cells and probed for GNE kinase expression by western blot. The wildtype ORF (GNE1-001) expressed well and set a threshold to be met by CpG reduced ORFs to justify further characterization. Candidates GNE1-005,GNE1-006, GNE1-007, GNE1-008, and GNE1-009 all expressed less efficiently thanGNE1-001 and were excluded from development. Control sequence GNE1-002 was CpG elevated through traditional codon optimization and obtained significantly higher expression than GNE1-001, as predicted. Interestingly, GNE1-004 contained zero CpGs, and achieved expression slightly greater than wildtype GNE1-001. This relative expression pattern was recapitulated in other relevant cell lines andGNE1-001, GNE1-002, and GNE1-004 ORFs were used to produce AAV vectors. The normalized risk factor scores for the three vectors were 7.62 for the wildtype GNE1-001ORF with 17 CpGs, 16.5 for the codon optimized GNE1-002 ORF with 40 CpGs, and3.7 for the GNE1-004 ORF with zero CpGs. We then exposed PBMCs isolated

from random healthy donors to antibody-opsonized vectors. We did not detect meaningful differences at MOI 1e4, but when we increased MOI to 1e5 up to 30-fold increases in GNE1-002 vector stimulated IFNαsecretion were observed compared to the GNE1-001 sequence. Surprisingly, the zero CpG containing GNE1-004 ORF vector was more than two-fold more immunostimulatory than the wildtype sequence. In a subsequent PBMC assay of the same vectors, we employed small molecule inhibitors verifying that IFNαsecretion was TLR9 dependent. This study highlights the unprecedented value of our novel biological assay to reveal immunological phenomena that cannot be predicted in silico and raises the intriguing possibility that CpGs in TLR9 inhibitory tetranucleotide contexts should be included in AAV ORFs to counter the immune stimulatory CpGs in AAVITRs.

1557. Innate Immune Avoidance by Extracellular Vesicle Associated AAV (EV-AAV)

Bradley A. Hamilton[1], Demetri de la Cruz[2], Rachana Patil[1], Ming Cheng[2], J. Fraser Wright[1], Casey A. Maguire[2]

[1]Pediatrics, Stanford School of Medicine, Stanford, CA,[2]Neurology, Harvard Medical School, Charlestown, MA

Immune responses to AAV vectors are a significant safetyconcern. Toll-like receptor 9 (TLR9) innate immune receptor has been implicatedas a potentiator of serious adverse events. Methods modeling human innateresponses to AAV have been lacking. However, we recently described an assay exposinghuman peripheral blood mononuclear cells (PBMCs) to AAV opsonized withengineered antibodies which stimulate the release of interferon-α (IFNα), a proxy signal for TLR9activation.Here we investigated extracellular vesicle associated AAV(EV-AAV) vectors, which are a subpopulation of vectors generated duringproduction in 293 cells that resists neutralizing antibodies. Additionally, EVsdisplay phosphatidyl serine on their surface, a lipid moiety that dampensinnate immune stimulation. We therefore hypothesized EV association would reducevector immune stimulation in our assay. We purified EV-AAV2 by size exclusionchromatography, and AAV2 by density gradient ultracentrifugation. Both vectortypes packaged an identical AAV2-CAG>GFP expression cassette. We isolated PBMCs from random healthy donors and exposed themto opsonized EV-AAV2 and controls. We did not detect IFNα secretion from untreated PBMCsor after AAV2 empty capsid exposure. Similarly IFNα stimulation by AAV2-GFP was undetectable,likely due to insufficient internalization by plasmacytoid dendritic cells(pDCs). However, opsonized GFP vectors induced IFNα secretion consistent with increasedpDC uptake. Opsonized AAV empty capsids did not elicit IFNα secretion, consistent with a lackof genomic TLR9 ligands. Importantly, EV association significantly reduced IFNα secretion from PBMCs isolatedfrom donor 63 (p=0.0369) and donor 64 (p=0.0020), compared to standard AAV2-GFP,while insignificant differences were observed with donor 62. Our data suggest that host-specific factors modulate theimmune response to EV-AAV. We therefore performed a neutralizing assay with reserveddonor plasma, revealing the rank order of IFNα response magnitudes correlated with donor neutralizingtiters (Donor 63 > Donor 62 > Donor 64). These data hint that an upstreamevent might both sensitize pDCs to AAV as well as increase neutralizing titers.Additional verification of endogenous antibody removal during PBMC isolationwill help answer this question. We hypothesized effector cell activation would also be reducedby EV association. After IFNα assaywe FACS gated PBMCs on CD19-CD3+CD8+ cytotoxic T lymphocytes (CTLs) and probed forCD25 and CD69 activation markers. Likely due to a short 24 hour post-exposureculture, the differences in CD25 expression between conditions were inconsequential(>2%). CD69 was expressed near baseline in each AAV-empty capsid conditionand in all conditions without opsonization. However, in opsonized AAV2-GFP conditions,CTLs expressing CD69 increased by 25%, 15% and 10% for donors 62, 63, and 64,respectively. Moreover, EV-association conferred 14%, 25%, and 48% reductions inCD69+ CTLs when compared to matched controls. EV-AAV holds potential for mitigating critical immuneresponse challenges now faced in AAV gene therapy. This study highlights thepotential of a novel PBMC assay to interrogate innate immune responses, andshows the innate immune avoiding potential of extracellular vesical associatedAAV in orthogonal assays. Future studies will describe the mechanism ofvesicle-mediated immune avoidance, be it through antibody evasion, phosphatidylserine-mediated inhibition, or both.

1558. Improvement of Transduction Efficiency of Adeno-Associated Vector (AAV) by Extracellular Vesicles (EVs)

Yasunari Matsuzaka

Division of Molecular and Medical Genetics, Center for Gene and Cell Therapy, The Institure of Medical Science, Tokyo, Japan

Introduction: AAV vectors are derived from non-pathogenic viruses and capable of transferring genes into non-dividing cells. In addition, gene expression is maintained in target cells for long periods of time. However, the gene transfer efficiency is reduced by the neutralizing antibodies against the viral capsid of the AAV vector. To overcome this problem, we are trying to improve the gene transfer efficiency of the AAV by constructing "vexosomes" which are nanoparticles in which AAVs are encapsulated in EVs. Materials and Methods: By using AAV and EVs extracted from the media of cultured cells, we tried to incorporate AAV particles into EVs by membrane fusion methods (post-loading methods). To compare the gene transfer efficiency between AAVs and vexosomes, these vectors were inoculated into cultured cells and the gene transfer efficiency was evaluated by luciferase activity. Results: Vexosomes outperform AAV in gene delivery efficiency to RD cells *in vitro*. In addition, the gene delivery rate of vexosomes increases with a higher number of EVs particles for a given amount of AAV genome copies. In the fusion experiments of EVs with AAVs using detergent, higher concentrations of the detergent were not correlated with higher encapsulation efficacy. These findings suggest that relatively mild encapsulation condition is an important factor for the integrity of proteins on EVs that play an important role in their binding to cells. Conclusions: The efficiency of gene transfer using vexosomes was higher than that of AAV *in vitro* experiments. The preparation of vexosomes by incorporating AAV particles into EVs using post-loading methods is an effective way to improve gene transfer efficacy. Gene transfer using vexosomes can improve therapeutic effects for various diseases.

1559. Immunosilencing of a Promiscuous T Cell Epitope in AAV9 Gene Therapy Vector by Rational Design Chimerism

Sojin Bing[1], Morten Seirup[2], Trish Hoang[2], Susana Najera[1], Collin Britten[3], Stephanee Warrington[4], Shian-Ling Chu[1], Ronit Mazor[1]

[1]FDA, Silver Spring, MD,[2]Promega Corporation, Fitchburg, WI,[3]Waters, Santa Barbara, CA,[4]CBER, Food and Drug Administration, Silver Spring, MD

Despite the high safety profile demonstrated in clinical trials, adeno-associated virus (AAV)-mediated gene therapy still faces considerable obstacles due to its immunogenicity. Here, we silenced the most immunodominant T cell epitope in AAV9 through rational design chimerism. The new chimeric vectors maintained their functions and potency, including yield, cellular specificity, in vitro and in vivo transduction efficacy, and biodistribution in mice, while not eliciting any cellular immune responses.

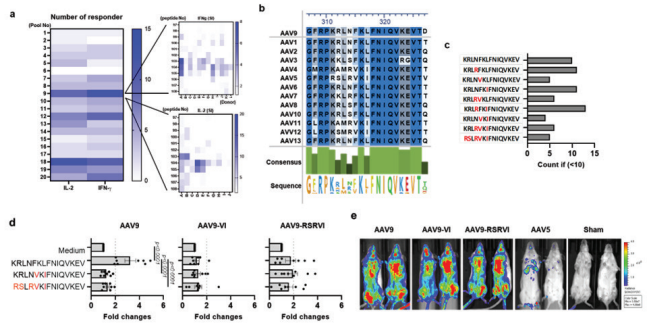


Fig 1. a. Responses of PBMCs obtained from 52 donors to peptide pools from AAV9 VP1 protein were measured by IL-2 and IFN-γ ELISpot assays. PBMCs from healthy donors were stimulated with empty AAV9 capsid for 14 days, followed by restimulation with peptide pools spanning the amino acid sequence of AAV9 VP1. After 24 hours, IL-2 and IFN-γ producing cells were detected by ELISpot assays. **b.** VP1 peptides 103-105 in AAV1-13 serotypes were aligned using DNAstar software showing a high degree of conservation with the exception of amino acids R312, L313, N314, F315, and L317. **c.** Binding affinity of peptides to 27 HLA class II alleles were predicted using the IEDB T cell epitope prediction tool. The number of alleles were counted if their percentile ranks predicted by the IEDB methods were below 10%. **d.** PBMCs were stimulated with the indicated AAV vectors for 14 days. Cells were re-stimulated with individual peptides, and the IFN-γ producing cells were measured by ELISpot assays. **e.** NanoLuc-expressing chimeric AAV9 variants (1E11 vg/mouse) were injected in BALB/c mice intravenously. Representative image of NanoLuc expression in the mice 8 days after vector administration.

1560. The Presence of CpG Dinucleotides in an AAV Transgene Induces a Plasmacytoid Dendritic Cell-Like Population Expressing the Fc Receptor Very Early After Vector Administration in Mice

Justin Glenn, Henos S. Negash, William Marcus Henry, Randolph Qian, Ye Liu, Joseph T. Bruder, Subha Karumuthil-Melethil, Olivier F. Danos

REGENXBIO Inc., Rockville, MD

AAV-mediated gene therapy is a promising platform for treating severe diseases; however, host-derived, antagonistic immune responses to the vector components still limit its use. Unmethylated CpGs in AAV vector DNA activate the innate immune response through TLR9 stimulation and enhance adaptive immune responses against both the AAV capsid and transgene product. We previously observed that CpG depletion increased the expression of a human IgG transgene product (TP) by 2-3-fold as early as 24 hours post-vector administration in mice. This was not accompanied by changes in AAV genome biodistribution or transgene mRNA levels, but instead with CpG-dependent differences in the abundance of a pDC-like population. This $PDCA^{lo/-}CD11b^{+}$ myeloid population was increased in the presence of CpG dinucleotides within the AAV genome. Here, we sought to determine a potential mechanism by which the induced myeloid cells reduce TP levels. *Ex vivo* studies revealed that these cells could directly bind and sequester the TP via Fc-FcγR engagement. This interaction was prevented by switching TP from IgG to an Fc-less, Fab form. Further characterization confirmed the inflammatory activation status of these cells by IRF7 positivity and revealed heterogeneity in their immune stimulatory potential. Thus, we extend the mechanisms of TLR9-mediated antagonism in AAV gene therapy to include the actions of a previously unreported pDC-like cell population.

1561. Identification of a Novel Strategy to Modify AAV Vector Capsid to Reduce Immunogenicity

Winston Colon-Moran, Alan Baer, Santosh Panthi, Nirjal Bhattarai

OTP/TVBB, FDA/CBER, Silver Spring, MD

AAV vectors are commonly used for vector-based gene therapies; however, host immune responses against AAV and/or transgene present a safety risk to subjects and can reduce therapeutic efficacy. To address this, we hypothesized that incorporating immunomodulatory factor(s) derived from naturally occurring human viruses into the AAV capsid may reduce AAV immunogenicity. To this end, we studied two immunomodulatory proteins expressed by the hepatitis B virus, e antigen (HBeAg) and s antigen (HBsAg), and its effect on human T cell activation (TCA). We found that HBeAg, but not HBsAg, inhibited T cell receptor-mediated TCA. Activation of human T cells with PMA and Ionomycin, which bypasses TCR and activates distal T cell signaling was not affected by HBeAg, suggesting that HBeAg specifically inhibits proximal TCR signaling. Deletion mutagenesis studies identified a short 11 amino acid peptide (11mer) derived from HBeAg that is sufficient to inhibit TCA. Mechanistically, we found that 11mer peptide inhibited activation of Interferon regulatory factor 3 (IRF3), potentially contributing to HBeAg-mediated inhibition of TCA. The 11-mer peptide was inserted into variable region (VR) III of the common VP3 region, which is exposed on the surface of the capsid. Importantly, *in silico* analysis showed that the insertion of the 11mer peptide did not affect the AAV capsid structure. Current studies are underway to assess quality, potency, and immunogenicity of the modified AAV vector and delineate mechanisms for the inhibition of T cell activation.

1562. Empty Capsid Immunization Prevents AAV8-Mediated Gene Transfer in a Dose-Dependent Manner in Cynomolgus Monkeys

Geoffrey DeCouto, Megumi Adachi, Nakyo Heo, Cristina Rodriguez Mateo, Su Liu, Bala Medicherla, Damir Simic, Carlos Fonck

Astellas Gene Therapies, South San Francisco, CA

Introduction. Immune responses against adeno-associated viruses (AAVs), which are used for gene therapy, have been reported to limit tissue transduction and diminish efficacy. In particular, antibody production, including IgGs against viral capsid proteins, is thought to block access to cells and contribute to faster clearance of viral vectors.

Despite the importance of understanding immune responses and how they interfere with AAV-based therapeutics, the field continues to struggle with establishing experimental models in which to induce and measure antibody levels that prevent successful AAV-based gene transfer. In this study we examined the impact of varying concentrations of empty AAV8 capsids on antibody production, transduction and transgene expression following AAV8 gene therapy in cynomolgus monkeys. *Methods*. Cynomolgus macaques, ~2.5 years old, were screened for pre-existing neutralizing antibodies (Nabs) against AAV8 capsids. Animals with Nab titers ≤1:5 were designated as immunologically naïve (IN), and animals with Nab titers >1:160 were designated as immunologically active (IA). Monkeys were distributed into the following groups: 1) IN that received vehicle control, 2) IN treated with an AAV8 gene therapy construct coding for a protein under the control of a muscle-specific promoter (AAV8-MSP, 1e14 vg/kg), and 3) IA treated with AAV8-MSP (1e14 vg/kg). Groups 4-7 were IN treated with increasing concentrations of empty capsids (equivalent to 1e7 - 1e13 vg/kg) followed by AAV8-MSP (1e14 vg/kg) 8 weeks later. Monkey sera were collected at various timepoints and analyzed for Nab and total antibodies (Tab). Twelve weeks after AAV8-MSP administration, monkeys were euthanized, and heart and skeletal muscles were analyzed for vector copy number (VCN), protein expression, and transgene protein enzymatic activity. *Results*. Vehicle control animals displayed no significant changes in Nab or Tab over time. Importantly, 8-20 weeks after receiving empty capsids, monkeys revealed a dose-dependent increase in Nab and Tab with temporal fluctuations consisting of a biphasic increase followed by a plateau. Heart tissue showed an empty capsid dose-dependent decrease in AAV8-MSP VCN and transgene protein enzymatic activity. Interestingly, groups 3 and 7 had similar and relatively low VCN and enzymatic activity over baseline, implying effective transduction blockade. In addition, heart enzymatic activity had an inverse correlation to antibody levels measured 8 weeks after empty capsid dosing (R^2: Nab = 0.57, Tab = 0.66). Compared to heart, lower values for VCN and enzymatic activity were observed in skeletal muscles including quadriceps, biceps, and triceps in all treatment groups. *Conclusions*. We evaluated the impact of AAV8 capsid immunization on transgene transduction and expression of an AAV8 therapeutic in cynomolgus macaques. The antibody response was dose-dependent and titratable; a high dose of empty capsids (1e13 vg/kg) prevented transduction and transgene protein enzymatic activity in target tissues, while a low dose (1e7 vg/kg) of empty capsids conferred no inhibitory activity. Our data also show that Tab titers appear to have slightly better correlation with VCN values and enzymatic activity compared to Nabs. Additionally, the relatively weak correlation between antibody levels and transduction and expression suggests that other factors not accounted for in this study may contribute to decreased transduction and expression of AAV8-based gene therapy. Nonetheless, this study identified AAV8 capsid doses and their corresponding Ab level increases that prevented effective AAV8 gene transfer.

Friday Posters: Lysosomal Storage Disorders

1564. Lentiviral Vector Optimization for the Preclinical Development of Hematopoietic Stem Progenitor Cells (HSPCs)-Gene Therapy(GT) for Mucopolysaccharidosis Type IVB (MPSIVB)

Stefania Crippa[1], Gaia Alberti[1], Laura Passerini[1], Giada De Ponti[2], Ludovica Santi[2], Raisa Hernandez[1], Ilaria Visigalli[3], Francesca Sanvito[4], Paola Albertini[4], Rossella Parini[5], Sean Russel[6], Leopoldo Staiano[7], Serena Scala[2], Silvia Adriana Gregori[8], Maria Ester Bernardo[2,9,10], Alessandro Aiuti[3,9,10]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milano, Italy,[2]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milan, Italy,[3]GLP - San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milan, Italy,[4]GLP - San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milano, Italy,[5]Rare Metabolic Disease Unit, San Gerardo Hospital, Monza, Italy,[6]Telethon Foundation, Rome, Italy,[7]Telethon Institute of Genetics and Medicine (TIGEM), Naples, Italy,[8]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, MILANO, Italy,[9]Università Vita-Salute San Raffaele, Milan, Italy,[10]Pediatric Immunohematology and Bone Marrow Transplantation Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy

Mucopolysaccharidosis type IVB (MPSIVB) is an ultra-rare inherited lysosomal storage disorder caused by b-galactosidase (b-GAL) deficiency, a lysosomal enzyme encoded by the *GLB1* gene. No approved therapy is available to treat MPSIVB patients who manifest severe skeletal alterations and neurological involvement. Based on recent data demonstrating the superior safety and efficacy of hematopoietic stem progenitor cell-gene therapy (HSPC-GT) to treat similar lysosomal storage disorders (LSDs), we aim to develop a HSPC-GT strategy for MPSIVB within the framework of an innovative platform approach for LSDs with skeletal involvement. We designed and tested different lentiviral vectors (LVs) to achieve b-GAL overexpression and release in human mobilized-peripheral blood (mPB) HSPCs. We found that the LVs bearing the human GLB1 cDNA (normal and codon-optimized) allowed the intracellular overexpression of the therapeutic enzyme, which, on the contrary, was present at low levels in the extracellular space of the transduced cell progeny (myeloid cells and osteoclasts), limiting the metabolic correction of patients' cells in in vitro cross-correction assays. We hypothesized that the low amount of b-GAL enzyme in the extracellular space was associated with a temperature-dependent stability of the human enzyme, which is low at 37°C. On the contrary, human mPB HSPCs transduced with the LVs encoding for the murine b-GAL released supranormal levels of the therapeutic enzyme in the absence of cell toxicity, which restored the enzymatic activity in patients' cells. Moreover, human HSPCs overexpressing the murine b-GAL efficiently engrafted and reconstituted the hematopoietic system, similar to untransduced and LV-human GLB1 transduced HSPCs upon transplantation into

NSGW41 mice. Importantly, we detected a significantly higher level of b-GAL activity in the bone marrow of mice transplanted with HSPCs transduced with the LV-murine GLB1. We further proved that the murine b-GAL was internalized and targeted into the lysosomal compartment of patients' cells as the human enzyme and excluded immunogenicity of the murine enzyme in short- and long-term in vitro assays. In conclusion, our data support the use of the murine *Glb1* transgene for HSPC-GT development at a pre-clinical level in *Glb1*-/- mice and for future clinical translation.

1565. Pre-Clinical Development of an Ex-Vivo Lentiviral-Based Hematopoietic Stem/Progenitor Cells (HSPC)-Gene Therapy (GT) for Mucopolysaccharidosis Type IVA (MPSIVA) as Part of an Innovative GT Platform Approach for LSDs with Skeletal Involvement

Stefania Crippa[1], Margherita Berti[2], Ludovica Santi[2], Giada De Ponti[2], Raisa Hernandez[1], Pamela Quaranta[2], Matilde Cossutta[1], Erika Zonari[1], Selene Ceriotti[1], Ilaria Visigalli[3], Luca Basso-Ricci[1], Paola Albertini[4], Francesca Sanvito[4], Antonella Paoli[5], Nicola Volpi[6], Amelia Morrone[7], Marina Radrizzani[1], Rossella Parini[8], Serena Scala[2], Alessandro Aiuti[2,9,10], Maria Ester Bernardo[2,9,10]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milano, Italy,[2]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milan, Italy,[3]GLP - San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milan, Italy,[4]GLP - San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), IRCCS San Raffaele Scientific Institute, Milano, Italy,[5]Department of Pediatrics, Meyer Hospital, Florence, Italy,[6]Department of Life Science, University of Modena and Reggio Emilia (UNIMORE), Modena, Italy,[7]Department of Pediatrics, Meyer Hospital, Florece, Italy,[8]Rare Metabolic Disease Unit, San Gerardo Hospital, Milano, Italy,[9]Università Vita-Salute San Raffaele, Milan, Italy,[10]Pediatric Immunohematology and Bone Marrow Transplantation Unit, IRCCS San Raffaele Scientific Institute, Milan, Italy

Mucopolysaccharidosis type IVA (MPSIVA) is a lysosomal storage disease (LSD) caused by the deficiency of the lysosomal enzyme N-acetylgalactosamine-6-sulfatase (GALNS), characterized by severe skeletal dysplasia, which the currently available therapies fail to cure. Our study aims at developing hematopoietic stem progenitor cells (HSPC)-gene therapy (GT) for MPSIVA as part of an innovative GT platform approach for LSDs, based on recent data proving the safety and efficacy of HSPC-GT for the treatment of other LSDs. To this aim, we generated a 3rd-generation lentiviral vector encoding for the GALNS enzyme (LV-GALNS), using the same LV construct of the MPSI-IH clinical trial (ClinicalTrials.gov, NCT03488394) to transduce human mobilized peripheral blood (mPB) CD34$^+$ cells. Transduced cells showed proper clonogenic and proliferative capability *in vitro* and significantly overexpressed and released the GALNS enzyme. Using the conditioned medium from LV-GALNS transduced myeloid culture, we restored GALNS expression and activity in patient-derived fibroblasts, mesenchymal stromal cells (MSCs), and MSC-derived osteoblasts. Importantly, LV-GALNS human mPB CD34$^+$ cell derived-osteoclasts secreted GALNS, possibly functioning as a resident source of enzyme for skeletal cross-correction. When transplanted in sub-lethally irradiated NSG mice, LV-GALNS mPB CD34$^+$ cells efficiently engrafted and repopulated the hematopoietic system. Importantly, we detected a significantly higher level of GALNS enzyme in the bone marrow of mice treated with HSPC-GT. To test the superior efficacy of GT compared to standard treatment, we have recently generated a GALNS knock-out animal model by CRISPR-Cas9 technology, which shows signs of skeletal disease, including shorter bones, reduced mineral density, and osteoblast number, in addition to a swollen, disorganized, and inflamed growth plate compared to age- and sex-matched wild-type controls. Finally, we tested the clinical-grade LV-GALNS in human HSPCs to identify the best-performing transduction protocol for future manufacturing processes. Altogether, our preclinical results show efficient transduction of human HSPCs without signs of toxicity, the release of supranormal levels of the therapeutic enzymes from the hematopoietic progeny of transduced cells, capable of metabolically cross-correcting patients' cells. The planned Proof-of-concept study in MPSIVA mice will further implement our data to support the clinical development of HSPC-GT for MPSIVA treatment.

1566. An Analysis of Biomarkers for the Early Diagnosis and Evaluation of Gene Therapy for Niemann-Pick Disease Type C

Chika Watanabe[1], Eriko Jimbo[1], Yoshie Kurokawa[1], Miyuki Watanabe[1], Karin Kojima[1], Kazuhiro Muramatsu[1], Keitaro Miyoshi[2], Masamitsu Maekawa[2,3], Chen Wu[4], Miki Igarashi[4], Yoshikatsu Eto[4], Katsuhito Asai[5], Tsuyoshi Teshima[5], Shin-ichi Muramatsu[6], Hitoshi Osaka[1], Takanori Yamagata[1,7]

[1]Department of Pediatrics, Jichi Medical University, Shimotsuke, Japan,[2]Faculty of Pharmaceutical Sciences, Tohoku University, Sendai, Japan,[3]Department of Pharmaceutical Sciences, Tohoku University Hospital, Sendai, Japan,[4]Advanced Clinical Research Centre, Institute of Neurological Disorders, Kawasaki, Japan,[5]Gene Therapy Research Institution Co., Ltd., Kawasaki, Japan,[6]Division of Neurological Gene Therapy, Jichi Medical University, Shimotsuke, Japan,[7]Tochigi Rehabilitation Center, Utsunomiya, Japan

Niemann-Pick disease type C1 (NPC1) is a congenital disorder caused by variants of the *NPC1* gene, which is involved in cholesterol transport in lysosomes. The clinical manifestations of NPC1 include liver failure, neurological and psychiatric symptoms, and pulmonary failure that occurs from neonatal to adult age. An onset in infancy results in rapid progression and a poor prognosis. With no essential treatment options available, we developed gene therapy for NPC1. An early diagnosis and treatment are crucial for improving the therapeutic efficacy. Therefore, it is necessary to establish specific biomarkers for the early diagnosis and treatment. **Methods:** We generated an adeno-associated virus (AAV) vector that expressed human *NPC1* under the cytomegalovirus (CMV) promoter (AAV-CMV-*hNPC1*) and injected it into the left lateral ventricle and cisterna magna on day 3 or 5 of life, with a total of 1.35×10^{11} vector genomes per mouse. We then explored novel biomarkers using a non-targeted metabolomic analysis of plasma from *NPC1*$^{-/-}$ and *NPC*$^{+/+}$ mice. In addition, biomarkers such as 7-ketocholesterol (7-KC), cholesterol, lyso-sphingomyelin (lysoSM),

and *N*-palmitoyl-O-phosphocholine-serin (PPCS) were analyzed in the mouse plasma and liver before and five weeks after vector injection into the ventricles and cisterna magna. **Results:** Our gene therapy prolonged the survival of treated homo-knockout (*NPC1*$^{-/-}$) mice by an average of 105 days, improved Rota-rod performance, and preserved cerebellar Purkinje cells. Of the 393 compounds identified, 17 were candidates for novel biomarkers, including guanidoacetic acid and N1-methylguanosine. In the plasma of NPC1 patients, no significant differences in compounds were found between the patients and controls. However, some compounds were found to be elevated in adult patients but not in children. PPCS levels in plasma and 7-KC, cholesterol, and lysoSM levels in the liver were elevated in *NPC1*$^{-/-}$mice, but no decrease was noted in AAV vector-treated *NPC1*$^{-/-}$mice. **Discussion:** The levels of biomarkers varied by age, so further research is needed to determine the appropriate biomarkers for newborn screening. The vectors injected into the central nervous system reached the liver. We continued to analyze the biomarkers at increasing dosages. Our gene therapy for NPC1 mice was effective for managing neurological symptoms, which are the leading cause of death. Therefore, we are currently preparing for a clinical trial.

1567. EXG110: Promising Gene Therapy for Fabry Disease - Achieving Complete Substrate Correction in Mice and Exceptionally Robust GLA Expression in NHPs

Shaoyong Li, Hong Wang, Pengfei Yu, Chunjuan Song, Mingwen Liu, Qingzeng Wang, Wei Hu, Xin Xin, Zhenhua Wu, Guo-jie Ye

Exegenesis Bio Inc., Lower Gwynedd, PA

Fabry disease (FD) is a rare metabolic disorder caused by deficiency of lysosomal enzyme α-galactosidase A (α-Gal A) activity due to deleterious mutations in the *GLA* gene. Accumulation of substrates within lysosomes results in cellular dysfunction and multiple organ damage. The current enzyme replacement therapy requires bi-weekly infusion, however, only minimal clinical effects in myocardial tissue were achieved. To provide a single administration with long-lasting effects, we developed a gene therapy product EXG110 for FD. EXG110 comprises a novel and highly efficient liver-muscle targeting Adeno-associated virus (AAV) capsid and an optimized human *GLA* transgene expression cassette. A single intravenous injection of EXG110 to 4-month-old male Fabry mice (*Gla* KO*)* assigned to 3 dose groups resulted in dose-dependent α-Gal A activity in the plasma throughout the duration of 8-week study. The efficacy of EXG110 treatment was evaluated by measuring the levels of substrate lyso-Gb3 with LC-MS/MS, and immunostaining of the substate Gb3 in the key tissues. The low dose (2e11 vg/kg) of EXG110 treatment corrected the lyso-Gb3 to near a normal level in the plasma, liver and heart, and significantly reduced the lyso-Gb3 level in the kidney as well. The Fabry mice treated with medium dose (2e12 vg/kg) exhibited complete substrates correction in the liver and heart, and with nearly normal lyso-Gb3 levels in the plasma and kidney. Furthermore, the renal cortex of Fabry mice treated with medium dose showed complete Gb3 clearance. As expected, the high dose (2e13 vg/kg) of EXG110 treatment resulted in complete correction of substrate levels in plasma and all key tissues including liver, heart and kidneys. An on-going 8-week dose-response study in Fabry mice was carried out to explore the minimum effective dose of EXG110. Dose-dependent α-Gal A activity and expression in the plasma were observed. To assess if EXG110 could mediate robust and stable α-Gal A expression and activity in non-human primates (NHPs), adult male Cynomolgus macaques were intravenously administrated with 3 doses of EXG110 in a staggered manner and monitored for at least 13 weeks. One monkey, dosed with 2e13 vg/kg, exhibited up to 1,750-fold increase of α-Gal A activity in the plasma compared to baseline level. The enzyme activities in the liver, heart, skeletal muscle of this animal were 54-fold, 80-fold, and 134-fold, respectively, over that of control animal. The monkeys dosed with 2e12 vg/kg and 6e12 vg/kg of EXG110 exhibited 221-fold and 629-fold higher plasma α-Gal A activity above baseline, respectively. Stable α-Gal A activity in the plasma has been observed for at least 18 weeks after the administration of EXG110. In summary, EXG110 demonstrated notable efficacy in Fabry mice and superior α-Gal A activity in NHPs. These preclinical findings support the ongoing clinical development of EXG110 for Fabry disease.

1568. Novel Ubiquitous Promoters Evade Transgene-Specific Immune Responses and Drive Widespread Therapeutic Transgene Expression in a Mouse Model of Pompe Disease

Baodong Sun

Pediatrics, Duke University School of Medicine, Cary, NC

Background: Systemic correction of muscle and the brain is desired for the treatment of Pompe disease, an inherited lysosomal storage disorder caused by acid alpha-glucosidase (GAA) deficiency. AAV-mediated gene therapy with current ubiquitous promoters often causes strong transgene-related immune responses that eliminate human GAA (hGAA) expression quickly in Pompe disease (GAA-KO) mice. We have recently identified a 356-bp human G6PC minimal promoter that displays unexpected high activity in mouse heart and skeletal muscle (the full length G6PC promoter is only active in liver and kidney). To further boost transgene expression in skeletal muscle, we have synthesized a hybrid promoter (CRM4-G6PC) by combining a 434-bp high potency human skeletal muscle-specific transcriptional cis-regulatory module (hSk-CRM4) with the 356-bp G6PC minimal promoter. Here we tested these two promoters in AAV-mediated gene therapy in GAA-KO mice. **Methods:** We constructed AAV9 vectors expressing hGAA driven by the G6PC promoter (AAV9-G6PC-hGAA) and the CRM4-G6PC hybrid promoter (AAV9-CRM4-G6PC-hGAA) and intravenously injected them at the same dose ($1x10^{13}$ vg/kg) into 14-week-old GAA-KO mice. Tissues were collected after 3 weeks to assess hGAA expression and glycogen reduction. For a longer-term (12-week) experiment, 19-week-old GAA-KO mice were intravenously injected with the AAV9-CRM4-G6PC-hGAA vector at a dose of $1x10^{13}$ vg/kg. **Results:** In the 3-week experiment, both AAV treatments led to significantly increased GAA activities in all tested tissues including the liver, heart, quadriceps, gastrocnemius, diaphragm, spleen, kidney, and the brain (**Figure 1**). The AAV9-CRM4-G6PC-hGAA treated mice had significantly higher GAA activities in the heart and limb muscles than the AAV9-G6PC-hGAA treated mice (**Figure 1**). Both AAV9-G6PC-hGAA and AAV9-CRM4-G6PC-hGAA vectors nearly cleared glycogen accumulation in liver, and both significantly reduced glycogen contents

in heart (-46% and -81%, respectively), quadriceps (-41% and -52%), gastrocnemius (-34% and -38%), and diaphragm (-53% and -55%). There was no difference in glycogen content in the brain between AAV-treated and untreated mice. Secreted hGAA protein was detected in the blood from mice treated with either vector, and there was no anti-GAA antibody detected in any of these mice. In the 12-week study, GAA activities were significantly increased in liver, heart and skeletal muscles of AAV-treated mice. Glycogen contents were significantly reduced by the AAV treatment in heart (- 94%), quadriceps (-19%), gastrocnemius (-40%), and diaphragm (-64%), accompanied by the improvement of muscle functions by treadmill and rota-rod tests. **Conclusion:** The G6PC minimal promoter is a unique ubiquitous promoter that drives high-level hGAA expression in liver, muscle, and many other tissues without causing anti-hGAA antibody response in GAA-KO mice. The addition of the muscle enhancer CRM4 greatly increases the promoter activity in the cardiac and skeletal muscles. These novel promoters can be broadly used for gene therapy for inherited disorders that affected multiple tissues including muscle.

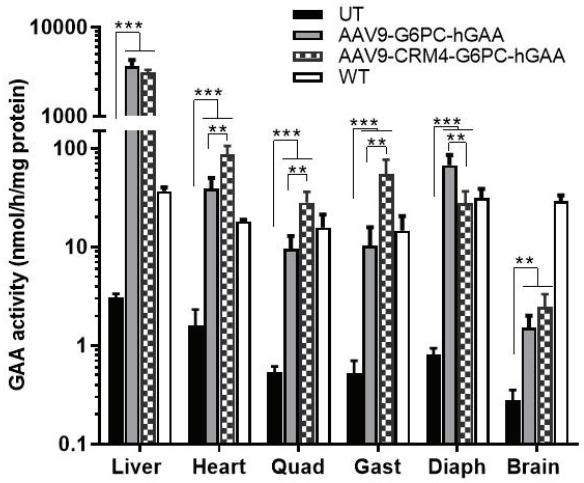


Figure 1. GAA activity in tissues of GAA-KO mice 3 weeks following AAV administration. n=6 for the untreated (UT) and AAV-treated mice; Data shown as mean ± SD. ** p<0.01, ***p<0.001. Wild-type mice (WT, n=5) were included as normal reference.

1569. Preclinical Studies of AAV Vectors with Tissue-Specific, Tandem, and Ubiquitous Promoters for Mucopolysaccharidosis Type IVA Mice

Shunji Tomatsu, Shaukat Khan, FNU Nidhi, Eliana Benincore

Nemours Children's Health, Wilmington, DE

Mucopolysaccharidosis type IVA (MPS IVA) is a lysosomal storage disorder caused by a deficiency of *N*-acetyl-galactosamine-6-sulfate sulfatase (GALNS), leading to the accumulation of storage materials (keratan sulfate and chondroitin-6-sulfate) and developed severe skeletal dysplasia. Enzyme replacement therapy and hematopoietic stem cell transplantation are in practice but have limited impact on skeletal lesions. Gene therapy using adeno-associated virus (AAV) is rapidly growing to treat metabolic diseases. We previously showed that liver-targeted AAV gene transfer of human GALNS expressed supraphysiological levels of the enzyme in blood and multiple tissues and partially improved storage materials and vacuolization of chondrocytes in bone lesions. In this study, we investigated 9 vectors; liver-specific AAV8 vectors with TGB or LSPX promoter, AAV8 and AAV9 vectors in liver-muscle tandem (LMTP), liver-bone tandem (LBTP), and ubiquitous (CAG) promoters in MPS IVA mice to compare therapeutic efficacy on the biochemical markers and bone pathology. All AAV constructs except AAV8-V2-TBG provided supraphysiological levels of GALNS enzyme activity in plasma of MPS IVA mice from 6 weeks to 16 weeks (at autopsy). AAV9-LMTP gave the highest enzyme activity on average. GALNS enzyme activities were also detected in various tissues, including bone. AAV8-CAG and AAV9-LMTP provided the highest enzyme activities in most tissues. All vectors normalized KS levels in plasma, liver, and bone. All vectors showed over 20 vector copy numbers (VCN) in liver, and AAV9-LMTP provided the highest VCN in bone and muscle. Anti-GALNS antibodies were raised in any vector at 16 weeks old. Pathological analyses demonstrated the reduction or complete absence of vacuolated cells in heart muscle and valves in any vector, while bone pathology was improved most with the AAV8-CAG and AAV-LMTP vectors. Overall, all vectors investigated indicated a substantial improvement in biochemical parameters and pathology. This summary of data moves to setting up the clinical trial for MPS IVA

1570. Optimization of Systemic AAV9 Gene Therapy in Niemann-Pick Type C1 Mice

Avani Mylvara[1,2], Cristin Davidson[1,2], Tansy Gu[2,3], Alana L. Gibson[2,4], Laura Baxter[2], Art Incao[2], Charles P. Venditti[2], William Pavan[2], Forbes D. Porter[1]

[1]National Institute of Child Health and Development, Bethesda, MD,[2]National Human Genome Research Institute, Bethesda, MD,[3]University of North Carolina, Chapel Hill, Chapel Hill, NC,[4]University of California San Diego, La Jolla, CA

Niemann-Pick disease, type C1 (NPC1) is an uncommon, fatal neurodegenerative disease resulting from pathological variations of the *NPC1* protein that encodes a lysosomal cholesterol transport protein. Despite extensive research over the past decades exploring potential therapies, individuals with NPC1 in the United States lack any approved regulatory therapy, and only a single approved therapy is available in other countries. Studies involving both systemic and direct central nervous system delivery of AAV9-*hNPC1* have demonstrated significant alleviation of the disease in murine NPC1 models. To assess the impact of dosage on efficacy of gene therapy in NPC1 mice, we administered three different doses of AAV9-*hNPC1* at the weaning age. The medium dose was further examined to assess a therapeutic window of efficacy and was administered at pre-, early, and post-symptomatic time points. Our findings indicate that higher doses administered systemically to *Npc1*^{m1N} mice and at earlier time points result in increased lifespans and delayed disease progression. Analysis of viral copy number and distribution of AAV9-*hNPC1* revealed that earlier administration of the gene therapy product led to greater transduction in the central nervous system. These results offer crucial insights for clinical trial design, suggesting that intervening earlier in a pre-symptomatic disease state with a sufficiently high dose likely will yield better outcomes for NPC1 individuals.

1571. Liver-Directed Gene Targeting as a Potential Therapy for Fabry Disease

Himanshi Saxena[1], Jessica Biasizzo[2], Rossana Domenis[2], Lorena Zentilin[3], Andrea Dardis[4], Andrés F. Muro[1]

[1]International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste, Italy,[2]Institute of Clinical Pathology, Department of Laboratory Medicine, University Hospital of Udine, Udine, Italy,[3]ICGEB, Trieste, Italy,[4]Regional Coordinator Centre for Rare Diseases, University Hospital of Udine, Italy, Trieste, Italy

Fabry disease (FD) is an X-linked inherited, lysosomal storage disorder caused by Alpha Galactosidase-A (GLA) gene mutations. This gene encodes for the GLA enzyme, responsible for glycosphingolipids (like globotriaosylceramide, Gb3) catabolism. Gb3 accumulation in lysosomes results in systemic clinical manifestations and reduced lifespan. Enzyme replacement therapy (ERT) and chaperone therapy are the available treatments for FD however, noncurative and with limitations. We developed a potential therapeutic approach based on the permanent genetic modification of hepatocytes to express the GLA enzyme by targeting the albumin locus *in vivo*. To model late-onset and early-onset FD, we treated juvenile (P30) and neonatal (P5) Fabry mice (Gla KO) with an AAV8 donor vector containing mAlb homology arms and a WT or codon-optimised (CO) version of the human GLA cDNA that improves translation about 4-6-fold over the WT sequence. To increase safety of the procedure avoiding concerns related to the use of programmable nucleases, we applied a nuclease-free approach coupled with the treatment with fludarabine, which enhances gene targeting rate. Juvenile Fabry animals were treated only with the donor vector (3.0E13 vg/kg) and fludarabine. resulting in increased plasma GLA activity compared to donor-only treated mice which was stable till the termination of the experiment at 5 months of age, accompanied by 80-95% of lyso-Gb3 clearance. As a comparison, the donor vector treatment was coupled with the CRISPR/SaCas9 AAV-mediated delivery (donor, 3.0E13 vg/kg; SaCas9, 6.0E12 vg/kg) to increase targeting efficiency resulting in elevated GLA enzyme activity, accompanied by a 70-80% reduction in Lyso-Gb3 accumulation in liver, kidneys and heart, compared to untreated mutant mice. The treatment of neonatal P5 Fabry mice with donor and SaCas9 AAVs was significantly more efficient than in juvenile animals due to the increased targeting rate in proliferating hepatocytes, reaching up to 8-9% with the highest dose (3.0E14 vg/kg). In fact, we completely cleared lyso-Gb3 in plasma and target organs with the highest dose, while 10x lower dose (3.0E13 vg/kg) resulted in 95-98% reduction in plasma and target organs, higher than that obtained in juvenile Fabry mice. A dose escalation study with liver-specific AAV-mediated episomal gene therapy was done as a proof-of-principle in juvenile Fabry KO mice. Animals treated at 3.0E12vg/kg and higher doses were able to reduce substrate accumulation by 98-100% in plasma and target tissues with the CO-GLA cDNA. Treatment with the lowest dose of the CO-GLA AAV vector (3.0E11vg/kg) resulted in the clearance of 85-95% lyso-Gb3 in the bloodstream and tissues proving the efficacy of the treatment for late-onset FD. ERT-treated animals were considered for comparative evaluation of the treatment, which were less efficient than the lowest AAV dose to clear lyso-Gb3 from plasma and tissues.We showed that treatment of early-onset FD is very effective with a genome targeting strategy, while non-integrating AAV-mediated liver gene therapy is very efficacious for late onset FD, even at low AAV doses. This data is inclined towards a promising one-shot therapy approach to ameliorate the phenotype irrespective of the mutation, targeting both the early and late-onset FD conditions. To test the translational applicability of this integrative strategy, AAV-LK03 vectors containing human ALB homology arms have been successfully tested in the HuH-7 human liver cell line and in primary cultures of human hepatocytes, and are being validated in humanized mice to generate consistent preclinical support.

1572. Development of a Hematopoietic Stem Cell Gene Therapy for Infantile GM1 Gangliosidosis

Linda Bucciarelli, Camilla Fabris, Elena Scarabello, Massimo Accardo, Valentina Poletti, Alessandra Biffi

Department of Pediatric Hematology and Oncology Center, Woman's and Child Health, Università Degli Studi Di Padova, Padova, Italy

GM1-gangliosidosis (GM1, OMIM #230500) is a rare, recessive Lysosomal Storage Disease caused by mutations in the *GLB1* gene encoding the β-galactosidase (β-gal) enzyme. The enzymatic deficiency induces several mechanisms of cell and organ damage resulting in severe and progressive neurodegeneration, and premature death of the patients. Effective LSD treatment requires a prompt and robust enzyme delivery both to the Central Nervous System (CNS) and the periphery. Carriers with ≥20% of residual activity are asymptomatic (Sopelsa et al. 2000), defining a minimal therapeutic goal. Hematopoietic Stem/progenitor Cell (HSC) Gene therapy (GT), based on the transplantation of autologous HSCs genetically-modified *in vitro* by lentiviral gene transfer, showed safety and transformative clinical efficacy in pioneer academic clinical trials for two rare LSDs: Metachromatic Leukodystrophy (MLD, Biffi et al. 2013, Sessa et al. 2016, Fumagalli et al. 2022) and MPS IH (Gentner et al. 2021). As HSC GT may represent a valuable therapeutic option also for GM1, we designed and developed an HSC GT strategy for GM1 gangliosidosis based on the use of a lentiviral vector (LV) encoding either a codon-optimized sequence of the human *GLB1* gene (hu*GLB1*co) or the murine (mu) *Glb1* cDNA. In GM1 patient-derived primary fibroblasts, transduction with both LVs determined a dose-dependent and supra-physiological enzyme expression, with the murine enzyme resulting ~6-fold more active than the human one. Both LVs produced a correctly processed β-gal, secreted and uptaken by target cells. hu*GLB1*-LV determined phenotypical cell correction, evaluated as rescue of lysosomal storage and swelling, with an average vector copy number (VCN) of ≥ 3. A proof-of-concept of efficacy study was conducted in the murine model of the disease, *i.e.* *Glb1*$^{-/-}$ mice, employing both LVs. The study included Busulfan-myeloablated 8-weeks old GM1 mice receiving *Glb1*$^{-/-}$ HSCs transduced with the therapeutic LVs (GT) or WT cells (HSC transplantation), and many control groups: GM1 mice receiving HSCs transduced with a control (GFP) LV, WT mice receiving mock-transduced cells and untreated WT and GM1 mice. At the end of the study (10 months post-transplant) enzymatic activity in the bone marrow (BM) of the *Glb1*$^{-/-}$ mice was rescued, reaching up to the WT range in mice receiving HSCs transduced with the mu*GLB1*-LV (with a mean VCN of 2 in the BM), and 41% of the WT level with the hu*GLB1*co-LV (with a mean VCN of 5 in the BM). In the brain, β-gal activity was also rescued up to ~28% and 20% of the WT level with the mu- and hu-LV, respectively; values that resulted therapeutic in clinical studies for other neuro-LSDs (Gentner et al. 2021, Fumagalli

et al. 2022). Biochemical correction was coupled to a significant amelioration of the neuromuscular phenotype evaluated by the rotarod, hanging and balance beam tests, in addition to an increased survival of GT-treated mice. In summary, we provided early proof of efficacy of HSC GT approach in relevant preclinical disease models to be potentially instrumental to a future clinical translation and identified in the murine cDNA a potentially novel therapeutic opportunity to be explored further.

1573. Real Time Tracking of AAV Gene Therapy via MR Imaging

Thomas J. Meade

Chemistry, Northwestern University, Evanston, IL

Challenge: The gene therapy field lacks disease-specific and robust biomarkers by which it can track therapeutic effects. **Solution:** We have developed a platform of MR imaging contrast agents that are conditionally activated by the product(s) of gene therapy in vivo **(Figure 1)**. The number of academic and biotech-led programs has significantly risen in the last few years, with over 2400 ongoing clinical trials by the end of 2023. It is an exciting and hopeful new era for the gene therapy field. However, ensuring effective clinical trials with shortened paths to approval will require robust biomarkers to track therapeutic effects. To date, there is a lack of non-invasive ways to determine biodistribution or activity levels of these therapies in the body and CNS in patients. This is a significant hindrance, leaving physicians guessing which organs or structures are effectively treated, and due to the lag time associated with clinical disease progression, this limitation ultimately impacts the evolution of treatment modalities. The need for non-invasive, disease-specific biomarkers that reflect treatment efficacy is paramount and not limited to gene therapy. Still, it applies to anything that augments the targeted enzyme activity (including enzyme replacement therapy (ERT), transcriptional read-through agents, and chaperone therapies. Here, we describe the development of a new MRI-based technology to track enzymatic activity enhancement in any organ, peripheral nervous system (PNS), or central nervous system (CNS) over time, and thus, it has the potential to play a critical role in the development and refinement of treatments for *many monogenetic enzymatic deficiencies*. Since these agents are specific to the disease, not the therapeutic modality, advancing this technology applies to the entire lysosomal disease field.

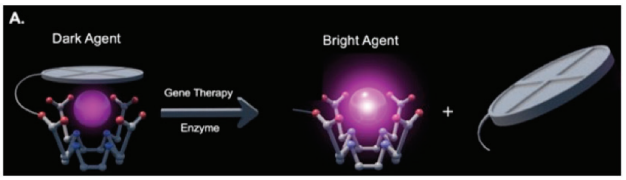


Figure 1A. Schematic of enzyme an *activated* MR contrast agents that monitor *in vivo* gene expression. These agents make possible quantitative, real-time tracking of gene therapy by MR imaging.

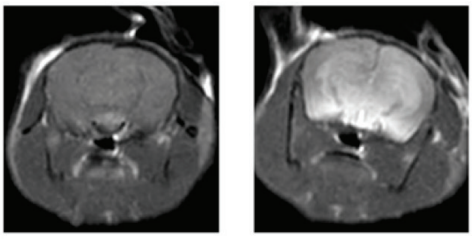


Figure 1B. Pre- and Post MR images of a knockout mouse that was treated by AAV gene therapy. **Left**: Pre-Contrast: **Right**: After intrathecal injection of contrast, no enhancement is observed in wild type animals, however after thalamic injection of gene therapy for β-gal, strong enhancement of the brain is observed, with the greatest in areas with increased CSF (lateral ventricles and ventral aspect of the brain). The significant increase in contrast indicates that the agent was "turned on" by the enzyme delivered by gene therapy.

1574. Peripheral and Behavioral Impacts of AAV Gene Therapy in a Mouse Model of Niemann-Pick Disease, Type C1

Benjamin E. Epstein, Gabrielle M. Soden, Arturo A. Incao, William J. Pavan

National Human Genome Research Institute, National Institutes of Health, Bethesda, MD

Niemann-Pick disease, type C (NPC) is a rare, fatal lysosomal storage disease affecting approximately 1:100,000 births in the United States. NPC is monogenic with autosomal recessive inheritance, and it is caused by mutations in either the *NPC1* or *NPC2* genes, which together function to export unesterified cholesterol from lysosomes. Primary clinical signs of NPC are neurodegenerative, including cerebellar ataxia and cognitive impairment, though visceral symptoms are prevalent as well, with 40% of NPC patients presenting with hepatosplenomegaly in their first year of life. No FDA-approved therapies currently exist

for NPC, though multiple clinical trials are in progress. Previous studies in mouse models of NPC have shown that adeno-associated virus (AAV)-based gene therapy can extend lifespans, slow symptom progression, and reduce neuronal death in the central nervous system (CNS). However, while neurological impacts have been well characterized, the impact of gene therapy on peripheral organs has not been thoroughly examined, particularly in mouse models administered highly efficacious gene therapy treatments. To establish the peripheral impacts of NPC in gene therapy-treated mice, we administered gene therapy in one of four different combinations of AAV serotype, age of delivery, and delivery method to cohorts of NPC1 mutant mice and compared disease outcomes, visceral pathology, and behavioral phenotypes. We found that while all treatments improved outcomes in mice, specific impacts on the periphery varied by treatment method. In particular, NPC1 mice with extended lifespans due to CNS-restricted AAV delivery displayed late-stage visceral pathology distinct from and more severe than normally observed at end stage in mice administered no treatment or less efficacious treatments. Further, while all methods improved behavioral phenotypes, different treatment methods had greater impacts on different individual behaviors assessed. These findings demonstrate the potential for previously unobserved disease manifestations to emerge late in the context of highly efficacious gene therapy treatment of NPC1, and they may suggest the possibility for further improved long-term efficacy by focusing on peripheral treatment in conjunction with the CNS.

1575. AAV-Mediated PPT1 Replacement and Cross Correction for Treating CLN1 Batten Disease

Suhail Alam, Apeksha Khatiwada, Daniel M. Cohen, Drew R. Peterson, John White, Melissa Derby, Bo Wang, Mohamad Nayal, Mallory Becker, Manju Benakanakere, Heena Beck, Charlie Li, Renee Gentzel, Jodi L. McBride, Maria Grazia Biferi, Elizabeth Ramsburg

Spark Therapeutics, Philadelphia, PA

CLN1 Batten disease is a progressive neurodegenerative disease caused by a biallelic loss of function mutation in a lysosomal enzyme called Protein Palmitoyl Transferase 1 encoded by the *PPT1* gene. Cells lacking PPT1 are unable to clear lysosomal contents which directly compromises cellular health resulting in a range of clinical symptoms including profound neurological dysfunction. Notably, CLN1 disease impacts the entire central nervous system. Regions exhibiting the most severe neuronal loss include the cerebral cortex, basal ganglia nuclei, thalamus, cerebellum, spinal cord, and retina. Children who develop the most aggressive infantile-onset form of CLN1 disease typically succumb to the disease in early childhood while those developing the disease later in life may survive to early adulthood. We are developing an adeno-associated virus (AAV)-mediated gene therapy approach with the goal to have ubiquitously expressed PPT1 throughout the brain and spinal cord of affected individuals. PPT1 is a secreted enzyme, which makes this a suitable candidate for maximizing efficacy through cross-correction. We will present data on the preclinical development of this approach, including the use of *in silico* tools to engineer PPT1 protein designs with enhanced secretion properties, the selection of lead candidates in cellular models, and the biodistribution results from preclinical animal studies. Studies in mice and sheep demonstrated that intra-cerebroventricular administration of vectorized PPT1 transgene in a proprietary AAV facilitates efficient vector distribution and transgene expression in crucial CNS regions affected by CLN1 disease. A long-term efficacy study conducted in the *Ppt1* deficient mouse model also revealed positive outcomes. Preliminary findings indicate that our treatment successfully preserves motor functions, prolongs survival, and is well tolerated. Taken together, these studies encourage continued preclinical exploration of this gene therapy-focused strategy for the treatment of CLN1 Batten disease.

Friday Posters: Heart, Lung, and Kidney Diseases

1576. KRT7 Lineage Expansion Following Bleomycin Injury in an IPF Ferret Model

Shuang Wu[1], Ian Driver[2], Meihui Luo[1], Hikaru Miyazaki[2], Jing Wang[1], Dimitry Popov[2], Smitha Shambu[2], Aleksandra Tata[3], Martin B. Jensen[2], Miao Yu[1], Ziying Yan[1], Douglas J. Bartels[1], Xingshen Sun[1], Purushothama Rao Tata[3], Xiaoming Liu[1], Vishwaraj Sontake[2], John F. Engelhardt[1]

[1]University of Iowa, Iowa City, IA,[2]Gordian Biotechnology, South San Francisco, CA,[3]Duke University School of Medicine, Durham, NC

IdiopathicPulmonary fibrosis (IPF) is a progressiveand irreversible disease with an unknown etiology, characterized by lungscarring and remodeling involving excessive deposition of extracellular matrix(ECM). It is currently an incurablelung disease with limited therapeutic options. Gene and cell therapies are a promisingapproach for treating a range of fibrotic diseases, including IPF. However, theprogress in developing these innovative therapies has been considerably impededdue to the absence of suitable IPF animal models, which hinders fullyunderstanding disease pathogenesis, a crucial foundation for approachinggenetic therapies. Recognized for their similarities in airway anatomy, lungcell biology, and physiology with humans, domestic ferrets stand out asexcellent models for respiratory disease research. Consequently, we undertookthe characterization of a bleomycin (BLM)-induced IPF ferret model, assessingalterations in histopathology, pulmonary function, biomarkers, and cell typecomposition of the IPF lung. The IPF ferret lung exhibited human analogoushistopathological features, such as honeycomb structures, proximalization ofthe distal airspaces, ECM deposition, a biomarker profile, and decreasedpulmonary compliance. Singlenuclei RNA sequencing (snRNA-Seq) analysis demonstrated that thetranscriptional profile and cell type composition of BLM-induced ferret lungwas closer to human IPF lung, compared to the BLM-induced murine lung. Differentiallyexpressed genes (DEGs) and cell type analysis further revealed that cytokeratin7 (KRT7) was strikingly upregulated in a fraction of transitional alveolartype-2 (AT-2) and type-1 (AT-1) cells and aberrant basaloid cells of the IPFferret lung, which was further validated by immunofluorescence staining. To betterunderstand the origins of KRT7 cells during disease progression, *KRT7*- CreERT2:*ROSA26*-mTmG doubletransgenic ferrets

were induced with tamoxifen to genetically label KRT7 cells EGFP+. BLM-injured *KRT7*-CreERT2:*ROSA26*-mTmG ferretsdemonstrated a dynamic change of KRT7-positive lineage cells in lung during theprogression of fibrosis. KRT7-positive cells within the alveolar regions of thelung expanded significantly during early phases of injury and then laterdeclined as terminal fibrotic stages were achieved, mirroring changes in KRT7expression evaluated by snRNA-seq. Prior to injury KRT7 expression cells in thelung appeared to express both AT-1 (AGER) and AT-2 (SFTPB) cell markers, but unidentifiedcell types were also observed. We are currently identifying and validating celltype transitions of KRT7 lineage cells following BLM injury by scRNA-seq and FISH.KRT7-positive lineages in the alveolar regions of the lung may represent a stemcell compartment that facilitates repair could be a therapeutic target for gene and cell therapy for IPF.

1577. Hermansky-Pudlak Syndrome: Gene Therapy Assessment for HPS-1-Associated Pulmonary Fibrosis in a Novel Hps1 Knockout Mouse Model

Gustavo Nieto-Alamilla[1], Molly Behan[1], Shachar Abudi-Sinreich[1], Marina Zieger[2], Payam Khoshkenar[3], Mahin Shaimonti Hossain[4], Resat Cinar[5], Bernadette Gochuico[1], Christian Mueller[6], William A. Gahl[7], May Christine Malicdan[8]

[1]Human Biochemical Genetics Section, Medical Genetics Branch, National Human Genome Research Institute, Bethesda, MD,[2]UMass Chan Medical School, Worcester, MA,[3]UMass Medical School, Worcester, MA,[4]Undergraduate Scholarship Program, OD, NIH, National Human Genome Research Institute, Rockville, MD,[5]National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD,[6]Sanofi Genzyme, Framingham, MA,[7]National Human Genome Research Institute, Bethesda, MD,[8]National Institutes of Health, Bethesda, MD

Hermansky-Pudlak syndrome type 1 (HPS-1) is a complex autosomal recessive disorder characterized by albinism and bleeding predisposition. It is part of a heterogeneous genetic syndrome with eleven types (HPS-1 to HPS-11), each associated with specific mutations in genes involved in lysosome-related organelles complex biogenesis. Current treatments, like lung transplantation, have limitations, necessitating alternative approaches. Previous work using a spontaneous murine mutant (Hps1ep/ep) elucidated disease mechanisms but lacked insights into spontaneous PF development. Gene therapy offers promise for HPS-PF treatment. This study introduces a novel Hps1 model and explores HPS1 gene augmentation. To understand HPS-1 and identify PF markers, we generated a CRISPR-Cas9-engineered knockout mouse model ($Hps1^{\Delta/\Delta}$), characterizing it at the cellular and molecular levels. AAV-based gene therapy (AAV5 and AAV8) aimed to identify predictive surrogate endpoints for HPS-PF outcomes. AAVs containing enhanced green fluorescent protein or mouse Hps1 gene were systemically delivered, with saline injections as controls. $Hps1^{\Delta/\Delta}$ mice exhibited clinical manifestations seen in HPS-1 patients, including reduced gene and protein expression, platelet abnormalities, and PF. Tropism analysis showed Hps1 expression in the lungs post-AAV treatment. AEC2 size was reduced in AAV-treated mice. AAV8-Hps1-treated mice exhibited significant pulmonary function improvements, indicating therapeutic potential. These findings highlight gene therapy advancements for HPS-1 PF, offering transformative treatment possibilities. Ongoing research is crucial to refine gene therapy, ensuring long-term safety and efficacy for HPS1-PF patients.

1578. Preclinical Optimisation of Lentiviral Delivery to Piglet Lungs for Gene Therapy of Surfactant Deficiencies

Craig McDougall[1], Cathy McLaughlin[1], Elisabeth Thornton[1], Christina Vrettou[1], Kamran Miah[2], Emily Castells[2], Rebecca Dean[2], Mariana A. Viegas[2], Stephen C. Hyde[2], Deborah R. Gill[2], Gerry McLachlan[1]

[1]The Roslin Institute, University of Edinburgh, Edinburgh, United Kingdom,[2]University of Oxford, Oxford, United Kingdom

Introduction. Surfactant protein deficiencies account for 10% of childhood interstitial lung disease and the prognosis depends on the specific protein that is deficient. Infants with severe surfactant protein B (SP-B) deficiency or double null mutations in ABCA3, a protein that transports surfactant lipids to lamellar bodies, present shortly after birth with respiratory distress and failure. Despite ventilation and treatment with synthetic surfactant, they rarely live beyond the first few months of life. An effective gene therapy for surfactant deficiencies has the potential to be transformative. **Methods**. A proprietary Simian Immunodeficiency Virus (SIV) based lentiviral vector, pseudotyped with Sendai Virus F/HN proteins and expressing EGFP, was delivered to the lungs of ventilated neonatal piglets. This was performed by multisite bronchoscopic instillation via an endotracheal tube to model clinical delivery of vector to the lungs of ventilated infants. At post-mortem, precision cut lung slices (PCLS) were prepared to assess the impact of vector dose and volume on the distribution and type of transduced cells. We used quantitative immunohistochemistry (IHC) with antibodies against EGFP as well as alveolar type I (ATI) and type II (ATII) pneumocytes. Transgene mRNA was quantified by qRT-PCR and localised by hybridisation chain reaction (HCR) assays to detect transgene mRNA, Surfactant Protein-C mRNA (ATII cells) or AGER-1 mRNA (ATI cells). **Results**. We hypothesised that delivery of a given vector dose in a larger volume can reduce the heterogeneity of transduction/transgene expression. Multisite instillation with small volumes of concentrated vector at two doses was compared with lavage-like delivery of a similarly high dose of vector in a larger volume. Multisite instillation of 5x 2e8 TU or 5x 1e9TU in small volumes (300ul per site) resulted in dose-dependent expression of EGFP but showed significant heterogeneity with approximately 50% of captured images showing no detectable EGFP, and efficiency ranging from 0 to 98% of alveolar epithelial cells in individual images. Delivery of the 5x 1e9TU dose in larger volumes (8ml per site) reduced the number of images without detectable EGFP (31% images and a range from 0 to 17% EGFP-positive cells per image). The overall average efficiency in the latter study was 4% EGFP+ alveolar epithelial cells. Increasing the dose further (5x 3e9TU) in the same volume (8ml per site) reduced the number of images with no GFP to 15% and increased the average expression to 8% of cells (Range 0-27%). The reduced heterogeneity from delivery in larger volume is supported by more consistent transgene mRNA levels in tissue samples across the lung. Expression of both mRNA and protein has been demonstrated in ATII cells. **Conclusion.** We have shown that multisite bronchoscopic instillation

of a lentiviral vector via a catheter can be optimised to provide more evenly distributed transgene expression throughout the lung and is compatible with treatment of a ventilated infant with surfactant deficiency. Importantly we have demonstrated transduction of target ATII cells for processing surfactants via lamellar bodies for secretion in the lung. In addition, the progenitor nature of ATII cells supports the notion of long-term efficacy from a single dose. Further studies in piglets will help to optimise dose and delivery protocols and will aid calculations of the required dose projected to reach the therapeutic level of expression in the target cells, in clinical trials.

1579. Lymphoid Enhancer Binding Factor 1 Inhibits Airway Glandular Basal Cell Differentiation into Epithelial Secretory Cells

Jing Wang, Shuang Wu, Meihui Luo, Miao Yu, John F. Engelhard, Xiaoming Liu

Department of Anatomy and Cell Biology, University of Iowa, Iowa City, IA

Asthma is a chronic pulmonary disease characterized by eosinophil-mediated airway inflammation, airway hyperresponsiveness (AHR), and airway remodeling. It involves hyperplasia of goblet cells and remodel of submucosal glands (SMGs), and excessive secretion of mucus. Among these features, aberrant differentiation of goblet cells and hyperplasia of SMGs are the disease hallmarks. The changes in cell-type composition and the imbalance in airway epithelial-cell function, the remodeling and dysfunction of SMGs are involved in asthma pathogenesis and airway remodeling. Therefore, components of molecular signaling pathways that regulate the regeneration of airway epithelia and the development of SMGs may be effective targets in gene and cell therapies for airway disease associated with asthma. A key signaling component during SMG development is the well-known transcription factor lymphoid enhancer binding factor 1 (Lef-1), which is also activated during epithelial repair and airway remodeling following airway injury. However, the mechanisms by which Lef-1 regulates the differentiation of secretory cells and the remodeling of SMGs in the context of asthma-associated inflammation have not been investigated. We have utilized lineage tracing mouse lines that carries either a *Lef-1* gene knockout (KO) or a knock-in (KI) to create ovalbumin (OVA)-induced asthma model. The mice were sensitized by applying OVA (1.0mg/kG in 8.0mg/mL of Alhydrogel) once weekly for three weeks, and then the response was boosted by intratracheal instillation of OVA (1.0mg/kG in saline) three times at one-week intervals. We then assessed changes in the cell type composition of airway epithelia and SMGs in a mouse model of asthma, as well as an air-liquid interface (ALI) culture model and an organoid culture model generated from SMG glandular cells isolated from these animals. Analysis of the lineage-tracing mouse linerevealed aberrant inflammation and the presence of Muc5ac- and Muc5b-positive secretory epithelial cells in the trachea and intrapulmonary airways. Analyses of the ALI and organoid cultures demonstrated that *Lef-1* overexpression strikingly inhibited the differentiation of secretory cells but induced that of ionocytes (assessed by staining for Barttin CLCNK Type Accessory Subunit Beta). In contrast, the IL-13, a central cytokine that drives airway epithelial dysfunction in the context of asthma, significantly induced the differentiation of secretory cells but inhibited that of ionocytes in the in vitro models. However, simultaneous overexpression of *Lef-1* and exposure of IL-13 had no impact on either the proliferation of airway basal cells or the differentiation of ciliated cells. Similarly, conditional KO of *Lef-1* had no effect on the differentiation of these cell types. These results clearly implicate *Lef-1* in regulating the differentiation of SMG cells into airway secretory cells vs ionocytes. We are currently evaluating the cell-type composition of tracheal and intrapulmonary airway epithelia, as well as SMGs, in the conditional *Lef-1* gene modified lineage tracing mice in the context of OVA-induced asthma. Given the role of *Lef-1* in regulating the differentiation of epithelial secretory cells and ionocytes, it represents a candidate target for genetic and cell therapy for asthma-associated airway disease.

1580. Mucin Targeted Gene Therapy for Asthma

Sahana Kumar[1], Maria Rife[1], Margaret Scull[1], Gregg Anthony Duncan[2]

[1]Biological Sciences, University of Maryland, College Park, College Park, MD,[2]University of Maryland, College Park, MD

Asthma is a globally burdensome respiratory disease, affecting millions worldwide. It is characterized by chronic airway inflammation, airway remodeling, and impaired mucociliary clearance (MCC). Mucus comprises gel-forming mucin proteins mucin 5B (MUC5B) and mucin 5AC (MUC5AC). Healthy airway mucus is predominantly composed of MUC5B, with reduced MUC5AC. However, in individuals with asthma, biochemical analysis of their mucus revealed a shift from MUC5B to MUC5AC as the predominantly secreted mucin. These changes to mucus composition have been shown to impair MCC and obstruct airways due to increased mucus plugs in asthma patients. Given the overproduction of MUC5AC in the asthmatic airway, we hypothesized delivery of MUC5AC siRNA to the airway epithelium, could improve MCC, reduce airway hyperreactivity, and improve asthma symptoms *in vitro* and *in vivo*. For *in vitro* studies to validate MUC5AC as a relevant therapeutic target in asthma, immortalized basal human airway epithelial (HAE) cells were differentiated into secretory and ciliated cells by culturing at air-liquid interface (ALI). To establish an asthma-like phenotype, ALI cultures were stimulated with cytokine interleukin-13 (IL-13). We utilized adeno-associated virus serotype 6 (AAV6), which shows tissue tropism for lungs, as the viral vector in these experiments. To determine if AA6 could penetrate the mucus barrier in asthmatic airways, multiple particle tracking (MPT) analysis was used to compare AAV6 diffusion in mucus from unstimulated and IL-13-stimulated ALI cultures. To characterize AAV6 transduction in HAE cells, AAV6 expressing enhanced green fluorescent protein was used to assess transduction efficacy. The efficacy of AAV6 carrying MUC5AC-siRNA (AAV6-MUC5AC-siRNA) to silence MUC5AC was evaluated using immunofluorescence and quantitative real-time polymerase chain reaction (qRT-PCR).MPT analysis of AAV6 in HAE mucus with and without IL-13 stimulation revealed that the mean squared displacement of AAV6 was similar in unstimulated and IL-13 stimulated mucus, indicating AAV6 can diffuse through asthma-like mucus barrier enriched with MUC5AC (Fig 1A). MPT analysis further revealed changes in mucus microstructure in IL-13-stimulated mucus, indicated by increased pore sizes (Fig 1B). AAV6 transduced unstimulated and IL-13 stimulated HAE cells at multiplicities of infection 10^4 and 10^5 (Fig 1C). Initial studies of AAV6-MUC5AC-siRNA showed promise, with expected trends of elevated

MUC5AC messenger RNA expression in IL-13-stimulated mucus and reduced expression in the AAV6-MUC5AC-siRNA treatment group (Fig 1D). Future and ongoing work includes evaluating MCC in MUC5AC-siRNA-treated HAE cultures and proof-of-concept *in vivo* studies using a mouse model of allergic asthma. If successful, this gene delivery approach could open avenues for mucin-targeted therapies aimed at asthma and other related chronic lung diseases.

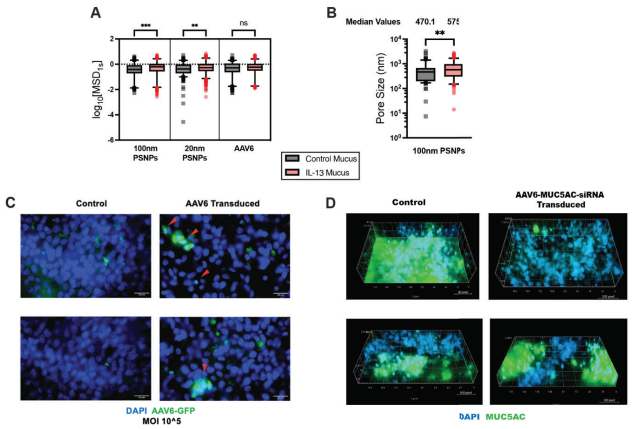


Figure 1. AAV6 penetrate mucus barrier and delivers siRNA.
(A) Box-and-whisker plots of log-based 10 of MSD at t=1s of 100nm PSNPs, 20nm PSNPs and AAV6 in untreated control (black) and IL-13 stimulated (red) HAE mucus are shown. Horizontal bars represent median values. Data sets statistically analyzed with One-Way ANOVA Kruskal-Wallis test: *** $p < 0.001$. **(B)** Calculated pore size in control (black) and IL-13 stimulated (red) HAE mucus based on 100nm PSNP diffusion in mucus. Data sets statistically analyzed with two-tailed Mann-Whitney test: ** $p<0.01$. (**C**) AAV6-eGFP transduction in differentiated IL-13 stimulated HAE cells 3 days post-infection at MOI 10^5 (scale bar=20μm). The red arrows indicate clusters of transduced cells. **(D)** Representative Z-stack images of HAE ALI cultures treated with and without AAV6-MUC5AC-siRNA. MUC5AC (green) is highly expressed in untreated controls while expression is reduced in treated groups.

1581. Transduction Efficiency of Adeno-Associated Virus and Lentiviral Vectors in Murine Lungs and Precision-Cut Lung Slices Derived from Various Species

Brenna A. Y. Stevens[1], Madison E. Hughes[1], Nicole Zielinska[1], Howard L. Erin[1], Jeff L. Caswell[1], Karimi Khalil[1], Bernard Thebaud[2], Yanlong Pei[1], Sarah K. Wootton[1]

[1]Pathobiology, University of Guelph, Guelph, ON, Canada,[2]Ottawa Hospital Research Institute, Ottawa, ON, Canada

Intro: Cystic Fibrosis and other monogenic lung diseases have long been a pertinent target for viral vectored gene therapies. The most clinically relevant viral vectors are adeno- associated viruses (AAV) and lentiviruses (LV). In this work, the potential of both vectors for lung gene delivery is evaluated in a murine model. Furthermore, it is widely observed that *in vitro* transduction by AAV-based vectors does not reliably correspond to *in vivo* transduction. Precision-cut lung slices (PCLS) offer a promising *ex vivo* model with many advantages over traditional cell culture. They retain similar diversity of cell types, structural architecture, and microenvironment, as lung tissue. It is hypothesized that PCLS can be used as a tool to more accurately predict *in vivo* viral-vectored transduction efficiency. **Methods:** Here, $4x10^8$ transducing units (TU) of three different pseudotyped LVs and $1x10^{11}$ vector genomes (vg) of AAV6.2FF or AAV9 carrying the reporter gene human placental alkaline phosphatase (AP) were administered intranasally to BALB/c mice (n=4) to evaluate each vector's efficiency as a gene therapy vector. Mice injected with PBS were used as negative controls. After 21 days, the lungs were harvested and either visually stained for AP expression or homogenized and run on AP enzymatic activity assays to quantify levels of AP expression. Histological slides were prepared, and AP stained to determine what cell types were being transduced by each vector. Additionally, PCLS were generated from BALB/c mice, pigs, cows, sheep, and rabbits. The slices were then transduced with either lentiviral and AAV vectors, or exclusively the AAV vectors. The PCLS were harvested 5 days post-transduction for staining and AP activity analysis. **Results:** The *in vivo* administration of AAV6.2FF-AP and AAV9-AP in mice resulted in robust and widespread expression of AP in the lungs, with a trend of AAV6.2FF outperforming AAV9. Similarly, in murine PCLS AAV6.2FF transduction produced high AP expression, while AAV9 transduction resulted in lower expression. In mice, the *in vivo* administration of VSV-G and ZebovΔmucin-pseudotyped LV induced little to no AP expression, while GP64-pseudotyped LV led to moderate AP expression. In murine PCLS, AP levels were low after all lentiviral pseudotype transduction. However, in porcine PCLS both AAV6.2FF and LV transduction resulted in high AP expression. AAV6.2FF transduction was effective in PCLS of all tested species. Transgene expression by GP64 pseudotyped LV was higher than expected; however, AAV6.2FF outperformed all LV vectors tested in both *ex vivo* PCLS and *in vivo* murine lungs. **Conclusions:** Due to the high correlation between PCLS and *in vivo* transduction results, it is evident that PCLS are a useful model to rapidly screen gene therapy candidates and bridge the gap between *in vitro* and *in vivo* data. AAV6.2FF also displayed a wide species tropism in PCLS. This is the first evidence of bovine and porcine lung transduction by AAV6.2FF. Given the availability of clinically relevant porcine models for cystic fibrosis, this supports the use of AAV6 and AAV6.2FF in the development of a gene therapy treatment for cystic fibrosis.

1582. Enhancing Airway Transduction Using AAV Equipped with Advanced Barcoding Methods for CF Gene Therapy

Ashley L. Cooney[1], Yonghong Chen[2], Brian Lewandowski[2], Priyanka K. Newase[1], Beverly L. Davidson[2], Paul B. McCray[1]

[1]University of Iowa, Iowa City, IA,[2]Children's Hospital of Philadelphia, Philadelphia, PA

Approximately ~10% of people with CF do not benefit from current CFTR modulators, including those with nonsense or splicing mutations and those intolerant of the medications. This motivates our investigations into genetic therapies including gene addition and gene editing. A key limitation to applying these and other emerging technologies to CF is effective therapeutic delivery. Adeno-associated virus (AAV) is a broadly useful vector for delivery to many cell types and is safe unless delivered at very high doses. Because of its persistence as an episome, expression lasts for months or years in slowly dividing cells. While past CF clinical trials using AAV2 were unsuccessful, many of the contributing limitations have been identified including poor transduction efficiency and inappropriate expression cassette design. Here, we address the inefficiency of current AAVs by taking advantage of an AAV library screen. Using advanced AAV barcoding methods and screening in multiple large mammalian species, we identified capsid variants that can transduce target cell types at doses 1 to 2 logs lower than currently available capsids. Additionally, libraries of

capsids from multiple serotypes were screened in primary air-liquid interface cultures of human airway epithelia (HAE) isolated from CF and non-CF individuals. One consistently enriched variant, BLD034, shows significantly enhanced transduction properties compared with the benchmark AAV2.5T capsid in HAE, even in the absence of the proteosome inhibitor doxorubicin (Dox). To test the transduction ability of BLD034 in a relevant airway model, HAE were transduced from the apical surface with AAV2.5T and AAV-BLD034 overnight in the presence or absence of Dox. By 21 days post-transduction, GFP expression in HAE transduced with BLD034 was robust relative to AAV2.5T in the absence of Dox. In the presence of Dox, these results were further amplified. Ongoing experiments are testing minimal effective dose and complementing CFTR in CF HAE. AAV capsids designed with advanced barcoding techniques provide opportunities for improved potency and may omit the need for concomitant proteasome modulation in airway gene therapy applications.

1583. COVID-19 and Its Associated Variants

Ghazala Rubi Rubi

Central Research Lab & Molecular Genetics Pathology, Lahore General Teaching Hospital, Lahore, Pakistan

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped RNA beta-coronavirus virus that is corona viridae-related. It originated in Wuhan, China in December 2019 and is known to cause COVID-19 illness. SARS-CoV-2 posed a major threat to the world in concern of health along with trade and medical facilities. COVID-19 patients experience comorbid conditions like severe fever, typhoid, myocarditis, and a fatal black fungus attack. World Health Organization declared COVID-19 as an emergency on 30th January 2020 and a pandemic on 11th March. As of right now, COVID-19 has been verified to have infected over 100 million people across 210 nations, and 2 million of those infections have resulted in death. RT-qPCR (Reverse transcriptase qualitative PCR) test is performed if any symptoms exist, and isolation is necessary for three to five days after the confirmation. In Pakistan, it was reported firstly on 26th February 2020 by the Pakistan government when two persons were found infected with the disease. SARS-CoV-2 changes its genome constantly and results in the emergence of different variants in different waves. Pakistan has faced 6 sequential waves since 2020 to date, each associated with different variants and overlapping variants. The first wave was associated with B.1, B.1.471, B.1.36, second with B.1.36.31, B.1.247, B.1.1.1, B.1.160, B.1.471, B.1.562, B.1.1.7, B.1.1.250, B.1.261, B.1.351, P.1, third with B.1.1.7, B.1.351, P.1, B.1.617.2, B.1.36, B.1.468, B.1.1.413, A.27, fourth with P.1, B.1.617.2, fifth with Omicron (B.1.1.529) and sixth with new sub variants of Omicron (BA.4 & BA.5). In terms of severity and infectivity, Omicron and its sub variants differ from Delta and other SARS-CoV-2 variants. In this review, we have discussed SARS-CoV-2 spike mutations, its variants along with their origin & association to wave(s), and its scenario in Pakistan.

1584. Demonstration of the Feasibility of AAV Vector-Mediated Gene Therapy for Alport Syndrome Using a Newly Established Floxed Mouse Model

Ranjan Das[1], Taisuke Furusho[1], Anusha Sairavi[1], Hideyuki Hakui[1], Shuhua Luo[1], Lena Li[1], Jeffrey H. Miner[2], Hiroyuki Nakai[1,3]

[1]Molecular and Medical Genetics, Oregon Health and Science University, Portland, OR,[2]Division of Nephrology, Washington University School of Medicine, St. Louis, MO,[3]Molecular Microbiology and Immunology, Oregon Health and Science University, Portland, OR

Alport syndrome (AS) is characterized by abnormalities in glomerular basement membranes (GBMs), leading to chronic kidney disease (CKD) in patients. This results from mutations in the *COL4A3*, *COL4A4*, or *COL4A5* genes, each of which encodes a different alpha chain of type IV collagen. Approximately 80% of individuals with Alport syndrome have the X-linked type (XLAS) due to a mutation in the *COL4A5* gene. Here, we establish a novel Col4a5-LoxSTOPLox-KnockIn (LSL-KI) XLAS mouse model using CRISPR-Cas9 and demonstrate therapeutic effects in XLAS mice following AAV-Cre vector administration at ages both before and after CKD is manifested. In this study, a LSL cassette containing an SV40pA-derived STOP signal was introduced into intron 1 present downstream of the 431-bp-long (contributing to the first 27amino acids) exon 1 of the mouse *Col4a5* gene located on the plus strand of chromosome X. Two founder mice were obtained from CRISPR-Cas9-treated embryos, and a colony was established. Male hemizygous mice developed significant proteinuria and elevated serum creatinine (sCr) and blood urea nitrogen (BUN), and died or reached an experimental endpoint for humane euthanasia around the age of 30 weeks due to renal failure. This newly developed mouse model manifested CKD in a manner similar to that of the well-established Col4a5 G5X XLAS mouse model. In a proof of concept (POC) study, bilateral renal pelvis injection was performed on young 5 to 6-week-old LSL-Col4a5 hemizygous male mice with either vehicle only or 3 x 10^{11} viral genomes (vg) of AAVKP1-CAG-Cre (n=2 in each group). Thirteen-weeks post injection, recovery of Col4a5 was clearly observed in GBM and tubular basement membrane (TBM) in one of the AAV vector-injected mice, where no expression of Col4a5 was detected in the vehicle treated group. Improvement of blood biomarkers was also evident in KP1 treated group (24-week-old) compared to vehicle group (26-week-old) (KP1 vs. vehicle -> BUN: 52.0 vs. 132.5 mg/dL, Cr: 0.2 vs. 0.8 mg/dL and Hb: 12.6 vs. 6.7 g/dL). In the second POC study, three Col4a5-LSL hemizygous male mice were intravenously injected with 6.8 x 10^{11} vg of AAV9-CAG-Cre vector at the age of 17 weeks, when these mice already manifested CKD. Ten-weeks post injection, one of the AAV vector-injected mice was assessed for Col4a4 and Col4a5 expression in kidney by immunofluorescence microscopy. In contrast to control hemizygous mice, vector-treated mice showed restoration of both Col4a4 and Col4a5 within the GBM. Surprisingly, the remaining two vector-treated mice did not exhibit loss of body weight until 36 weeks of age and survived for more than 39 weeks, surpassing the age by which all untreated XLAS mice would succumb or reach the endpoint. In macroscopic observations, AAV vector-treated XLAS mice demonstrated visual improvements in kidney structure. The kidneys of untreated mice were shrunken, and irregular and granular on the surface. On the other hand, the kidney surfaces of AAV vector-treated

counterparts were relatively smooth. Moreover, the kidney size and shape were normal in these treated mice, closely resembling those of wild-type mice. These observations demonstrate that KP1 and AAV9 vectors effectively transduced podocytes, leading to the restoration of heterotrimeric type IV collagen expression and its incorporation into the GBM. Thus, we demonstrate for the first time the efficacy of AAV vector-mediated gene therapy for Alport syndrome, and importantly the therapy is effective even after the establishment of CKD.

1585. Direct Delivery of a Novel AAV Gene Therapy to the Kidney Enables Podocyte Targeting for the Treatment of Glomerular Diseases

Sam Illingworth[1], Kritima Gurung[1], Valeryia Kuzmuk[1], Hannah Wood[1], Demetris Taliotis[1,2], Gavin Walsh[1,2], Moin Saleem[1,2], Alice Brown[1]

[1]Purespring Therapeutics, London, United Kingdom,[2]Universtity of Bristol, Bristol, United Kingdom

Adeno associated vector (AAV) gene therapy has the potential to treat a wide range of kidney diseases via targeted delivery of genes to podocytes, a kidney cell type instrumental in renal function. Despite the potential to impact many kidney conditions, podocyte targeted AAV gene therapy has proven challenging. This is due to technical and physiological hurdles limiting the ability of AAV to be effectively and specifically delivered to podocytes. We have developed a novel AAV gene therapy and local administration procedure able to deliver transgenes effectively, specifically and safely to podocytes. The podocyte is the central player in the primary function of the kidney, filtration of the blood, and dysregulation of podocyte proteins is implicated in numerous monogenic and non-monogenic kidney disorders. We have exemplified our approach through pre-clinical development of PS-001, a novel locally delivered AAV gene therapy that delivers the podocin gene, *NPHS2*, to the podocyte. Mutations in the *NPHS2* gene are the most common cause of monogenic paediatric nephrotic syndrome, a severe kidney disease which results in renal failure and requirement for kidney transplantation in childhood. PS-001 consists of a capsid that is highly effective at transducing human podocytes and promoter that drives gene expression of podocin specifically in the podocyte. Functional gene expression of podocin was demonstrated in *in vitro* studies in podocytes from *NPHS2* nephropathy patients. PS-001 efficacy, safety and potential for clinical translation has been robustly demonstrated in rodent disease models and in large animal studies in Gottingen Minipigs. Murine *in vivo* proof of concept studies were conducted in a knock-in model of a mutated *nphs2* gene variant most commonly associated with disease in humans ($nphs2^{R140Q/-}$). Using a murine analogue of PS-001, podocin expression was measured in kidney glomeruli and significant efficacy demonstrated via rescue of kidney function. This was shown by resolution of severe proteinuria (mean ACR ug/mg 6049 ± 2803 vs 192 ± 51 in PS-001 treated animals, n=11), improved serum albumin (g/L 27.50 ± 3.33 g/L vs 30.75 ± 2.46 in PS-001 treated animals, p=0.021) and improvement in kidney histopathology (mean sclerosed glomeruli 81% ± 19 vs 18.75% ± 12 in PS-001 treated animals, P<0.0001). The methods to translate the approach to the clinical setting using local administration were established in pigs. Administration of PS-001 resulted in transgene mRNA expression across 89% ± 11% of kidney glomeruli as assessed by RNAScope (n=3 pigs), and transgene derived podocin could be specifically detected in podocytes as assessed by immunofluorescence analysis. Using qPCR, RT-PCR and ELISA it was shown that following local administration, podocin gene expression was restricted exclusively to the kidney glomerulus, where podocytes are located, with gene expression undetectable in off-target organs including the liver. Additional toxicological assessments of kidney and other organs, urine and blood analyses, and post-study histopathological assessments revealed no treatment-related safety concerns. Collectively, we present data showing development of an AAV gene therapy and method of administration for the delivery of transgenes specifically to kidney podocytes. We have shown transgenes can be efficiently targeted to podocytes to replace defective genes or to use the podocyte as a protein factory to modulate glomerular biology. This technology therefore provides an important novel modality for potentially treating a broad range of kidney disease.

1586. AAV1 CFTR Gene Therapy Successfully Reduces Cysts in a Mouse Model of Autosomal Dominant Polycystic Kidney Disease

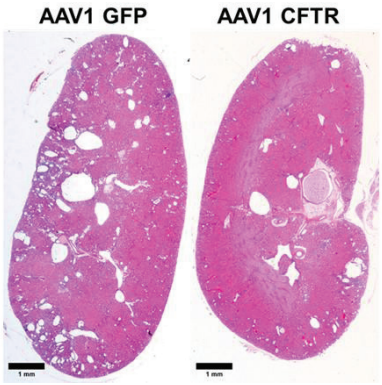
Cristian Ciobanu, Liudmila Cebotaru

Medicine and Physiology, Johns Hopkins University, Baltimore, MD

Autosomal dominant polycystic kidney disease (ADPKD) is characterized by the formation of multiple large fluid-filled cysts that displace normal renal tissue and lead to end-stage renal disease by the sixth decade of life. Current potential therapies for ADPKD involve treatment of the symptoms associated with kidney disease and/or organ transplantation. Tolvaptan, a new drug in clinical use, is limited by side - effects. Cyst growth involves fluid secretion into the cyst lumen through the action of CFTR, located at the luminal membrane. We showed that CFTR correctors such as VX-809 alter the location of CFTR in cystic epithelia from the apical to the basolateral membrane, thereby reducing cyst formation. Here, we propose the novel hypothesis that a gene therapy based on CFTR can prove effective in reducing kidney damage in ADPKD. One of the most commonly discussed challenge is the size exclusion by the glomerulus that retards movement of virus particles into the glomerular filtrate. We would argue that the kidney is a highly vascular organ with networks of peritubular capillaries supplying blood to the cortical regions and the vasa recta supplying blood to the medulla. To provide data showing that kidney transduction via systemic delivery is feasible, approximately 1-month-old, $Pkd1^{R3277C/R3277C}$ ($Pkd1^{RC/RC}$) mice were injected intraperitonially with 2×10^{12} particles/kg of AAV1 containing either GFP or Δ27-264-CFTR (referred to as GFP or CFTR-vector). Δ27-264-CFTR is a truncated version of CFTR that increases expression of endogenous CFTR via transcomplementation. Animals were necropsied 2 months after treatment. Remarkably the cyst area and size were less in the CFTR- compared to GFP-vector treated animals. We were able to detect vector genomes at $>4x10^5$ vector genomes/ug DNA and mRNA expression at $4\text{-}8x10^4$ copies/ug DNA after vector instillation. To determine the location of the vector induced transduction we performed confocal microscopy. Tissues were co-stained with either NHE3 or ENaC to highlight proximal or collecting duct expression, respectively. Expression of GFP and CFTR above background levels consistent with protein expression were

generated by both vectors. We detected more GFP in the vector-treated cystic cells compared to surrounding tissue suggesting that AAV1 is more specific for cysts than the cells surrounding the cysts. We detected more CFTR IF at the basolateral membrane in AAV1-CFTR treated kidneys suggesting that CFTR was restored to its normal location. These experiments demonstrate convincingly that kidneys of RC/RC animals can be transduced by AAV1 and that phenotypic correction of defective function can be achieved.

AAV1 CFTR reduces area and number of the cysts.



Friday Posters: Neurologic Diseases

1587. Characterization of Brain Transduction Capability of a Peptide-Displaying AAV Vector in Mice, Rats and Non-Human Primates

Maria Bunuales[1], Miguel Chillon[2], Assumpcio Bosch[3], Manuela Gonzalez-Aparicio[1], Angeles Garduno[1], Maria Espelosin[1], Marta Garcia-Gomara[1], Ana Garcia-Osta[1], Mar Cuadrado-Tejedor[1], Jose L. Lanciego[4], Ruben Hernandez-Alcoceba[1]

[1]DNA and RNA Medicine, University of Navarra. CIMA, Pamplona, Spain,[2]Biochemistry and Molecular Biology, Institut de Neurociències. Universitat Autònoma de Barcelona and Vall d'Hebron Research Institute. Institució Catalana de Recerca i Estudis Avançats (ICREA), Barcelona, Spain,[3]Biochemistry and Molecular Biology, Institut de Neurociències. Universitat Autònoma de Barcelona and Vall d'Hebron Research Institute, Barcelona, Spain,[4]DNA and RNA Medicine. Pathology, Anatomy and Physiology Department, School of Medicine, University of Navarra. CIMA, Pamplona, Spain

Different screening methods are being developed to generate adeno-associated viral vectors (AAV) with the ability to cross the blood-brain barrier (BBB) upon intravenous administration. Recently, the AAV9P31 stood out as the most efficient version among a library of peptide-displaying capsids selected in C57BL/6 mice using RNA-driven biopanning. In this work we have characterized in detail its biodistribution in different mouse strains (C57BL/6J and Balb/c), as well as Sprague Dawley rats and non-human primates (Macaca fascicularis). Using GFP and NanoLuc reporter genes, we confirmed homogeneous infection and transgene expression across the cerebrum, cerebellum and brainstem of mice injected intravenously with AAV9P31. A more restricted pattern was observed upon intracerebroventricular or intraparenchymal injection. Peripheral organs transduced upon intravenous administration included lung, liver, peritoneum, heart and skeletal muscle. Transgene expression in the brain was observed as early as 72 hours after intravenous administration in 2 weeks-old C57BL/6J mice, and it was stable during more the 3 months follow-up. However, the ability of AAV9P31 to cross the BBB was not observed in rats and macaques. Brain penetration was also limited upon intracerebroventricular (icv) administration in macaques, whereas direct injection into the susbtantia nigra led to local neuronal transduction. We conclude that the AAV9P31 capsid is an excellent tool for transduction of mouse brain.

1588. Innovative Device for Concomitant Neural Recordings and Drug Delivery into the Brain - Feasibility Bench and *In Vivo* Studies

Guadalupe Zepeda[1], Su-youne Chang[2], Jiwon Kim[2], Inyong Kim[2], Maria F. Porto Cruz[1], Alfonso Chavez[1], Mary McNeil[1], Maria Vomero[1], Camilo Diaz-Botia[1], Dave Rosa[1], Gregory A. Worrell[2], Aura Kullmann[1]

[1]NeuroOne Medical Technologies Corporation, Eden Prairie, MN,[2]Mayo Clinic, Rochester, MN

Convection enhanced delivery (CED) of therapeutic compounds (e.g. drug, gene or stem cells) into the brain has been increasingly used in clinical trials for a variety of neurological conditions, including Parkinson's disease, epilepsy and others. The existing CED devices consist of cannulas able to deliver infusate, but unable to record neural activity. This severely limits the ability to evaluate compound effects both in acute settings (e.g. impact of delivery on physiological cell activity) and long-term settings (e.g. evaluation of therapy efficacy for therapies intended to alter neural activity). We propose a new device that combines delivery with on-site recordings. This device consists of a stereoelectroencephalography (sEEG) electrode equipped with a unique accessory catheter that can be connected to a syringe infusion pump. The sEEG electrodes are FDA-cleared for stereotactic implantation in desired brain regions and indicated for temporary (<30 days) monitoring, recording and stimulation of neural activity. Currently these electrodes are routinely used in clinical practice in the evaluation of patients with drug resistant epilepsy. The feasibility of this prototype system was demonstrated in bench and in vivo testing in a rat model. Bench testing was conducted to evaluate infusion rates, reflux and infusion/diffusion volume relationship. Experiments performed in 0.6 % agarose gel using 1% Methylene Blue dye showed CED can be achieved. In vivo testing was conducted in a rat model to demonstrate feasibility of neural recordings pre- and post- drug delivery. The sEEG electrode was implanted into the hippocampus. Baseline neural activity was recorded for ~30 min, after which penicillin (5000 units/µl; 5 µl) was infused at rate of 0.5 µl/min. This elicited seizure-like episodes consisting of large amplitude bursts of coordinated activity interspaced by quiet periods. Histological examination of the brain tissue using hematoxylin and eosin showed an ellipsoidal shape diffusion volume. These experiments provide proof of concept that the prototype is able to perform the desired functions, monitor neural activity and deliver therapeutic compounds. The availability of drug delivery capability in an sEEG can potentially have significant impact on the screening, safety

and efficacy of the treatments. In acute settings, real time monitoring of the effect of therapy on cellular activity can be used as a screening and/or safety indication (e.g. does the compound and/or delivery impact neuronal activity, does it have negative impact on the cells). In long term settings, it can be used to directly assess the onset, mechanism of action and efficacy of therapeutic compounds, especially for therapies intended to alter neural activity (e.g. for epilepsy).

1589. Optimal AAV Serotype / Promoter Combinations to Target Specific Cell Types in the Marmoset Brain

Hirokazu Hirai[1,2], Ayumu Konno[1,2], Yasunori Matsuzaki[1,2]

[1]Department of Neurophysiology and Neural Repair, Gunma University, Maebashi, Japan,[2]Viral Vector Core, Gunma University, Maebashi, Japan

To express transgene with high specificity and efficiency in specific cell types, it is crucial to combine a serotype that is highly infectious for the target cell type with a target cell type-specific promoter. However, the tropism of different serotypes to marmoset brain cells has not been systematically investigated. In this study, we investigated the tropism of nine different serotypes for various neuronal and glial cells in the marmoset cerebral cortex.Nine serotypes of AAV (AAV1, 2, 5, 6, 7, 8, 9, rh10, and DJ) that express green fluorescent protein (GFP) under the control of the ubiquitous chicken β-actin hybrid (CBh) promoter were injected into the cerebral cortex of marmosets. Four weeks after the AAV injection, ratios of GFP-expressing cell types to total GFP-labeled cells were immunohistochemically examined.While the proportions of neurons and glia to total cells in the marmoset cerebral cortex were approximately 55% and 45%, respectively, majority of the GFP-expressing cells (82-97%) in the AAV-treated cortex were neurons, indicating that all AAV serotypes tested, especially AAV2 (97.0 ± 0.6%) and AAV-rh10 (96.9 ± 0.8%), have a marked tropism for neurons. All AAV serotypes tested caused GFP expression in both excitatory and inhibitory neurons, with AAV-rh10 showing a preference for excitatory neurons and AAV2 showing a preference for inhibitory neurons.All nine serotype AAVs transduced glial cells with significantly lower efficiency, and most of transduced glia were oligodendrocytes. Among nine serotypes examined, AAV5 most efficiently transduced oligodendrocytes, which accounted for approximately 18% of all GFP-labeled cells.In addition to the CBh promoter, AAVs with other ubiquitous cytomegalovirus (CMV) or CAG promoter also failed to transduce astrocytes: however, all nine AAV serotypes, especially AAV7, with the astrocyte-specific human GfaABC1D promoter efficiently and selectively transduced astrocytes, suggesting that every AAV serotypes tested can infect astrocytes, but the incorporated ubiquitous promoters are not functional in astrocytes. Moreover, we assessed AAVs carrying cell type-specific promoters, in terms of specificity and efficiency of target cell transduction, when packaged using serotypes with infectious to target cells and those with low infectivity to target cells. Our results indicate that serotypes that achieve efficient target cell transduction in the marmoset cerebral cortex are AAV-rh10 for excitatory neurons, AAV2 for inhibitory neurons, AAV5 for oligodendrocytes, and AAV7 for astrocytes.

1590. AAV-Mediated Gene Augmentation Therapy for NEK1-ALS

Katharina E. Meijboom[1], Desiree M. Baron[1], Debra Cameron[1], Erinn Ives[1], Aparna Sreeram[1], Ana Rita Batista[2], Janice A. Dominov[1], Cathleen Lutz[3], Robert H. Brown[1], John E. Landers[1]

[1]UMASS Chan Medical School, Worcester, MA,[2]UMass Chan Medical School, Worcester, MA,[3]The Jackson Laboratory, Bar Harbor, ME

Amyotrophic lateral sclerosis (ALS) is a terminal neurodegenerative disease that affects upper and lower motor neurons, leading to progressive muscle weakening and respiratory failure. Loss-of-function mutations in NIMA-related kinase 1 (NEK1) constitute a major genetic cause of ALS in both familial and sporadic ALS cases, accounting for 2 to 3% of all cases. To develop and test a NEK1 gene augmentation therapy, we created a mouse model with a nonsense mutation in the mouse NEK1 gene. This mutation resulted in a complete absence of NEK1 protein in homozygous mice. Preliminary data show that homozygous mice are noticeably smaller and have a significantly reduced bodyweight measured from three weeks onwards compared to heterozygous and WT littermates. Mice are currently 16 weeks old; any effect on lifespan has yet to be determined. We will further establish other NEK1 phenotypes, such as motor disturbances, DNA damage, cilia formation and nuclear envelope integrity. In NEK1 patient-derived fibroblasts and induced motor neurons, we observed DNA damage and diminished nuclear envelope integrity. To rescue any behavioral and molecular phenotypes *in vitro* and *in vivo*, we are designing AAV-mediated NEK1 augmentation strategies. Thus far, heterozygous neonatal NEK1 mice received intracerebroventricular injections of AAV-PHP.eb-hNEK1 driven by the MSCV promoter. This resulted in 11.6 and 1.8 vg/diploid genomes and significant expression of hNEK1 transcripts in the brain and spinal cord, respectively. We are exploring diverse NEK1 augmentation strategies, including different NEK1 transcript variants, AAV serotypes, promoters, and regulatory elements. Our objective is to establish effective therapeutic interventions for NEK1-related ALS, evaluating outcomes in both homozygous NEK1 mice and patient-derived cells.

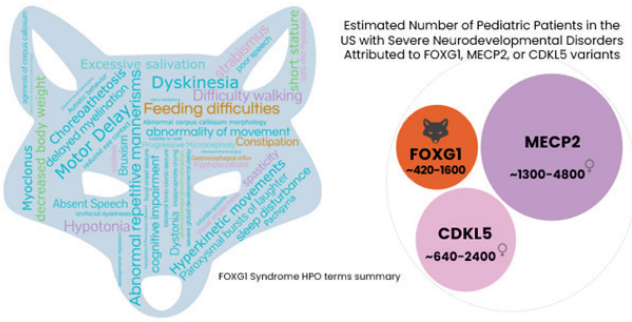
1591. Estimating Patient Prevalence for Complex Neurodevelopmental Disorders: The Emerging Face of FOXG1 Syndrome

Karen E. Malone[1], Katarina Mueller[2], Mary Grace Shine[2], Jae Lee[2], Soo-Kyung Lee[2,3]

[1]GeneScape, Leiden, Netherlands,[2]Department of Biological Sciences, The State University of New York at Buffalo, Buffalo, NY,[3]FOXG1 Research Foundation, San Francisco, CA

Background and objectives: Understanding the scale of unmet need for specific severe neurodevelopmental disorders (NDD) remains very challenging, in part due to the many overlapping clinical features associated with global and developmental delays, and the variability of epilepsy phenotypes. FOXG1 Syndrome is a prime example of this challenge and requires definitive genetic diagnosis to be identified. We provide the first prevalence estimates of FOXG1 Syndrome based on genetic surveys of severe NDD patients. **Methods:** We conducted a systematic literature review and meta-analysis of studies performing genetic testing panels on cohorts of severe NDD within the last ten

years. We compiled 13 studies (after omitting one outlier enriched for FOXG1 patients), totaling nearly 36,000 severe NDD patients. **Results:** The proportion of severe NDD attributed to FOXG1 was 0.20% [95% CI: 0.16 - 0.25%]. For comparison, MECP2 and CDKL5 patients accounted for 0.55% [95% CI: 0.48 - 0.64%] and 0.34% [95% CI: 0.28 - 0.40%], respectively. This corresponds to an estimated prevalence of 0.6 - 2.2 FOXG1 patients per 100,000 children or ~420 - 1600 patients in the United States. The estimated prevalence of CDLK5 patients was 1.8 - 6.6 cases per 100,000 female children, and the estimated prevalence of MECP2 patients was 3.5 - 13.2 cases per 100,000 female children. **Conclusions:** FOXG1 Syndrome was previously considered an ultra-rare indication potentially occurring in ~1 per million children. Our study based on genetic testing demonstrates the FOXG1 patient population is expected to be approximately one third the size of the MECP2 patient population which is primarily associated with Rett Disease. Uncoupled from Rett Disease clinical criteria, both FOXG1 Syndrome and CDKL5 Deficiency represent distinct and sizable patient populations. This shows the value of genetic testing in diagnosing these complex diseases and paints a more up-to-date picture of the scale of unmet need, that will support further drug development strategies.



1592. Synthetic Morphogenesis to Improve Early Brain Organoid Models

Catherine A. Hamann, Cheryl Quartey, Ethan S. Lippmann, Jonathan M. Brunger

Vanderbilt University, Nashville, TN

Introduction: During development, signaling centers organize cellular differentiation via coordinated morphogen production. As ectodermal cells undergo neurulation, morphogen networks shape the neural tube and early structures of the central nervous system. Notochord-derived sonic hedgehog (SHH) specifies formation of the floor plate, which serves as a source of SHH to pattern cells of the neural tube and define the dorsal-ventral axis. A number of diseases and disorders, such as nonsyndromic holoprosencephaly, are caused by SHH and other early developmental genetic mutations. However, few brain organoid protocols focus on incorporating signaling centers, instead relying on small molecules and proteins applied with little to no spatial organization. Protocols have been developed to generate drug-inducible organoids consisting of a floor plate-like signaling center using human pluripotent stem cells (hPSCs), which have resulted in markers of successful dorsal-ventral patterning. **Methods**: Leveraging artificial gene circuits, we take a synthetic morphogenesis approach to uncover the design rules required to program hPSCs to autonomously form a floor plate region that serves as an organizing center to further elaborate neural tube formation in an organoid. Thus, we engineered hPSCs that produce SHH under the control of the customized signaling receptor synNotch. Like native Notch (**Figure 1Ai**), synNotch (**Figure 1Aii**) responds productively to artificial juxtacrine cues, such as transmembrane ligands on neighboring cells, leading to highly localized transgene expression. We engineered synNotch hPSCs to produce SHH, and our previous results show via qRT-PCR that successful juxtacrine binding of synNotch hPSCs potently upregulates SHH in response to the synNotch transmembrane ligand, GFP. **Results**: SynNotch induction results in domains of high FOXA2 expression (floor plate marker) while also showing low PAX6 (dorsal tissue marker), as assessed by immunofluorescence (**Figure 1B**). Following successful tissue formation in 2D, we generated 3D organoids whereby a small region of the organoid consists of SHH-producing cells, which can be activated by surrounding GFP-presenting cells, making up the remainder and majority of cells within the organoid (**Figure 1C**). SynNotch induction and subsequent SHH expression results in regions of high FOXA2 expression polarized at one end of the organoid (**Figure 1D**), presumably in and around the artificial cell signaling center. **Conclusions**: Future work will expand on this synthetic morphogenesis approach to build a floor plate organizing center and analyze additional markers of dorsal-ventral patterning. This work has the capacity to study early developmental events and can be used as a tool to study preclinical gene and cell therapies, including those related to SHH and early embryonic development.

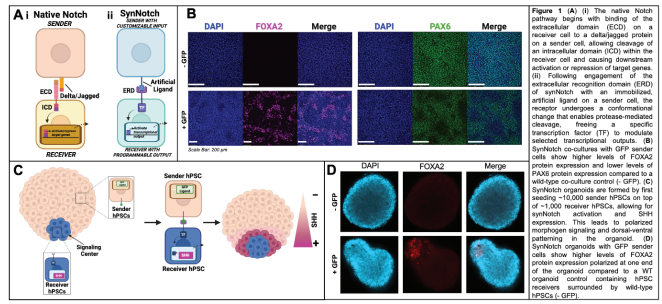


Figure 1 (A) (i) The native Notch pathway begins with binding of the extracellular domain (ECD) on a receiver cell to a delta/jagged protein on a sender cell, allowing cleavage of an intracellular domain (ICD) within the receiver cell and causing downstream activation or repression of target genes. (ii) Following engagement of the extracellular recognition domain (ERD) of synNotch with an immobilized, artificial ligand on a sender cell, the receptor undergoes a conformational change that enables protease-mediated cleavage, freeing a specific transcription factor (TF) to modulate selected transcriptional outputs. (B) SynNotch co-cultures with GFP sender cells show higher levels of FOXA2 protein expression and lower levels of PAX6 protein expression compared to a wild-type co-culture control (- GFP). (C) SynNotch organoids are formed by first seeding ~10,000 sender hPSCs on top of ~1,000 receiver hPSCs, allowing for synNotch activation and SHH expression. This leads to polarized morphogen signaling and dorsal-ventral patterning in the organoid. (D) SynNotch organoids with GFP sender cells show higher levels of FOXA2 protein expression polarized at one end of the organoid compared to a WT organoid control containing hPSC receivers surrounded by wild-type hPSCs (- GFP).

1593. Identification of Artificial microRNAs Targeting C9orf72 Sense and Antisense Transcripts for ALS Treatment

Gabriela C. Toro[1], Sha Zhu[1], Hongxing Wang[1], Weiqun Zhang[1], Na Liu[1], Joan Shen[1,2], Lingzhi Zhao[1,2]

[1]NeuShen Therapeutics, Lexington, MA,[2]Co-corresponding authors, Lexington, MA

Hexanucleotide repeat expansion (HRE) in the C9orf72 gene is the most common genetic cause of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Pathogenic mechanisms involve a toxic gain of function of RNA and/or dipeptide repeats from both C9orf72 sense transcript and anti-sense transcript as well as C9orf72 loss of function. A recent clinical trial utilizing an oligonucleotide antisense (ASO, BIIB078) targeting the G4C2 sense repeat RNA assessed the hypothesis of gain of toxic function. The results showed a substantial reduction in sense RNA foci and dipeptides; however, no clinical benefits were observed, suggesting that reducing sense C9orf72 alone may not suffice to alleviate C9orf72 HRE toxicity. Furthermore, a recent study demonstrated that expression of C4G2 antisense repeats,

rather than G4C2 sense repeats, impairs TDP-43 function in induced pluripotent stem (iPS) neurons. This underscores the potential role of antisense C4G2 RNA repeats in the pathomechanisms of C9orf72 HRE toxicity, suggesting that lowering antisense C4G2 repeats or both antisense and sense repeats may be necessary. However, the expression of C9orf72 antisense is notably low in cell lines including immortalized neuronal cell lines and iPSC-derived neurons, which poses a significant challenge in the discovery of effective antisense silencers. To address this, we embarked on constructing a series of assays with the goal of identifying microRNAs (miRNAs) targeting both antisense and sense C9orf72 transcripts. For rapid screening, we adapted a Renilla/Firefly dual luciferase reporter cell-based assay and developed a method capable of quantifying sense and HRE-containing antisense C9orf72 expression simultaneously in a single run. Using this assay, we identified several potent sense and antisense miRNAs, demonstrated by a robust reduction (> 85%) in C9orf72 reporter signal when co-transfected with a reporter plasmid carrying sense and antisense strands. Moreover, when constituting one sense and one antisense miRNA into a dual miRNA format, we observed an enhanced decrease in the reporter signal compared to either the sense miRNA or the antisense miRNA alone, indicating a potential synergic action by sense and antisense miRNAs. Next, we designed customized sense- and antisense-specific droplet digital PCR (ddPCR) assays, enabling the quantification of very low copies of antisense and sense C9orf72 expressed in C9orf72 ALS patient-derived lymphoblastoid cell line (LCL) cells. Using the ddPCR assay, we have validated a few active sense and antisense miRNAs, previously screened from the reporter assay. Our preliminary characterization indicates a potential advantage in concurrently expressing miRNAs targeting both sense and antisense strands to lower C9orf72 expression. Dual silencers demonstrating efficacy in both assays are selected for future development of Adeno-Associated Virus (AAV) gene therapy to treat C9orf72 HRE-caused ALS.

1594. Development of a Disease Modifying Adeno-Associated Virus-Based Combination Gene Replacement Therapy for Multiple System Atrophy

Yezheng Tao, Wei Cheng, Pengjuan Ma, Shin-Shay Tian, Xiaoping Zhao

Shanghai Vitalgen BioPharma Co., Shanghai, China

Multiple system atrophy (MSA) is a neurodegenerative disorder clinically characterized by autonomic failure, parkinsonism, and ataxia, resulting from the selective deterioration and loss of neurons in the striatonigral pathway, olivopontocerebellar pathway, and autonomic systems. MSA is a rapid progression and life-threatening disease with an urgent need for disease-modifying therapies that can halt the disease progression and improve the patients' life span. The precise etiology of MSA is unclear. Based on the disease pathogenesis, the major pathogenic mechanisms include the formation of toxic oligodendroglial cytoplasmic inclusions (GCIs) containing filamentous α-synuclein (oligodendrogliopathy) and mitochondrial dysfunction-induced oxidative stress and neuronal damages. These two mechanisms are thought to collaborate to accelerate the loss of functional neurons. To address its complex disease mechanisms, we developed an AAV9-based MSA combination gene replacement therapy VGN-R12a, designed to reduce GCIs and slow down neurodegeneration simultaneously by targeting the different aspects of MSA pathology as described above. To test the *in vivo* efficacy of VGN-R12a, we established an in-house mouse model to mimic the oligodendrogliopathy and mitochondrial dysfunction of MSA. We first generated a transgenic mouse in which mutated human α-synuclein (α-synuclein A53T) was specifically expressed in oligodendrocytes using the proteolipid promoter (PLP-Syn-A53T). These mice exhibited significant increases of the α-synuclein oligomers in their midbrain, striatum, cerebellum, and brainstem, accompanied by motor deficits in open field task. 3-nitropropionic acid (3-NP), a mitochondrial toxin, was used to induce mitochondrial dysfunction in these mutant mice to accelerate the decline of motor function and enhance neurodegeneration, closely mimicking the MSA pathology. VGN-R12a was tested in the MSA mouse model described above through intracerebroventricular injection. We found that VGN-R12a could dose-dependently alleviate the MSA phenotypes, as well as reduce the levels of phosphorylated α-synuclein, a biomarker for synucleinopathies. In conclusion, our data suggest that VGN-R12a has the potential to be an effective disease-modifying therapy for MSA.

1595. vMiX, an Innovative AAV-Based RNA Interference Platform: First Applications to Treat ALS

Romain Joubert[1], Zoe Walker[1], Deborah Ojutalayo[1], Alice Gennari[1], Louise Reilly[1], Yalem Bekele[1], Joana Furtado[1], Catia Andreassi[1], Olivier Brock[2], Amrita Abhilash[1], Paniz Hosseini[1], Samamtha Correia[1], Philip Coan[1], Carlos Miranda[1], John Isaac[1], Christopher E. Shaw[1,2]

[1]AviadoBio, London, United Kingdom,[2]King's College London, London, United Kingdom

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder due to the degeneration of motor neurons in the brain and spinal cord. It causes a progressive loss of voluntary muscle control, resulting in paralysis and fatal respiratory failure. Mutations in many genes have been implicated in disease pathogenesis but the mis-accumulation of TDP-43 drives disease pathogenesis in ~95% of patients. Expanded polyglutamine repeats in the Ataxin-2 gene (*ATXN2)* >30 are a risk factor for ALS and Frontotemporal Dementia (Glass 2022). Knockout and knockdown of ATXN2 in mice significantly slows disease progression in TDP-43 overexpressing mouse models (Becker 2017), thus, *ATXN2* silencing has emerged as a therapeutic strategy to treat ALS. To enable RNA interference, we generated a novel adeno-associated viral vector expressing a transcript encoding micro-RNA (miRNA) that can rapidly be modified to generate guide sequences that target any transcript in any species. miRNA processing involves various enzymes that sequentially cleave primary miRNA (pri-miRNA) transcripts to generate guides within the RNA-induced silencing complex capable of binding to specific mRNA sequences leading to their degradation of suppression of translation. Our vector has a hybrid miRNA scaffold designed to to optimise miRNA processing by Drosha and Dicer and enhance guide strand selection. Moreover, the pri-miRNA expression employs pre-mRNA 3' end cleavage in its processing, demonstrating for the first time that an RNA of interest can be expressed outside the canonical promoter - transcript - termination signal design. The performance

of our vMiX vector was compared to commercially available miR155 miRNA constructs with identical guides and assessed using luciferase reporters. The miR155 vector exhibited variable levels of knockdown (30-75%) depending on the targeted mRNA region (5'UTR, coding sequence, 3'UTR), while our vMiX vector, expressing the same miRNAs consistently achieved an 80% knockdown. To test the specificity of our new vector, the knockdown was evaluated against a wild-type and a codon-optimized mRNA coding for the same protein (3 mutations outside the seed sequence). Using protein analysis, vMiX showed an 81% knockdown of the wild-type target but had no activity against the codon-optimized one, demonstrating that, unlike endogenous miRNA, vMiX requires a full sequence match to induce silencing, thereby increasing specificity and reducing the risk of off-target effects. vMiX was developed as an RNAi platform. Using *in silico* design we generated multiple miRNA candidates targeting genes implicated in ALS pathogenesis including superoxide dismutase 1, *C9orf72*, and *ATXN2* and assessed their potency for knockdown and processing by quantifying guides and guide:passenger ratio across multiple species (human, primate, mouse, and pig). Two separate *in vivo* studies were carried out using miRNA targeting human *ATXN2* in a mouse model expressing the human transgene with 72 CAG repeats. Intrathalamic injections of an AAV9-miR-ATXN2 in adult mice revealed a dose-dependent response in *ATXN2* mRNA knockdown. vMiX demonstrated highly specific and robust *in vitro* and *in vivo* knockdown, and efficient targeting in multiple cell types. While the vMiX vector designed to target *ATXN2* holds promise for treating sporadic ALS its potential extends to silencing other targets of interest for addressing various disorders in brain and other organs. *(Glass et al., Brain 2022. Becker et al., Nature 2017.)*

1596. Systemic Gene Replacement Therapy Corrects Neurological Phenotype in a Mouse Model of NGLY1 Deficiency

Ailing Du[1], Xuntao Zhou[2], Lingzhi Ren[1], Nan Liu[1], Chen Zhou[1], Jialing Yang Liang[1], Guangping Gao[1,2], Dan Wang[1,3]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Department of Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA,[3]RNA Therapeutics Institute, UMass Chan Medical School, Worcester, MA

NGLY1 deficiency (OMIM #615,273) is an ultrarare autosomal recessive deglycosylation disorder caused by biallelic mutations in the *NGLY1* gene encoding cytoplasmic peptide: N-glycanase (NGLY1). NGLY1 is ubiquitously expressed and normally functions as a de-N-glycosylating enzyme that, together with the 26S proteasome, degrades misfolded N-glycosylated proteins in the cytoplasm. Patients suffer from multisystemic involvement including developmental delay, intellectual disability, scoliosis, hypotonia, motor deficit, and liver dysfunction; the neurological manifestations represent the major disease burden. Currently, there is no treatment for this disease. Unlike in humans, NGLY1 deficiency in C57BL/6J mice is embryonically lethal, posing a significant challenge to studying the disease mechanisms and testing AAV-based gene therapy *in vivo*. In this study, we obtained a *Ngly1* inducible knock-out (iKO) C57BL/6J mouse model. This iKO mouse is homozygous for a loxP-flanked *Ngly1* exon and carries a tamoxifen-inducible, ubiquitously expressed *cre* transgene that enables tamoxifen-dependent *Ngly1* knockout after birth. We showed that tamoxifen treatment induced 80-90% knockdown of *Ngly1* expression in multiple tissues including the liver, heart, skeletal muscle, spinal cord, and brain, resulting in symptoms recapitulating human disease, including elevation of the biomarker GlcNac-Asn (GNA), motor deficits, kyphosis, Purkinje cell loss, and gait abnormalities. We designed a codon-optimized human *NGLY1* transgene cassette driven by a chicken beta-actin promoter and packaged it into two capsids, AAV9 and AAV.PHPeB. Systemic administration of the AAV.PHPeB vector to iKO mice after disease onset led to higher transgene expression in the central nervous system (CNS) than AAV9, and better correction of multiple disease features at 8 weeks post-treatment. Furthermore, another cohort of AAV.PHPeB-treated animals were monitored over a year and showed near-complete normalization of the neurological aspects of the disease, demonstrating the durability of gene therapy. Our data suggest that brain-directed *NGLY1* gene replacement via systemic delivery is a promising therapeutic strategy for NGLY1 deficiency. Although the superior CNS tropism of AAV.PHPeB vector used in this study does not translate to primate, emerging AAV capsids with enhanced human CNS tropism will enable future translational studies. G.G. and D.W. are co-corresponding authors

1597. SOD1 Gene Repression Mediated by Zinc Finger Repressors (ZFRs) as a Therapeutic Approach for SOD1-Mediated ALS

Andrew Young, Kenneth Kim, Jill Cifrese, Asa Hatami, Jisoo Lee, Patrick Dunn, David Paschon, Marian Glynn, Cecelia Paculba, Toufan Parman, Emily Tait, Stephen Lam, Sarah Hinkley, Alicia Goodwin, Alex M. Ward, Kathleen Meyer, Bryan Zeitler, Amy M. Pooler

Sangamo Therapeutics, Inc., Richmond, CA

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that leads to degeneration of motor neurons in the spinal cord and brain. This loss of motor neurons results in loss of muscle control, paralysis, and eventual death. Superoxide dismutase 1 (*SOD1*) is a gene that codes for an enzyme that protects cells from free radical damage. Mutations in *SOD1* account for 10%-20% of inherited forms of ALS. These mutations are thought to manifest ALS symptoms through a mechanism whereby toxic aggregates of misfolded SOD1 protein accumulate in motor neurons leading to neuron dysfunction and loss. Targeting SOD1 for repression using antisense oligonucleotides such as Tofersen, which recently received conditional approval, improved clinical biomarkers of SOD1 ALS in human patients, suggesting SOD1 repression as an attractive therapeutic target. Zinc finger proteins (ZFs) are DNA-binding proteins that can be engineered to specifically bind any genomic sequence. When fused to a transcriptional regulatory domain such as KRAB, these zinc finger repressors (ZFRs) can reduce the expression level of a targeted gene. In a proof-of-concept study, we engineered ZFRs with human SOD1 repression activity for use as potential therapeutics for SOD1 mutation-associated ALS. We designed a library containing 100s of ZFRs targeting human sequences near the *SOD1* transcriptional start site. The library was initially screened in human and rodent neuronal cell lines. Lead ZFRs were then packaged into AAV and screened in human iPSC-derived GABAergic and mouse primary cortical neurons *in vitro*. We identified ZFRs that repressed human *SOD1* mRNA in neurons by more than 50% over a wide dose

range with minimal to no detectable off-target activity as measured by global transcriptomics analysis. These results show a proof-of-concept that ZFRs may provide a potential novel therapeutic approach for preventing or slowing the progression of ALS.

1598. Disruption of Aberrant Splicing of STMN2 by Gene Editing with a Type V CRISPR-Cas Enzyme as a Potential Treatment for ALS

Priscilla D. Negraes[1], Peter D. Buckett[1], Jace Jones-Tabah[1], Anthony J. Garrity[1], Mishti Bose[1], Sevda Lule[1], Andrew Park[1], Nolan Sheppard[1], Linh B. Truong[1], Qiyuan Yang[1], Ivan A. Kristanto[1], Maximiliano F. Presa[2], Jennifer Ryan[2], Erin Merkel[1], Christina Noe[1], Jenny V. Tobin[1], Lijun Liu[1], Florie Borel[1], Cathleen Lutz[2], John E. Murphy[1], Chee Yeun Chung[1]

[1]Arbor Biotechnologies, Cambridge, MA,[2]The Jackson Laboratory, Bar Harbor, ME

TDP-43 is a nuclear RNA-binding protein that regulates RNA processing. In many neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTD), TDP-43 is mis-localized and accumulated in the cytoplasm, a pathological hallmark of these conditions. The lack of nuclear TDP-43 in disease contexts results in aberrant splicing and processing of numerous genes. One of the most highly down-regulated is STMN2, which encodes for a microtubule-binding protein that controls axonal outgrowth and is highly expressed in motor neurons. Prior studies suggest that nuclear depletion of TDP-43 results in the aberrant splicing of STMN2 and loss of full-length functional STMN2, which may contribute to the pathology in ALS. To explore a potential treatment for ALS, we used gene editing with a Type V CRISPR-Cas enzyme to disrupt aberrant splicing of STMN2 by creating larger deletions of ~10 base pairs. We first screened in HEK cells multiple type V CRISPR nucleases and guides spanning the 310 base pair aberrantly spliced novel exon of STMN2 (exon 2A) that includes cis-acting elements such as a splice acceptor site, a TDP-43 binding region and a polyadenylation site. Nuclease/guide pairs with efficient indel formations from the HEK screening were tested in SH-SY5Y cells under TDP-43 knockdown to identify the pairs that were capable of reducing aberrant splicing and promoting an increase of full-length STMN2 mRNA. Several nuclease/guide pairs disrupting the splice acceptor site resulted in the effective reversal of aberrant splicing and increase of full-length STMN2 mRNA, but not the other cis-acting elements, thus defining the splice acceptor as the relevant target. These findings were replicated in human induced pluripotent stem cell (iPSC)-derived motor neurons. Moreover, the disruption of aberrant splicing using the nuclease/guide pairs in human motor neurons reversed the axonal loss caused by TDP-43 knockdown. Finally, the most potent nuclease/guide pairs, ABR-001 variant and gX, and ABR-004 variant and gY, were packaged into a rh10 serotype AAV vector and injected into neonatal mice transgenic for the human STMN2 exon 2A locus. In these mice, efficient editing as well as the reversal of STMN2 aberrant splicing were observed. In summary, we have identified multiple type V nuclease/guide pairs that reverse the aberrant splicing of STMN2 and the disease-relevant phenotypic consequences due to TDP-43 nuclear depletion in a cell line, in motor neurons and in a mouse model. Together, these findings have the potential to pave a path towards a CRISPR gene editing therapeutic for ALS.

1599. Leveraging *In Silico* and *In Vitro* Modeling for Optimization of Intrathecal AAV9 Delivery to the Brain: Validation in Nonhuman Primates and Translation to Human Rett Syndrome Patient Anatomy

Bryn Martin[1], Lucas Sass[1], Mohammadreza Khani[1], Ostin Arters[2], Stuart Sater[1], Howard Dobson[3], Scott Haller[4], Richard Watts[5], Kathrin Meyer[6], Deep Singh[7]

[1]Alcyone Therapeutics, Lowell, MA,[2]Alcyone Therapeutics, Inc., Moscow, ID,[3]InVicro, Needham, MA,[4]Charles River Laboratory, Wilmington, MA,[5]Yale University, New Haven, CT,[6]Nationwide Childrens Hospital, Columbus, OH,[7]Alcyone Therapeutics, Waltham, MA

Background Targeted, consistent, and safe delivery of genetic therapies to the brain is critical for effective treatment of CNS disorders. To date, researchers are attempting to optimize CSF delivery by choice of injection location. There are currently no validated tools for prediction of CSF solute transport in nonhuman primates (NHP) or transformation to humans. It is often overlooked that NHP CNS anatomy lacks similitude to humans. Thus, NHP delivery optimization may not equate to humans. Alcyone Therapeutics' Falcon™ platform includes novel computational modeling and a lumbar-access intrathecal intra-CSF delivery system that can target drugs to the brain, spinal cord or brain and spinal cord together, while being safe and clinically scalable. We used Falcon™ to optimize AAV9 delivery to the CNS and then validate NHP model predictions using AAV9.CB.GFP administered by either Falcon™ or lumbar puncture (LP). We also developed the first Rett Syndrome disease-specific model to optimize AAV delivery taking into consideration disease-related alterations of CSF flow that may impair drug transport. **Methods** CSF geometry and flow data was collected from NHPs via MRI. These measurements defined the 3D computational fluid dynamics (CFD) simulation and 3D printed models representing details of NHP brain and spinal cord anatomy including CSF flow and cisterns. We used this system to compare and optimize delivery parameters for widespread brain and spinal cord targeting and compared various protocols to LP injections. To validate the model predictions, seronegative NHPs (3-6 yrs) were co-infused with gadolinium and AAV9.CB.GFP via LP (n=5) or the optimized Falcon™ delivery (n=5). Biodistribution was analyzed 3w later by immunohistochemistry, ddPCR (DNA/RNA) and western blots. Drug flow was compared via gadolinium concentration. Following NHP model validation, we developed the first model systems of Rett syndrome patient anatomy using the same methods and optimized AAV delivery to the human disease anatomy to improve dosing in future clinical trials. **Results** We validated the model systems with in vivo NHP biodistribution confirming model predictions. Falcon™ was significantly superior, reaching the cranial space 1-5 min post-injection, while LP required 30-60 min. At 30 mins post injection, gadolinium concentration was significantly greater throughout the cortical gray matter and basal ganglia with Falcon™ vs. LP. In vivo PD showed Falcon™ vg per diploid genome was significantly higher throughout brain regions compared to LP. The Rett disease-specific

model showed significant alterations in terms of neuroanatomy and CSF flow that would impact AAV delivery to the brain. **Conclusion** Alcyone Therapeutics' Falcon™ platform includes a novel state-of-the-art modeling system including precise and anatomically detailed models of NHPs, healthy humans and humans with Rett Syndrome, as well as a lumbar-access intrathecal intra-CSF delivery system. The modeling predictions were validated using AAV9.CB.GFP in NHPs showing faster and broader distribution of AAV9 in the brain with Falcon™ which led to significant improvement in biodistribution. The improved targeting of the CNS was also reflected by significantly reduced liver off target effects. We optimized the approach for Rett Syndrome for upcoming clinical studies. Importantly, our delivery system and models can also be used to optimize repeat dosing of small molecules, ASOs and RNA gene therapy as it is designed for permanent implantation.

1600. PMP22 Gene Repression Mediated by Zinc Finger Repressors (ZFRs) as a Therapeutic Approach for CMT1A

Esmeralda Ponce, Odessa Yabut, Daniel Chung, Ken Kim, Victoria Chou, David Ojala, Annemarie Ledeboer, Andrew Young, Amy Pooler

Sangamo Therapeutics, Inc., Richmond, CA

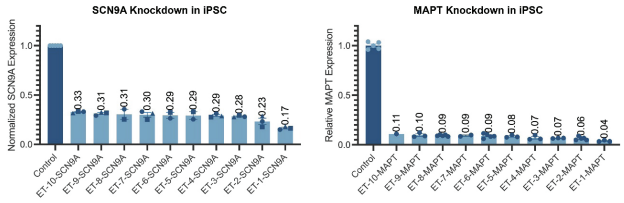
Charcot-Marie-Tooth disease 1A (CMT1A) is an inherited, progressive peripheral neuropathy. Symptoms of CMT1A include weakness and atrophy of muscles within the legs and arms impairing movement and balance. Symptoms typically appear in early adolescence and continue to progress throughout the patient's life and there are currently no disease modifying treatments available. The cause of CMT1A is duplication of the PMP22 gene which codes for a peripheral myelin transmembrane glycoprotein. Overexpression of the PMP22 gene leads to an inability of Schwann cells (peripheral myelin producing cells) to properly myelinate large axons, leading to disruption of myelination, dysfunction in peripheral nerve conduction and eventual axonal loss. Zinc finger proteins (ZFs) are DNA-binding proteins that can be engineered to specifically bind any genomic sequence. When fused to a transcriptional regulatory domain such as KRAB, these zinc finger repressors (ZFRs) can reduce the expression level of a targeted gene. In a proof-of-concept study, we engineered ZFRs targeting PMP22 repression with sequences conserved between mouse, NHP and human for use as potential therapeutics for CMT1A. We designed a library containing 100s of ZFRs near the PMP22 transcriptional start site. The library was initially screened in both neuronal and Schwann cell lines from humans and rodents. Taking advantage of the tunability of our zinc finger platform we selected ZFRs capable of partial PMP22 repression to avoid any potential negative consequences associated with a complete loss of PMP22 expression. Lead ZFRs were then packaged into AAV vector and screened in CMT1A patient-derived fibroblasts in vitro. Select ZFRs demonstrated greater than 50% repression of total PMP22 transcript in patient derived fibroblasts. The data presented here support continued development of PMP22-targeting ZFRs as a potential therapeutic approach for the treatment of CMT1A.

1601. Identification of Potent and Selective AAV-miRNA Candidates to Knockdown Non-Monogenic Neurological Targets *SCN9A* (Pain) and *MAPT* (Tauopathies)

Chao Tai, Veda Tatavarty, Szu-Ying Chen, Sawyer Randles, Mirella Rivera-Velazquez, Steven Tan, Puja Dhanota, Serena Liu, Alex Diaz de Arce, Pingping Jia, Sammy Liang, Keiko Takahashi, Mark Elegue, Sirika Wood, Suresh Poda, Stephanie Tagliatela

Encoded Therapeutics, South San Francisco, CA

Rationale: Adeno-associated virus (AAV)-mediated microRNA (miRNA) expression provides potential long-term knockdown of target genes following a one-time administration. This is an attractive strategy for central nervous system (CNS) applications when compared with chronic repeat dosing required for short interfering RNA (siRNA) or antisense oligonucleotides (ASOs). Utilizing Encoded's vector engineering platform, we identified several potent and target-specific miRNA candidates to effectively knockdown two clinically relevant non-monogenic neuronal targets: *SCN9A* and *MAPT*. *SCN9A* encodes the voltage-gated sodium channel alpha subunit 1.7 ($Na_V1.7$), which is associated with pain sensation. Clinically, *SCN9A* variants lead to hypoalgesia or hyperalgesia phenotypes (secondary to loss- or gain- of-function mutations, respectively). *MAPT* encodes tau, which regulates microtubule dynamics in neurons. Pathologic *MAPT* variants are associated with tauopathies, and in Alzheimer's disease, increases in tau pathology are closely associated with disease progression. **Methods:** Using computational methods, we identified over 7,000 potential miRNAs within *MAPT* and *SCN9A* pre-mRNA. We selected the top 50 miRNA candidates based on cross-species sequence homology (for future *in vivo* testing), minimal to no predicted off-target effects per *in silico* predictions, and favorable miRNA stability and processing features. Here we present *in vitro* knockdown potency in HEK-293T cells and two lines of human iPSC-derived neurons for the top miRNAs selected. **Results: *SCN9A* knockdown:** The top 10 of 50 tested miRNA candidates achieved a 66-83% knockdown of the endogenous *SCN9A* gene in cell lines tested. While exhibiting substantial knockdown of the target gene, these candidates exhibited minimal off-target effects on related sodium channels (*SCN1A*, *SCN2A*, *SCN3A*, *SCN8A*, *SCN10A*) across all cell types tested. This profile of selectivity is desirable since small molecule inhibitors face challenges with selective $Na_V1.7$ inhibition. ***MAPT* knockdown:** Similarly, the top 10 of 50 tested miRNA candidates achieved 89-96% knockdown of endogenous *MAPT* in cell lines tested. Off-target analysis of the top miRNA candidates is currently underway. **Conclusion:** Taken together, our miRNA platform enables the discovery of potent and specific candidates that knockdown disease-relevant genes in the CNS with the potential to significantly impact the treatment landscape for various neurological and genetic disorders. **Figure 1.** miRNA candidate screening yields robust endogenous *SCN9A* and *MAPT* knockdown in human iPSC-derived neurons. Cells were treated with AAV-based-miRNA candidates. *SCN9A* expression assessed reveals up to 83% knockdown (left) and *MAPT* up to 96% knockdown (right). Data points are biological replicate means, with error bars indicating SEM.



1602. Intravenous Administration of BBB-Penetrant AAV Gene Therapy Provides Broad and Robust CNS TAU Lowering in a Tauopathy Mouse Model

Hechen Bao, Shiron J. Lee, Cansu Colpan, Elisabeth R. Knoll, Ching-Lien Fang, Barbara S. Bueno, Gretchen Rivera, Maneesha A. Paranjpe, Brian C. Ezell, Kyle Grant, Rajeev Sivasankaran, Dinah W. Y. Sah, Todd A. Carter, Wencheng Liu

Voyager Therapeutics, Lexington, MA

Progression and spread of tau pathology in the human brain correlates well with cognitive decline in Alzheimer's disease (AD). A potential therapeutic approach for AD is to reduce tau (MAPT, microtubule associated protein tau) protein levels in the central nervous system (CNS). Anti-sense oligonucleotides (ASOs) have been shown to reduce tau expression and tau pathology in preclinical and clinical studies. However, this approach requires repeated administration of the ASO via intra-ventricular or intrathecal administration. Here, we demonstrate that a single, intravenous (IV) administration of a blood-brain barrier (BBB)-penetrant, self-complementary adeno-associated virus (scAAV) gene therapy reduces tau expression in the CNS of a tauopathy mouse model expressing human tau. In silico and in vitro screening for MAPT mRNA reduction led to the identification of candidate siRNA sequences that robustly lowered MAPT mRNA. This was followed by insertion of candidate siRNA sequences into proprietary, primary artificial microRNA (pri-amiRNA) backbones for AAV delivery. The efficacy of one pri-amiRNA candidate, TAU-2023, delivered by a BBB penetrant AAV capsid, was then thoroughly evaluated in vivo in hTau mice, which express all six isoforms of human MAPT. The results demonstrated a dose-dependent increase in vector genomes and concomitant MAPT mRNA knockdown, in multiple brain regions of AAV-treated hTau mice, four weeks post-injection. MAPT mRNA reductions ranged from 70% to 90% relative to the vehicle-treated hTau mice. Total tau protein levels were also significantly reduced, with average reduction ranging from 50% to 70%, in multiple brain regions. Treatment with TAU-2023 was well-tolerated with no notable test article related changes in body weight or cage-side observations up to two months post dose. Additionally, small RNA sequencing demonstrated favorable efficiency and precision of pri-amiRNA processing. Current research aims to evaluate the effectiveness of TAU-2023 in reducing pathological tau in a tauopathy mouse model. Results to date indicate that the combination of a potent and well-tolerated, MAPT targeting pri-amiRNA and a BBB-penetrant capsid could represent a promising one-time, IV treatment option for AD and other tauopathies.

1603. Preclinical Assessment of JAG201, a Clinical Stage Gene Therapy for Severe Neurodevelopmental Disorders Caused by Mutations or Deletions in *SHANK3* Including Phelan-McDermid Syndrome (PMS) and Autism Spectrum Disorder (ASD)

Michael Hughes, Janet Do, Daniel McCoy

Jaguar Gene Therapy, Lake Forest, IL

Background: The SH3- and ankyrin repeat (SHANK) protein family are major scaffolds of the post-synaptic density of excitatory synapses, where SHANK3 contributes to synaptic integrity by connecting ion channels and glutamatergic receptors to the cytoskeleton and proteins that facilitate intraneuronal signaling pathways. In cases of *SHANK3* haploinsufficiency, caused by loss of function mutation or chromosomal deletion encompassing *SHANK3*, patients present with a debilitating neurodevelopmental disorder characterized by severe intellectual disability, impaired or absent speech and language, severely disrupted sleep, reduced motor function, behavioral challenges, and autism and/or autistic features. Genetic sequencing studies indicate that *SHANK3* mutations or deletions may be present in ~1% of patients with ASD, equating to about 30,000 patients in the US. JAG201 is as an investigational AAV9-based gene therapy intended to deliver a functional version of *SHANK3* to treat PMS and *SHANK3* haploinsufficiency caused by loss of function mutation or deletion. Here, we present efficacy data following intracerebroventricular (ICV) administration of JAG201 in a mouse model of *Shank3* deficiency. *Methods:* On postnatal day 14 (±2 days), *Shank3*$^{\Delta 4\text{-}22}$ mice received either a single unilateral ICV administration of vehicle or JAG201 at a dose range of 2.40E09 to 2.75E11 vg/animal. Animals were followed for up to 10 months post-treatment, with assessments of established neurobehavioral phenotypes carried out to characterize therapeutic efficacy of JAG201. Postmortem analyses included assessment of JAG201 protein expression and functional binding partners in synaptic membrane preparations, biodistribution of JAG201 vector DNA and RNA via droplet digital PCR analysis, and RNA fluorescence *in situ* hybridization. *Results:* In mice, JAG201 led to improvements in several assessed neurobehavioral deficits at dose levels of ≥6.00E10 vg/animal. Specifically, (i) EEG evaluation of a restorative sleep marker deficit demonstrated significant improvement in *Shank3*$^{\Delta 4\text{-}22}$ mice treated with JAG201 at the 6.00E10 and 1.20E11 vg/animal dose levels; (ii) Open field test, evaluating motor and explorative behavioral deficits, demonstrated a significant increase in distance travelled of *Shank3*$^{\Delta 4\text{-}22}$ mice treated with JAG201 at doses of ≥6.00E10 vg/animal; and (iii) Rotarod test, evaluating motor-coordination deficits, demonstrated a significant increase in the latency to fall of *Shank3*$^{\Delta 4\text{-}22}$ mice treated with JAG201 at the 6.00E10 and 2.75E11 vg/animal dose levels. Synaptic membrane preparation analysis demonstrated a dose-dependent increase in JAG201 protein expression levels in the brains of treated *Shank3*$^{\Delta 4\text{-}22}$ animals. Finally, neurobehavioral improvements correlated with a significant increase in Homer1 levels in the synaptic membrane of JAG201-treated *Shank3*$^{\Delta 4\text{-}22}$ animals, demonstrating an improvement in a synaptic physiological deficit via recruitment of a SHANK3 functional binding partner. JAG201 DNA and RNA biodistribution assessed at 3- and 10-months post-treatment showed widespread rostrocaudal transduction and persistent transgene expression

throughout the CNS in a dose-dependent manner in *Shank3*$^{\Delta 4\text{-}22}$ mice treated with JAG201. *Conclusion:* These data demonstrate therapeutic benefit of ICV administered JAG201 on established neurobehavioral and synaptic physiological phenotypes in an animal model of *Shank3* deficiency. Unilateral ICV administration of JAG201 also results in widespread biodistribution and persistent transgene expression throughout the CNS. A first-in-human clinical study with JAG201, an AAV9-based gene therapy targeting *SHANK3* mutation- or deletion-related disorders, is slated to begin this year.

1604. The Mouse Aspa Promoter Contains Tissue-Specific Enhancers and Silencers

Thomas Leland[1], Mengtian Cui[1], Tapan Sharma[1], Suk Namkung[1], Sophia Liu[1], Jackson McGowan[1], Fang Zhang[1], Yue Zhang[1], Touraj Farzani[1], Guangping Gao[1,2], Phillip Wl Tai[1,2]

[1]Horae Gene Therapy Center, UMass Chan Medical School, Worcester, MA,[2]Microbiology and Physiological Systems, UMass Chan Medical School, Worcester, MA

Canavan disease (CD) is a lethal inherited leukodystrophy resulting from loss-of-function mutations in the *aspartoacylase (ASPA)* gene. In preclinical mouse studies performed in our lab, a CD gene therapy using a ubiquitously expressed human *ASPA* transgene (*CMV-CBA*-h*ASPA*) packaged into an adeno-associated virus (AAV) vector rescued the disease phenotype of a CD mouse model. At full dose, ubiquitous ASPA expression also resulted in enhanced rotarod performance. This phenotype was recapitulated by astrocyte-restricted overexpression of ASPA, suggesting that in addition to rescuing the disease phenotype, ASPA expression in astrocytes enhances motor performance. Gene therapies primarily rely on capsid tropism and the regulatory elements present in the transgene cassette to drive the transduction of target cells. Ideally, the set of transduced cells is comprised entirely of those that are in need of the therapy, but off-target expression is commonplace. Moreover, there may be reasons why a constitutive promotor is a better choice for a given vector, but it is important to explore endogenous promoters as they show potential for limiting levels of off-target expression. Challenges remain with such promotors as their sequence boundaries are often poorly defined, limiting our ability to mimic the complex regulatory circuits governing native gene expression.We are currently investigating the mouse *Aspa* promoter to understand the function of its putative enhancers, initially defined by epigenetic marks found 5' of the gene. We hypothesized that the endogenous promoter would transduce fewer off-target tissues and cell-types than *CMV-CBA*, and would retain the expression dynamics of the endogenous *Aspa* gene. We cloned a set of *Egfp* vector plasmids bearing m*Aspa* promoter variants with each defined region deleted individually or in combination, and packaged them into AAV9 for systemic injection into neonatal C57Bl/6 mice. m*Aspa* promoter activity exceeded that of the *CMV-CBA* control in brown fat, was equivalent in liver, and lower in heart and brain. In whole-brain, deletion of a putative central nervous system (CNS) enhancer did not decrease activity. In liver, heart, and brown adipose tissue, the deletion increased promoter activity, suggesting that the CNS enhancer acts as a repressor in these tissues. Deletions of a putative peripheral tissue (PT) enhancer led to diminished promoter activity, but only in the presence of the CNS enhancer. This finding suggests that the CNS enhancer acts to modulate the activity of the PT enhancer in a tissue dependent manner.These data demonstrate that the native *Aspa* promoter exhibits a complex regulatory program which remains functional when incorporated into an AAV vector and delivered outside of its normal chromosomal context. The putative PT enhancer appears to be broadly active, and the putative CNS enhancer appears to repress activity in off-target tissues. We are continuing to investigate the cell- and tissue-dependent activities of the m*Aspa* promoter elements to gain further insight into the mechanisms of *Aspa* transcription. G.G. and P.T. are corresponding authors

1605. Shank3 Gene Activation Mediated by Zinc Finger Activators (ZFAs) as a Therapeutic Approach for Phelan-McDermid Syndrome

Daniel Chung[1], Siavash Fazel Darbandi[1], Seemin Ahmed[2], Esmeralda Ponce[1], Jason Eshleman[1], Patrick Dunn[1], Toufan Parman[1], Sookhee Park[2], Andrew Fitzgerald[2], Gibbs Nasir[2], Bob Radeke[1], Jisoo Lee[1], Giulia Cisbani[3], Tim Kröcher[3], Chiara Melis[3], Sarah Zander[3], Mirjam Zachow[3], Claudia Schober[3], Michael Helwig[3], Sarah Hinkley[1], Alicia Goodwin[1], Alaric Falcon[1], Eloise Hudry[2], Katie Worringer[2], Gregory Davis[1], Bryan Zeitler[1], Andrew Young[1], Amy Pooler[1]

[1]Sangamo Therapeutics, Inc., Richmond, CA,[2]Novartis, Cambridge, MA,[3]Evotec SE, Hamburg, Germany

Phelan-McDermid syndrome (PMS) is a rare genetic condition characterized by clinical features with varying severity, including intellectual disability, absent or delayed speech, and autism spectrum disorders (ASD). PMS is caused by a deletion or structural change in chromosome 22 or a pathogenic variant of the *SHANK3* gene. *SHANK3* encodes a synaptic protein localized at excitatory synapses to organize scaffolding proteins crucial for proper synapse formation or dendritic spine maturation. Mutations or loss of a *SHANK3* allele due to copy number variations can lead to SHANK3 haploinsufficiency causing synaptic and circuitry deficits associated with PMS. We are utilizing our proprietary Zinc Finger Activator (ZFA) platform to selectively upregulate gene expression by tethering Zinc Finger Proteins to a trans-activation domain. In a proof-of-concept study, we demonstrated Shank3 upregulation at mRNA and protein levels *in vitro* with zero to minimal off-targets in primary cortical neurons derived from wildtype (WT) and *Shank3*$^{(+/\Delta C21)}$ mutant mice. Further, we confirmed AAV-ZFA dose-dependent upregulation of *Shank3 in vivo* in relevant brain regions of WT mice by bulk tissue and single-cell mRNA and protein expression analyses. Thus, our results support the development of ZFAs to specifically restore SHANK3 to physiological levels, providing a potential precision therapy for PMS. These data support further development and investigation of ZFAs as a therapeutic option for neurodevelopmental disorders caused by genetic haploinsufficiency.

1606. Titratable Control of Neuronal Activity Using Precision Genetic Neuromodulation

Calvin Chad Smith, Alba Guijarro Belmar, Sylvain Gigout, Hanna Luniak, Miranda Mathews, Panayiota Demosthenous, Riccardo Privolizzi, Zinicola Zinicola, Brandon Roberts, Andrew Murray, Robert Brownstone
Sania Therapeutics, London, United Kingdom

Many neurological diseases can be attributed to abnormal electrical activity of specific neural circuits. If we could normalize the electrical activity of selective populations of neurons, we could develop a new generation of treatments for a wide range of neurological disorders. Genetic therapies provide a potentially powerful route to this kind of treatment if they could be targeted to the malfunctioning neural circuits and if they provided a mechanism for titrating neural activity in a way that could be controlled by the patient and physician. This type of precision therapy requires two key components: (a) a targeting vector system so that neuronal activity can be corrected selectively in dysfunctional circuits without altering the properties of neighbouring cells and brain regions; and (b) a tunable physiological system to increase or decrease neuronal activity through an easily administered (e.g. oral) medication. Sania Therapeutics is developing a precision therapy for neural circuits by leveraging a combination of intersectional technology platforms that allow for the targeted delivery of chemogenetic proteins through engineered AAV vectors. This approach will allow us to selectively modulate neural circuit activity in disease states while minimizing adverse effects associated with current therapies. Chemogenetics refers to the alteration of electrical activity of a neuron via the use of a genetically delivered protein that responds to a systemically delivered ligand. From a therapeutic perspective, the ideal chemogenetics system would have several properties: i) minimal effects on baseline neuronal activity in the absence of an activator; ii) potent and titratable effects on neuronal activity in response to an activator; (iii) activated by a drug that can be readily administered orally and has minimal effects on non-expressing cells; and iv) validated efficacy in humans. Here we present data showing the efficacy of our lead chemogenetic ion channel candidate (SRx-C490) for modulating excitability in two distinct sub-types of human iPSC derived neurons, motor neurons (MN) and sensory neurons (SN). We show that SRx-C490 can be delivered to MNs and SNs using AAVs and activated by low doses of an FDA approved oral medicine to produce titratable reductions in activity. We first assessed population level spontaneous activity using high density microelectrode arrays, showing that SRx-C490 had no effect on baseline (no activator) spontaneous activity of MNs. However, administration of the activator induced dose-dependent reductions in network activity for SRx-C490 transduced MNs. We next used patch clamp electrophysiology to assess the effect of SRx-C490 on high resolution MN firing characteristics and excitability at baseline, and at a range of activator doses. We show that AAV-mediated expression of SRx-C490 has minimal effect on baseline MN firing characteristics, but the activator induced reductions in excitability at doses that were sub-threshold for control neurons. Specifically, nanomolar activator doses reduced motor neuron excitability by 80% in SRx-C490 transduced motor neurons, while GFP transduced motor neurons were unaffected. We also evaluated the efficacy of SRx-C490 in hIPSC SNs with patch clamp electrophysiology. Similarly to MNs, SRx-C490 had minimal effect on baseline activity in SNs but the activator reduced excitability by up to ~75% at low doses. In summary, we show that AAV-delivered SRx-C490 is effective at reducing excitability in two distinct neuronal subtypes when activated by an FDA approved medicine. This novel chemogenetics system, in combination with Sania's cell-type specific AAV capsids, demonstrates the potential of our platforms to generate precision therapies for multiple neurological disorders.

1607. Intracerebroventricular AAV9 Delivery of Creatine Transporter Restores Peripheral Creatine in a Mouse Model of SLC6A8 Disorder

Troy B. Webster[1], Robyn T. Binsfeld[1], Ilona Tkachyova[2], Melissa Mitchell[1], Steven J. Gray[3], Andreas Schulze[2], Jagdeep S. Walia[1]
[1]Queen's University, Kingston, ON, Canada,[2]The Hospital for Sick Children, Toronto, ON, Canada,[3]University of Texas Southwestern, Dallas, TX

SLC6A8, or creatine transporter, is an active transporter responsible for cellular creatine uptake. SLC6A8 deficiency is an X-linked genetic disorder induced by loss of function mutations in *SLC6A8,* resulting in a severe depletion or absence of creatine in the central nervous system. The most notable role of creatine is as a phosphate buffer for ATP, increasing the energy storage capacity of high energy cells such as neurons. Disease manifestation includes a wide range of symptoms, including developmental delay, intellectual disability, motor function impairment, seizures, and hyperactivity. The primary cause of the disease is thought to be primarily due to the absence of creatine in neurons. However, creatine plays an important role in many peripheral organs, and supplementation is ineffective at restoring cellular creatine in the absence of a transporter. Previously, we shared the proof-of-principle that AAV9 delivery of a codon-optimized *SLC6A8*, via intracerebroventriular (ICV) route, can fully restore brain creatine and improve behavioral phenotypes. However, SLC6A8 disorders have also been shown to have effects on the peripheral organs, such as abnormalities in the heart, muscle, and immune system, as well as in the brain. Here, we administered self-complementary AAV9 encoding codon-optimized SLC6A8 at postnatal D0 at three different dosages via ICV injections. Five weeks post-injection, we measured creatine, creatinine, arginine, and guanidinoacetate in the heart, muscle, brain, liver, and spinal cord via mass spectrometry. Treatment resulted in significant restoration of creatine and creatinine to both the heart and muscle tissues compared to vehicle controls. Liver creatine was not significantly elevated, and guanidinoacetate and arginine were constant across all treatments. This data shows that gene therapy may be able to improve some of the peripheral phenotypes of SLC6A8 disorders in addition to the previously shown effects on the brain.

1608. Intravenous Gene Therapy Provides a Long-Term Correction of White Matter Vacuolation and Motor Deficits in a Mouse Model of Megalencephalic Leukoencephalopathy with Subcortical Cysts

Alejandro Brao[1,2], Ángela Sánchez[1,2], Irina Rodríguez[1,2], Esther Prat[3,4], Virginia Nunes[3,4], Miguel Chillón[1,2,5], Raúl Estévez[3,4], Assumpció Bosch[1,2,6]

[1]Departament de Bioquímica i Biologia Molecular & Institut de Neurociències, Universitat Autónoma de Barcelona (UAB), Bellaterra, Spain,[2]Unitat Mixta UAB-VHIR, Vall d'Hebron Institut de Recerca (VHIR), Barcelona, Spain,[3]Departament de Ciències Fisiològiques, Institut d'Investigació Biomèdica de Bellvitge (IDIBELL) - Institut de Neurociències, Universitat de Barcelona, L'Hospitalet de Llobregat, Spain,[4]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), ISCIII, Madrid, Spain,[5]Institut Català de Recerca i Estudis Avançats (ICREA), Barcelona, Spain,[6]Centro de Investigación Biomédica en Red de Enfermedades Neurodegenerativas (CIBERNED), ISCIII, Madrid, Spain

Megalencephalic leukoencephalopathy with subcortical cysts (MLC) is an infantile onset leukodystrophy characterized by white matter oedema for which there is no efficient treatment. Most MLC patients (~75%) suffer from a biallelic loss-of-function of *MLC1* and develop progressive white matter swelling and vacuolation, leading to early-onset macrocephaly, cerebellar ataxia, spasticity and muscle stiffness, epileptic seizures, and mild cognitive decline. While the physiological role of MLC1 is not fully elucidated, it is known that it is enriched at the astrocyte-astrocyte cell junctions, where it interacts with a myriad of water and ion channels, G protein-coupled receptors, and other membrane-bound proteins, regulating their localization, activity, and derived signaling pathways. Hence, mutations in MLC1 result in the deregulation of its interacting partners as well as the unbalancing of brain water and ion homeostasis. Our goal is to design and optimize a gene therapy strategy using the *Mlc1*$^{-/-}$ mouse model, but also to identify novel indicators that can be used to eventually validate its therapeutic effect. As a proof of concept, we intravenously injected $1{\cdot}10^{12}$ vg of AAV9P31 carrying the human *MLC1* cDNA under the control of the human astrocyte-specific promoter gfa2 (AAV9P31-gfa2-MLC1) to 10-month-old *Mlc1*$^{-/-}$ mice, 5 months after the onset of white matter vacuolation, a hallmark of the disease. This resulted in a long-term astrocyte-driven expression of MLC1 as later as 1 year after viral vector administration in all brain areas analyzed, as confirmed at the RNA and protein levels. In vivo magnetic resonance imaging (MRI) revealed a complete normalization in signal abnormalities associated with brain water accumulation, including a significant decrease in T_2 and mean diffusivity (apparent diffusion coefficient), two key parameters altered in MLC patients. Histopathological analyses showed a full rescue of cerebellar white matter vacuolation, hence confirming in vivo MRI findings. On the other hand, our gene therapy halted the progression and even abolished the motor deficit we first describe in this model, despite the late age of administration. Briefly, *Mlc1*$^{-/-}$ mice develop a progressive hindlimb impairment as seen in motor tests specifically used for cerebellar ataxia-like behaviors in mice, such as hindlimb clasping or hanging wire tests. Treated mice experimented a steady, but prolonged recovery of this phenotype and behaved like healthy controls 1 year after AAV administration. Taken together, our results not only demonstrate the long-term effectiveness of our treatment, but also provide new, valuable insights into the pathophysiology of MLC.

1609. Development of a Robust Zinc Finger Activation Platform for Treatment of Neurological Disorders

Irene Tan, Gillian Houlihan, Jenny Hodges, Siavash Darbandi, Heather Kozy, Esme Ponce, Dan Chung, Bhakti Kadam, Yolanda Santiago, Andrew Olin, Qi Yu, Bryan Zeitler, Annemarie Ledeboer, David Paschon, Gregory Davis

Sangamo Therapeutics, Inc., Richmond, CA

Many neurological disorders are caused by haploinsufficiency, or a heterozygous genotype leading to loss of function in one allele of a gene. These mutations can lead to neurodevelopmental impairments that can affect learning, self-control, and cognition. One strategy to address such disorders is the targeted upregulation of the wild-type allele using sequence specific transcription factors, such as zinc finger activators (ZFAs), to restore normal protein levels in the brain. However, long-term and high-level expression of certain published transactivation domains (TADs) in neurons can lead to undesired levels of differentially expressed genes (DEGs) and poor tolerability in vivo. To overcome this limitation, we have assessed a large panel of human TADs that, when linked to Sangamo's proprietary engineered ZF proteins, showed favorable profiles in various screening cell lines, including high specificity and selective, dose-dependent activation of therapeutically relevant target genes. These ZFAs were then packaged into AAV, transduced into neurons, and assessed for their activity and specificity in long-term experiments. Robust on-target activation was observed along with varying levels of genome-wide gene dysregulation which was TAD dependent. Importantly, several ZFAs were identified that elicited little to no gene dysregulation , whereas ZFAs utilizing conventional TADs exhibited a high number of DEGs. Finally, a four-week in vivo study was performed in which a highly-specific, genome orthogonal ZF was linked to the top-performing TAD candidates, packaged into a blood-brain penetrant AAV (AAV.PHP.B), and systemically delivered to the brain of wild-type mice via intravenous injection. Mice transduced with AAVs delivering ZFAs with our highly-specific TAD candidates survived to the end of the study with no neuroinflammation or neuronal loss observed in the brain or spinal cord, confirming the in vivo tolerability of these ZFAs and underscoring the importance of careful specificity assessments in vitro. These data demonstrate the power of the ZF activation platform Sangamo has developed and its potential to treat a variety of neurological diseases.

1610. Reinstatement of *Tcf4* in Juvenile Pitt-Hopkins Syndrome Model Mice Suggests the Importance of Early Intervention for TCF4 Reinstatement Strategies

Lucas M. James, Eric B. Gao, Benjamin D. Philpot

UNC Chapel Hill, Chapel Hill, NC

Pitt-Hopkins syndrome (PTHS) is a severe neurodevelopmental disorder caused by haploinsufficiency of the Transcription factor 4

(*TCF4*) gene. PTHS clinically presents with intellectual disability, lack of speech, motor delay, breathing disruption, seizures, sleep disturbance, hypotonia, and GI problems. Currently, there are only FDA-approved treatments for symptom management and recent studies have focused on developing therapeutics to treat PTHS at its genetic cause via gene therapy. PTHS symptoms emerge in early life with hypotonia and missed developmental milestones; however, diagnosis requires genetic testing which often does not occur in young individuals. TCF4 is most highly expressed during development, peaking perinatally, and is important for early brain development. TCF4 is particularly important in the forebrain with key roles in progenitor proliferation, neuronal migration, and laminar organization. Therefore, we expect that reinstatement of *TCF4* early in development would confer the greatest therapeutic benefit. While studies in mouse models and human brain organoids demonstrate the transformative potential of an intervention strategy, the critical period for reinstatement of *TCF4* has not been well defined in preclinical models. Research has focused on early postnatal mice and organoids which best model a late-embryonic reinstatement of TCF4 in humans. PTHS is not screened for prospectively prenatally or in infants, so it is critical to determine whether reinstatement of *TCF4* later in life offers therapeutic benefit. We used the conditional-reinstatement mouse model Tcf4-LSL (TCF4 Lox-Stop-Lox) developed by our lab to model a later-in-life reinstatement of *Tcf4*. This mouse model shares phenotypes with other TCF4 haploinsufficiency models, with the added advantage of a Cre-recombinase removable stop cassette to reinstate *Tcf4*. Wildtype and Tcf4-LSL mice were injected retro-orbitally at postnatal day 14 with AAV.PHP.eB-hSyn1-Cre to reinstate biallelic *Tcf4* expression in neurons throughout the brain or AAV.PHP.eB-hSyn1-mCherry as a negative control. Our lab has previously shown that reinstatement of TCF4 expression in neurons with this vector at postnatal day 1 (P1) improved behavioral phenotypes in the Tcf4-LSL model. Following injection at P14, mice began behavioral testing at 9-10 weeks through several behavioral assays previously identified as TCF4-sensitive including open field, elevated plus maze, novel object-location memory, and nest-building assays. Our preliminary data suggests that early reinstatement of *TCF4* holds the greatest potential to treat behavioral symptoms of PTHS; however, our study also encouragingly suggests that cognitive behaviors such as learning and memory may be amenable to reinstatement of *TCF4* later in life. Ongoing work is focused on determining the extent of synaptic and transcriptional improvement following reinstatement of *Tcf4* in juvenile mice.

1612. AAV Mediated Delivery of IL-10 to Demyelinating Lesions in an Animal Model of Multiple Sclerosis Does Not Suppress Disease but Results in Increased Active Lesion Formation

Geoffrey D. Keeler
University of Florida, Gainesville, FL

Demyelination is the destruction of the myelin sheath, or the protective coating surrounding neurons. Demyelination is considered a hallmark characteristic of Multiple Sclerosis and can lead to axonal damage, axonal death, and progressive disability in late disease stages. To date, there is no FDA approved treatment for demyelination. Current, treatment strategies for MS focus on decreasing neuronal inflammation. These treatments have little effect on demyelination and in fact allow continued formation of new lesions. Remyelination is the regenerative process in which the body repairs the myelin sheath. In MS, remyelination occurs but often fails in the majority of lesions. Thus, the induction of remyelination has been postulated to be an effective treatment option for MS, either alone or in conjunction with current treatments. The demyelinating lesion consist primarily of activated microglia and astrocytes. Demyelinating microglia and astrocytes are responsible for producing an inflammatory microenvironment that is hostile to remyelination. Thus, altering the microenvironment of the lesion represents a strategy to increase remyelination. Previously, we showed peripherally injected AAV efficiently crosses the blood-brain barrier and results in robust transgene throughout the brain and spinal cord in an animal model of MS. Here we show that our AAV platform successfully targets lesions sites (Fig1A-D). Next, we investigated the effects of AAV mediated delivery of IL-10 on disease outcomes. We found that our AAV.IL-10 treatment had no effects on the clinical outcome (Fig1E). Interestingly though, we found there to be more active lesions in IL-10 treated animals as compared to PBS and AAV.eGFP treated animals as visualized via Neutral Red Staining (Fig1F-H). These results show that IL-10 exacerbated active lesion development in the CNS of SJL mice suffering from relapsing-remitting MS like disease.

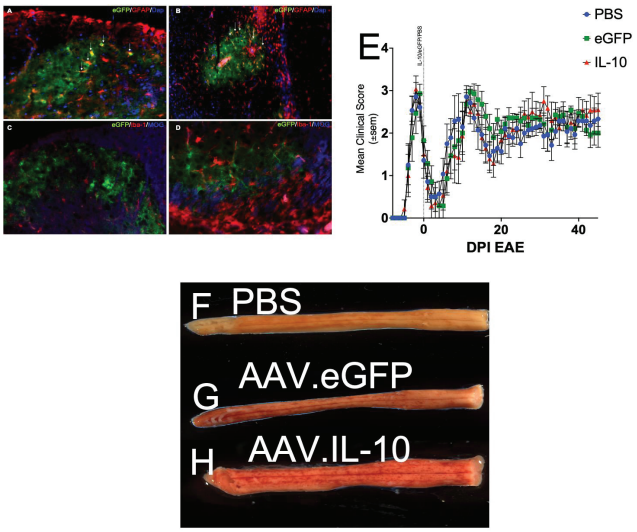


Figure 1: (A-F) Images showing AAV.eGFP targeting demyelinating lesions in brain and spinal cord as indicated by the presence of eGFP in the absence of MOG. (E) Mean Clinical Scores of animals treated with either PBS, AAV.eGFP, or AAV.IL-10, (F-H) spinal cords stained with Neutral Red (red areas represent the presence of active lesions).

1613. *In Vivo* Precise Nuclease-Free Genome Editing of the Murine MeCP2 Gene

Swati Bijlani[1], Ka Ming Pang[1], Sampath Rangasamy[2], Lakshmi Bugga[1], Vinodh Narayanan[2], Saswati Chatterjee[1]
[1]City of Hope, Duarte, CA,[2]TGen, Phoenix, AZ

Rett Syndrome is an X-linked, acquired, neurodevelopmental disorder that leads to loss of cognitive and motor abilities. It is associated with de novo mutations in the MECP2 gene and is almost exclusively seen in females. Males with pathogenic MECP2 mutation rarely

survive. MECP2 is a multifunctional methyl CpG binding protein that mediates transcriptional regulation, chromatin modification and mRNA processing. MeCP2 is expressed in all tissues with highest levels in the brain. It is critical for neuronal development. Over 95% of MECP2 mutations associated with Rett syndrome map to Exons 3 and 4 and alter binding to methylated DNA and interaction with transcription regulators, chromatin modifiers and RNA. It is critical to regulate MeCP2 expression as both under- and over-expression are pathogenic. Recent advances to restore regulated MeCP2 expression have therefore focused on modifying MECP2 gene transfer vectors to include the 3' untranslated region containing miRNA binding sites. Two early-stage clinical trials of gene therapy for Rett syndrome using similar vectors are underway. However, the efficacy and durability of this approach remains unknown. Moreover, due to size limitations, gene therapy vectors do not include all required regulatory elements. In contrast to gene therapy which provides additional episomal copies of the transgene, genome editing corrects the pathogenic MECP2 mutations directly in the genome. We hypothesized that permanent correction of mutations in the genome will retain all native regulatory elements and result in sustained systemic physiologic expression thus avoiding overexpression-related toxicity. Stem cell derived AAVHSCs mediate precise nuclease-free genome editing via homologous recombination (HR) and are not associated with additional mutagenesis unlike nuclease-based editing platforms. AAVHSCs are known to cross blood brain barrier. Thus, systemic administration should treat both peripheral organs as well as the central nervous system. Here, we evaluated the efficacy of HR-mediated editing of the MECP2 gene in vivo. An AAVHSC genome editing vector was designed to correct mutations in Exon 4 of the murine Mecp2 gene and insert a promoterless luciferase open reading frame (ORF) immediately downstream of Exon 4. Successful editing of the Mecp2 gene is expected to insert the promoterless luciferase ORF downstream of Exon 4, with expression driven by the chromosomal Mecp2 promoter. Luciferase expression would then serve as a surrogate for MECP2 expression. The murine Mecp2 editing vector was packaged in AAVHSC15 and injected intravenously in wild type mice at 2.2E+11 vg/mouse. Luciferase expression was followed by serial bioluminescent imaging. Systemic luciferase expression was observed by Day 7 with the highest levels found in the liver, likely due to liver tropism of AAVHSC15. Notably, the expression was stable throughout the duration of study, to 24 weeks post-injection with no associated toxicity. Sequence analysis is underway to further evaluate biodistribution and in vivo editing efficiency. These studies are expected to define the optimal dose range required for widespread restoration of Mecp2 expression in Rett models after systemic delivery. In vivo genome editing studies are also underway to evaluate the correction of an Mecp2 mutation in a mouse model of Rett syndrome. Overall, we demonstrated that in vivo administration of an AAVHSC15 editing vector resulted in durable systemic editing of the Mecp2 gene. Thus, we conclude that the AAVHSC genome editing vectors have the potential to permanently correct MECP2 mutations and restore endogenously regulated protein expression.

1614. Development of a Self-Regulating AAV Gene Therapy for the Safe and Effective Treatment of Rett Syndrome

Monique Otero

UMass Chan Medical School, Worcester, MA

Rett Syndrome (RTT) is a debilitating X-linked neurodevelopmental disorder that affects 1:10,000 girls. RTT is characterized by a period of normal development until 6-18 months, followed by rapid loss of previously acquired skills, resulting in severe neurological and physical disability. RTT patients often lose the ability to walk and talk, but with symptom management can live to middle age or older. A hallmark of the disorder is the loss of functional hand use with stereotypic or repetitive hand movements. RTT is caused by mutations in *MECP2* which encodes methyl-CpG binding protein 2 (MeCP2), an epigenetic regulator that is highly abundant in neurons and critical for normal neurodevelopment, maturation, and function. RTT primarily affects girls, who are heterozygous for the mutation, but in rare cases hemizygous boys are affected and have severe neonatal encephalopathy that is fatal in infancy. RTT is not neurodegenerative and genetic restoration of *MECP2* expression reversed neurological defects and rescued survival in adult RTT mice. In previous studies, AAV delivery of the *MECP2* transgene did not reverse symptoms and resulted in toxic over-expression leading to another neurological disorder: MeCP2-duplication syndrome. Recently the use of truncated or instability prone AAV-MeCP2 transgenes, which attenuate expression, have been successful in extending survival in RTT mice. *MECP2* is a dosage sensitive gene and to keep transgene expression within the therapeutically beneficial window, our lab developed self-regulating, AAV-MeCP2 vectors for the gene therapy of RTT. MeCP2 expression homeostasis requires the upstream positive and negative regulators, Brain-Derived Neurotrophic Factor (BDNF) and microRNA-132 (miR-132), respectively. Our vector delivers the MeCP2 transgene with miR-132 recognition elements to exploit this negative feedback loop. We have generated a series of scAAV9-MeCP2 vectors carrying target sites for miR-132 in combination with various other elements. We are comparing the therapeutic efficacy of these vectors delivered by intracerebroventricular (ICV) injection in neonatal *Mecp2*$^{y/-}$ mice. *Mecp2*$^{y/-}$ males have neurological abnormalities and a survival expectancy of 8-12 weeks. We currently have AAV-treated *Mecp2*$^{y/-}$ mice alive at 30 weeks of age in several treatment groups (Fig. 1). We continue to monitor their body weight and are conducting phenotypic scoring. Additional animals are being enrolled in short-term studies to analyze MeCP2 expression by immunoblotting and immunofluorescence of CNS as well as in peripheral tissues.

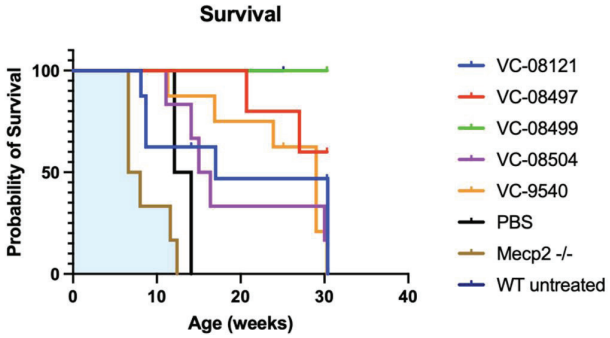


Figure 1: Improved survival of Mecp2y/- male mice after neonatal ICV treatment with scAAV9-Mecp2 vectors

1615. Rescuing STXBP1 Haploinsufficiency in Patient Derived iPSCs Using AAV Mediated Gene Replacement

Sakshi Arora[1,2], Elisa A. Waxman[1,2], Lea V. Dungan[1,2], Deborah L. French[1,2], Benjamin L. Prosser[2,3], Beverly L. Davidson[1,2]

[1]Center for Cellular and Molecular Therapeutics (CCMT), Children's Hospital of Philadelphia, Philadelphia, PA,[2]Center for Epilepsy and Neuro Developmental Disorders (ENDD), The Children's Hospital of Philadelphia, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA,[3]Department of Physiology, Pennsylvania Muscle Institute, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA

Syntaxin-binding protein 1 (STXBP1, also known as MUNC18-1) plays an essential role in synaptic vesicle fusion and is required for neurotransmitter release, and mutations in *STXBP1* are a leading cause of developmental epileptic encephalopathy. Multiple mutations in *STXBP1* result in premature termination codons, causing transcript nonsense mediated decay and STXBP1 haploinsufficiency. Induced pluripotent stem cells (iPSCs) generated from isolated patient blood samples, and then differentiated into neurons, retain phenotypes of patient neurons, providing a relevant model for screening potential therapies. Given the inherit inefficiencies with standard AAVs in transducing neurons, we first assessed a range of newly discovered AAVs variants emerging from capsid screens in non-human primates (NHP) brains for their abilities to more robustly transduce iPSCs derived neurons. Several variants in this screen showed 2-3 log improved transduction relative to their respective parent serotypes. Next, we cloned and packaged full length STXBP1 cDNA into AAV variant BD004 and BD005, and transduced iPSC-neurons derived from STXBP1 patients. We showed 3-30-fold upregulation of *STXBP1* mRNA and 2-3-fold upregulation of STXBP1 protein in haploinsufficient neurons. Additionally, when applied to patient-derived excitatory cortical neurons AAV-STXBP1 rescued the aberrant electrophysiological phenotypes noted by multi-electrode array (MEA) analysis. Together we present new AAVs for robust transduction of iPSC derived neurons from patient cells, and show their utility for assessing efficacy of AAV-STXBP1 for STXBP1 haploinsufficiency disorders. (S.A. and E.W. contributed equally to this work).

1616. Epigenetic Regulation of Human Prion Expression as a Potential One-Time Treatment for Prion Disease

Shih-Wei Chou[1], Kimberly Marlen[1], David Ojala[1], Patrick Dunn[1], Giulia Cisbani[2], Finn Peters[2], Chiara Melis[2], Jing Hu[1], Qi Yu[1], Matthew R. Tiffany[1], Sarah Mueller[1], Sarah Hinkley[1], Mohad Mehrabian[1], Toufan Parman[1], Marina Falaleeva[1], Alaric Falcon[1], Marian Glynn[1], Kathleen Meyer[1], Amy Pooler[1], Bryan Zeitler[1]

[1]Sangamo Therapeutics, Inc., Richmond, CA,[2]Evotec SE, Hamburg, Germany

Prion disease is a severe, rapidly progressing and invariably fatal neurodegenerative disorder that affects an estimated 1000 new patients per year in the US and Europe. It is caused by the misfolding of the cellular prion protein, PrP, encoded by the *PRNP* gene. There are currently no approved or clinical-stage disease-modifying therapies for the prevention or treatment of prion disease. We have previously shown that a single-administration epigenetic regulation approach using an AAV.PHP.B-delivered zinc finger repressor (ZFR) targeting the mouse *Prnp* gene can achieve >50% reduction of PrP throughout the mouse brain and cerebrospinal fluid (CSF) and result in an unprecedented survival benefit in an aggressive mouse model of Prion disease. Here, we report the development of human *PRNP*-targeted lead ZFRs, delivered intravenously using an engineered blood-brain barrier penetrant capsid. Using cultured human iPSC-derived neurons, we identified highly potent ZFRs that reduced prion mRNA >90% with no detectable off-target activity. Next, we tested top performing ZFRs for target engagement and tolerability in a transgenic mouse model harboring the full-length human *PRNP* locus. The lead candidate ZFRs were well tolerated during the 8-week study, with no significant neuroinflammatory marker elevation or microscopic findings in the brain. Dose-dependent expression of the ZFR and repression of *PRNP* mRNA expression was observed in micro-dissected brain regions. At the bulk level, ZFR treatment reduced PrP protein by 50-80% in various brain regions and the CSF. Multiplexed single-cell analysis demonstrated potent *PRNP* repression in transduced neurons across the brain, including the cortex, thalamus, pons, and midbrain of the treated mice. A lead ZFR is currently under evaluation in nonhuman primates using an engineered AAV capsid with the potential ability to cross the BBB. These results support the accelerated development of a ZFR-based genomic medicine for the treatment of prion disease.

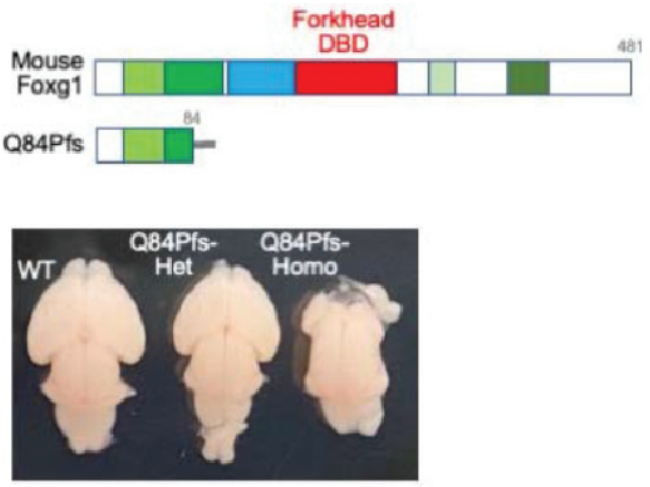
1617. FOXG1 Frame Shift Mutant Mouse Model Revealed Phenotype of FPXG1 Syndrome

Jaein Park[1], Dongjun Shin[2], Shin Jeon[3], Soo-Kyung Lee[1], Jaewon Lee[1]

[1]Biological Science, FOXG1 Research Center / SUNY Buffalo, Getzville, NY,[2]FOXG1 Research Center / SUNY Buffalo, Buffalo, NY,[3]FOXG1 Research Center / University of Pennsylvania, Philadelphia, PA

Single allelic mutations in the gene encoding the forebrain-specific transcription factor FOXG1 lead to FOXG1 syndrome (FS). Patient-specific animal models are needed to understand the etiology of FS, as FS patients show a wide spectrum of symptoms correlated with location and mutation type in the FOXG1 gene. Here we report the first patient specific FS mouse model, Q84Pfs heterozygous (Q84Pfs-

Het) mice, mimicking one of the most predominant single nucleotide variants in FS. Intriguingly, we found that Q84Pfs-Het mice faithfully recapitulate human FS phenotypes at the cellular and brain structural levels. Importantly, Q84Pfs-Het mice exhibited myelination deficits like FS patients. Further, our transcriptome analysis of Q84Pfs-Het cortex revealed a new role for FOXG1 in synapse and oligodendrocyte development. Together, our study revealed the crucial postnatal role of FOXG1 in neuronal maturation and myelination and elucidated the essential pathophysiology mechanisms of FS.



1618. Long-Term Relief of Neuropathic Pain by AAV Expressing Multispecies-Compatible siGCH1

Heesue Chang[1], Minkyung Park[1], Kyoung Jin Lee[2,3], Ji Hyun Kim[2,3], Im Kyung Kang[2,3], Hyochan Park[4], Keerang Park[5], Chan Hee Chon[4], Heuiran Lee[2,3], Ha-Na Woo[3,6], Hyun Ho Jung[1]

[1]Neurosurgery, Yonsei University College of Medicine, Seoul, Korea, Republic of,[2]Microbiology, University of Ulsan College of Medicine, Seoul, Korea, Republic of,[3]Bio-Medical Institute of Technology, University of Ulsan College of Medicine, Seoul, Korea, Republic of,[4]Inventage Lab, Seongnam, Korea, Republic of,[5]Cedmogen Co, Cheongju, Korea, Republic of,[6]Biochemistry & Molecular Biology, University of Ulsan College of Medicine, Seoul, Korea, Republic of

Introduction: BH4 (tetrahydrobiopterin) is involved in the synthesis of various neurotransmitters affecting homeostatic balance. Although tightly regulated under normal conditions, its intracellular concentration is impaired after nerve injury. GTP cyclohydrolase I (GCH1) has been identified as a critical enzyme responsible for regulation of BH4 levels and is dramatically upregulated in the dorsal root ganglion (DRG) in the course of neuropathic pain. Despite advances in the development of new drugs that address the persistent effects of neuropathic pain, traditional analgesics often lacked specificity and failed to target the underlying causes of neuropathic pain, providing only partial relief. This study aims to develop and evaluate an RNA interference (RNAi)-based AAV designed to specifically downregulate GCH1 mRNA from multiple species and subsequently downregulate BH4 levels, ultimately mitigating neuropathic symptoms in a rat model. **Methods & Results**: We first designed u-siGCH1 and selected most effective multispecies-compatible u-siGCH1 through RT-qPCR and western blot. We then constructed AAV expressing u-siGCH1 (AAV-u-shGCH1) and verified its ability downregulating GCH1. Male Sprague-Dawley rats (200g-220g) were used and grouped into normal (NM), spared nerve injury (SNI), AAV-u-shCON, and AAV-u-shGCH1. Spared nerve injury model was applied and the virus was injected on the post-operative day (POD) 16. Behavior tests were conducted before surgery, post-injection day (PID) 3, 7, 14, 21 and 28. Rats were sacrificed on PID 28 and DRG tissues and blood were collected for immunohistochemical analyses and for examining BH4 concentration, respectively. In vitro assessments showed that the selected u-siGCH1 was remarkably effective in downregulating both GCH1 mRNA and protein levels in human (HeLa), monkey (Vero), and rat (C6) cells. In neuropathic pain model, it was found that AAV-u-shGCH1 group showed a gradual increase in mechanical withdrawal threshold until PID 28, which was statistically significant compared to NM, SNI, and AAV-siCON group. Not only u-siGCH1, but also AAV-shGCH1 effectively downregulated BH4 levels in HeLa cells. **Conclusion**: This study demonstrated that this novel therapeutic approach can provide a long-term strategy to alleviate neuropathic pain through a single treatment, and suggested the potential of gene therapy in the field of neuropathic pain.

1619. Transcriptomics and Proteomics Unravel Molecular Safeguards in Klotho Gene Therapy for ALS

Sergi Verdés[1,2,3], Rubén Guerrero-Yagüe[1,2], Núria Gaja-Capdevila[1,4,5], Judith Sauleda[1,2], Javier Del Rey[1,2], Joan Roig-Soriano[1,2,3], Guillem Chillón[1,2,6,7], Marc Leal-Julià[1,2,3], Laura Rodríguez-Estévez[1,2,3], Miguel Chillón[1,2,3,8], Mireia Herrando-Grabulosa[1,4,5], Xavier Navarro[1,4,5], Assumpció Bosch[1,2,3,5]

[1]Institute of Neuroscience, Universitat Autònoma de Barcelona (UAB), Cerdanyola Del Vallès, Spain,[2]Department of Biochemistry and Molecular Biology, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès, Spain,[3]Mixed Unit UAB-VHIR, Vall d'Hebron Research Institute, Barcelona, Spain,[4]Department of Cell Biology, Physiology and Immunology, Universitat Autònoma de Barcelona (UAB), Cerdanyola del Vallès, Spain,[5]Centro de Investigación Biomédica en Red Enfermedades Neurodegenerativas (CiberNed), Instituto de Salud Carlos III, Madrid, Spain,[6]Center for Cellular and Molecular Therapeutics, Children's Hospital of Philadelphia (CHOP), Philadelphia, PA,[7]Department of Pathology and Laboratory Medicine, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA,[8]Institut Català de Recerca i Estudis Avançats (ICREA), Barcelona, Spain

Amyotrophic lateral sclerosis (ALS) gradually weakens and wastes muscles due to the degeneration and loss of motoneurons (MNs). In our previous work, we systemically administered MyoAAV vectors coding for α-Klotho (KL) to SOD1^{G93A} mice. We aimed to enhance KL secretion by skeletal muscles, targeting both MN terminals and muscle endplates. Overexpressing KL in early and symptomatic disease stages preserved neuromuscular function, locomotion, and strength. KL-treated SOD1^{G93A} mice exhibited more surviving spinal and cortical MNs and reduced glial reactivity in the spinal cord. Positive effects correlated with increased neuromuscular junction occupancy, maintained muscle mass, and prolonged survival. Despite

KL being recognized as a pleiotropic protein, its precise protective mechanisms against muscle denervation and MN death in the ALS mouse model remained unclear. To address this, we conducted comprehensive RNA sequencing and proteomic studies in the lumbar spinal cord and gastrocnemius muscles of sKL- and mock-treated animals. Transcriptomic and proteomic profiling of the spinal cord revealed fewer dysregulated genes and proteins in KL-treated $SOD1^{G93A}$ mice compared to mock controls. KL secretion enriched synaptic activity and plasticity, mitigated inflammatory and oxidative responses, and decreased mitochondrial and apoptotic alterations in $SOD1^{G93A}$ mice. In the muscles, KL overexpression had a robust effect, restoring gene expression patterns, particularly increasing the expression of endogenously downregulated proteins in ALS muscles. Predominant corrected pathways were related to muscle contraction and development, as well as the inhibition of fibrosis and atrophy. Klotho upregulated the expression of genes to counteract oxidative stress, calcium buffering proteins, axonal pathfinding factors, and mitochondrial proteins. The inflammatory state induced by ALS was also ameliorated in muscles overexpressing KL. In both tissues, novel roles for KL in promoting ubiquitin-mediated proteolysis and correcting RNA disturbances were discovered. Remarkably, KL induced changes in gene expression patterns only in $SOD1^{G93A}$ animals but not in wild-type-treated mice, emphasizing its specificity in pathological conditions. Overall, functional omics demonstrated the multiple beneficial effects of KL on the altered pathological mechanisms in ALS. Our studies identify new roles for KL and offer insights for future interventions in ALS and other neurodegenerative disorders.

Friday Posters: Ophthalmic and Auditory Diseases

1620. Molecular Mechanism and Gene Enhancement Therapy of Leber Congenital Amaurosis Caused by RPE65 Point Mutations in Patient iPSC Derived RPE Cells

Xiufeng Zhong, Ke Ye, Suai Zhang, Ping Xu, Xiaojing Song, Yuan Wang

State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-sen University, Guangzhou, China

Purpose: RPE65 plays an important role in the isomerization process of vitamin A in the visual cycle. The loss of function caused by its gene mutation is one of the main causes of Leber congenital amaurosis (LCA). With the development of gene sequencing technology, new mutation sites of RPE65 gene have been continuously discovered, but the pathogenic mechanisms remain largely unknown. We identified a LCA patient carrying heterozygous mutations c. 200T>G and c. 430T>C in the RPE65 gene in clinical practice. This study aims to evaluate the mechanism of retinal degeneration caused by these two novel mutations and explore the effect of gene therapy.Method: An in vitro non-RPE visual cycle pathway was constructed by transfecting plasmids for stable and transient expression of wild-type and mutant RPE65 proteins in HEK293T cells. The expression differences of mutant RPE65 were analyzed by qPCR and Western blot (WB), and the mechanism of down-regulation of mutant RPE65 proteins was investigated. LC-MS was used to evaluate the function of wild-type and mutant RPE65 proteins. In addition, patient-specific hiPSCs were obtained using reprogramming method, and retinal pigment epithelial (iRPE) cells with this heterozygous mutation were obtained by 2D differentiation. The differences between patient-specific iRPE cells and normal iRPE cells/RPE65 knockout (KO) iRPE cells were analyzed by light microscopy, electron microscopy, immunostaining, and WB. RNA-seq was performed to analyze its potential pathogenic mechanism at the transcriptional level. Finally, the therapeutic effect of AAV-mediated gene augmentation using different promoters in patient-specific iRPE cells was evaluated.Results: In the non-RPE system, we detected that the two point mutations reduced protein expression without affecting RPE65 transcription levels by accelerating RPE65 protein degradation under the autophagosome-lysosome pathway. The isomerization activity was abolished because of the mutations. Using a 2D differentiation system, we obtained a large number of normal iRPE, RPE65 KO iRPE and patient-specific iRPE. All three types of iRPE cells exhibited polygonal cell morphology and expressed specific proteins of RPE. However, immunofluorescence and WB analysis showed that this heterozygous mutation significantly reduced RPE65 protein level. RNA-seq analysis showed that mutations of RPE65 did not affect the expression of other genes related to the visual cycle. Additionally, analysis of transcriptomic data suggesting that the reduction of RPE65 protein levels caused by this heterozygous mutation may be related to the function of autophagosome-lysosome pathway. Finally, we designed an AAV vector with a broad-spectrum promoter and an AAV vector that specifically expresses in RPE cells. Both viruses can increase RPE65 protein levels in patient-specific iRPE, but the former is more efficient.Conclusion: The heterozygous mutations c. 200T>G and c. 430T>C in the RPE65 gene affect the stability of the RPE65 protein, leading to abnormalities in the visual cycle pathway and further causing retinal degeneration. This process may be related to the activation of the autophagosome-lysosome pathway. In addition, this study preliminarily evaluated the effect of AAV vectors with different promoters on RPE65 expression, providing a basis for the design of gene therapy vectors.

1621. HUB101 Gene Therapy for Blindness Caused by Pediatric Lebers Congenital Amaurosis (LCA16)

Pawan K. Shahi[1], Meha Kabra[1], Allison Spillane[1], Yu Chang[1], Divya Sinha[2], David M. Gamm[2], Susan Macdonald[3], Jeff Sabados[3], Bikash R. Pattnaik[1]

[1]Pediatrics, University of Wisconsin-Madison, Madison, WI,[2]Waisman Center, University of Wisconsin-Madison, Madison, WI,[3]Hubble Therapeutics, Boston, MA

Inherited retinal disorders (IRD) in children cause progressive loss of vision due to mutations in genes crucial for retinal function. Channelopathies represent a broad category of IRD due to the mutations in genes encoding ion channels that are required for visual information decoding and cellular communication. Understanding the molecular mechanisms of ion channel dysfunction is critical for targeted therapies leading to functional restoration. The retinal pigment epithelium (RPE) is a single layer of cells between the choroid and light-sensitive photoreceptors. These cells express the Kir7.1 ion

channel encoded by the *KCNJ13* gene. Mutations in the *KCNJ13* gene lead to a form of Leber congenital amaurosis (LCA16) and snowflake vitreoretinal degeneration (SVD). In this study, we focused on LCA16 caused by nonsense mutations in the *KCNJ13* gene. We used a preclinical AAV5 to deliver a VMD2 promoter-driven healthy copy of human *KCNJ13* open reading frame (here referenced as HUB-101, from Hubble therapeutics) to RPE cells. HUB-101 transduction of LCA16 patient-derived iPSC-RPE cells resulted in the expression of the Kir7.1 channel and restoration of channel function as measured by single-cell patch-clamp experiments. In our conditional knock-out mouse model lacking the Kir7.1 protein in RPE cells, we delivered HUB-101 by subretinal injection. It not only expressed the protein, but it also enhanced the c-wave amplitude in electroretinography (ERG). The c-wave represents light-evoked RPE cell response attesting to functional restoration in vivo. With the recent approvals of gene transfer therapy for clinical trials for other diseases, HUB-101 is designed for treating LCA16 blindness. Beyond treating visual impairment, HUB-101 gene therapy holds promise for genetic ion channelopathies such as neurological diseases.

1622. AXV-101, a New Codon-Optimised *BBS1* AAV9 Vector Halts Photoreceptor and Outer Nuclear Retinal Layer Organisation Degeneration in a Dose-Dependent Manner

Oriane Guillermin[1], James Grey[1], Gabriela Carreno[1], Nertiyan Elangkovan[1], Nicole Jeffrey[1], Chiara Sidoli[1], Andres Crespo[1], Emma Mustafa[1], Phil Beales[1,2], Elisa Cuevas[1], Victor Hernandez[1,3]

[1]Axovia Therapeutics Ltd, London, United Kingdom,[2]Ggm, GOSICH UCL, London, United Kingdom,[3]Brunel University London, London, United Kingdom

Bardet-Biedl syndrome (BBS) is an autosomal recessive disorder associated with primary cilia dysfunction, presenting with retinal dystrophy and progressive visual loss, amongst other clinical features. *BBS1* is the most common mutated gene found in BBS, with the missense *BBS1* M390R mutation being the most common allele. We have previously demonstrated that different AAV8 vectors, expressing the wild-type BBS1 cDNA under the regulation Rhodopsin Kinase (AAV8-RK-BBS1) and Cytomegalovirus (AAV8-CMV-BBS1) promoters, are safe and able to halt the retinal degeneration in the *Bbs1*$^{M390R/M390R}$ (M390R) mouse. In this study we have improved the efficacy and safety profiles with AXV-101, an AAV9 capsid vector expressing a human codon-optimised BBS1 cDNA sequence (hCOBBS1) under the regulation of the CAG promoter (AAV9-CAG-hCOBB1). To test safety, efficacy, and potency of AXV-101, 1.5µl of three doses of vector were administered subretinally and unilaterally in one bleb at p7-9: low dose ($6.67x10^{11}$vg/ml), medium dose ($3.33x10^{12}$vg/ml) and high dose ($6.67x10^{12}$vg/ml). M390R and Wild-type (WT) groups were dosed, including untreated (contralateral eyes) and subretinal injected retinae with phosphate-buffered saline (PBS) and AXV-101 formulation buffer. Functional and structural readouts were captured at 2.5-, 4- and 6-months of age and included photopic and scotopic electroretinogram (ERG) and retinal Optical Coherence Tomography (OCT). At 6 months the degenerated retina in M390R untreated mice are too thin (ONL) to be accurately measured by automated OCT image segmentation. The ONL thickness of WT retinae treated with either low, medium or high doses maintain a normal structure with a stable ONL thickness at 6 months. At 2.5 months, M390R treated eyes display a dose response in ONL thickness, where PBS and buffer averages are 18 mm and 23 mm respectively, low dose at 25 mm, medium at 28 mm and high dose at 31mm. The dose response continues at 6 months for the medium and high dose while the ONL is missing for the PBS, buffer and low dose treated eyes. At 6 months medium and high doses treated M390R retinae have a 99.3% and 98% chance of retaining the ONL. Overall, there is rescue in ONL thickness from an early timepoint in M390R treated eyes with sustained efficacy up to at least 6 months. These results were corroborated with the histological analysis at 6-month after treatment. Buffer-treated eyes displayed an ONL throughout the entire retina about 2 nuclei thick, However, four out of five M390R eyes treated with medium dose exhibit a rescue patch where the ONL is visibly thicker with a thickness between 9 and 10 nuclei observed, compared with the 11 to 12 nuclei of WT controls. ERG responses show a high variability. When comparing trends of M390R treated with AXV-101 with buffer treated eyes, some retinal function rescue is observable. Notably, rod photoreceptor activity measured by scotopic a and b wave amplitude, is significantly improved in both medium and high dose treatment groups when compared to buffer treated at 4 months. At 6 months, the rescue is maintained for medium and high dose treated eyes for scotopic b wave measurements. We also observed a dose response correlation observed at 6-months when assessing hCOBBS1 transgene expression with low, medium to high dose treatments, correlating with the histological and functional rescue observed. In summary, these results indicate that AXV-101 can halt functional and structural retinal degeneration in the M390R (Bbs1) mutant retinae in a dose dependent manner, with the medium dose ($3.33x10^{12}$vg/ml) the minimum and sufficient dose to sustain photoreceptor survival without any safety concerns.

1623. Development of a Novel Recombinant AAV Gene Therapy rAAV-AIPL1 for Leber's Congenital Amaurosis 4 (LCA4) Disease

Zhuoyu Ni, Fangting Han, Fei Wu, Zicheng Song, Lei Zhang, Shan Fang, Anni Ou, Zeyu Liu, Lei Du, Yitong Hou, Xingxin Chen, Fenghua Wang

Innostellar Biotherapeutics, Shanghai, China

Leber's congenital amaurosis (LCA) is a serious early-onset congenital blinding retinopathy, accounting for more than 5% of hereditary retinal diseases. Affected individuals are diagnosed at birth or in the first few months of life with nystagmus, severely impaired vision or blindness. To date, mutations in about 20 different genes cause LCA. Among which, LCA4 is caused mainly by mutations of acryl hydrocarbon receptor interacting protein like-1 (AIPL1) gene. AIPL1 gene is expressed in photoreceptors and the pineal gland. In the retina, AIPL1 acts as a specialized chaperone of HSP90 and cyclic nucleotide phosphodiesterase PDE6 complex, which is an essential enzyme effector in the phototransduction pathway. Mutations in AIPL1 gene disrupt AIPL1 interaction with HSP90 and prevent assembly of the PDE6 holoenzyme. Reduced PDE6 function leads to intracellular cGMP elevation and prolonged opening of the cyclic nucleotide gated channels and excessive Ca2+ influx, and photoreceptor degeneration and vision deterioration follows. Currently, there is no approved

treatment for LCA4. We developed a novel recombinant adeno-associated virus AAV (rAAV) based gene therapeutics. Capsids from our Capstellar platform can effectively transduce retina cells and deliver human AIPL1 gene driven by a photoreceptor-specific promoter. rAAV-hAIPL1 can be correctly expressed in rodent model and human iPSC-derived retinal organoid (RO) models. The AIPL1 knockout ROs show aberrantly high cGMP level in the photoreceptors due to reduced PDE6 function, while rAAV-hAIPL1 treated RO exhibits relatively normal cGMP concentration, indicating a recovery of PDE6 holoenzyme function. In the aipl1-/- mouse model at the age of 3 weeks, its retina is severely atrophied with photoreceptor cell layer nearly disappeared. When rAAV-hAIPL1 is administered in the subretinal space of aipl1-/- mice, hAIPL1 protein expression is recovered along with significant retention of photoreceptor cell layer and related biomarker expression. In summary, we developed a novel rAAV-hAIPL1 gene therapeutic modality. It can specifically express human AIPL1 protein in photoreceptors. The functional proof-of-concept was also demonstrated in mouse models and human retinal organoid disease models that rAAV-hAIPL1 treatment recover correct hAIPL1 expression and its interaction with its normal chaperon protein HSP90 and recoup PDE6 holoenzyme function, hence, delaying photoreceptor cell degeneration and vision loss. Further studies are underway to support future clinical development.

1624. Nonclinical Evaluation of CTx001, a Gene Therapy for the Treatment of Geographic Atrophy in Age Related Macular Degeneration

Mustafa M. Munye[1], Athanasios Didangelos[1], Sofiya Pisarenka[1], Suhnrita Chaudhuri[1], Rachel Green[1], Peter Emery-Billcliff[1], Nakul Patel[1], Pamela Whalley[1], Parisa Zamiri[1], Simon J. Clark[2], Paul N. Bishop[3], Dennis Keefe[4], Rafiq Hasan[1]

[1]Complement Therapeutics, London, United Kingdom,[2]Eberhard Karls Universitat Tubingen, Tubingen, Germany,[3]The University of Manchester, Manchester, United Kingdom,[4]Complement Therapeutics, Bedford, MA

Introduction: Geographic atrophy (GA) is a leading cause of blindness in the US and Europe and strongly associated with genes that regulate the alternative complement pathway. Poor regulation of the C3 amplification loop results in generation of the potent opsonin C3b leading to inflammation, membrane attack complex (MAC) formation, RPE/photoreceptor cell death and vision loss. Two complement pathway inhibitors have recently been approved by the FDA having slowed disease progression in patients, validating the pathway as a key driver of disease. However, the slowing of disease progression has been modest and to date has not translated to vision preservation. Both treatments also require frequent intravitreal injections impacting adherence. **Results**: CTx001 is an AAV gene therapy encoding a novel soluble and potent complement regulatory protein, mini-CR1, for the treatment of GA. Mini-CR1 is derived from the membrane bound complement receptor 1 (CR1) protein. We show that mini-CR1 supports potent suppression of the C3 amplification loop acting as a co-factor to the Factor I mediated cleavage of C3b with an IC50 of 125nM in human serum. We demonstrate that in contrast to other co-factors being evaluated for the treatment of GA, mini-CR1 supports proteolysis of C3b to iC3b and then to the terminal C3dg fragment thereby reducing levels of the potent C3b and iC3b opsonins. There is growing appreciation of the need for GA treatments to address complement overactivation in both retinal and choroidal compartments, separated by the selective Bruch's membrane. Mini-CR1 successfully crossed enriched Bruch's membrane derived from human donor eyes *ex vivo* and maintained co-factor activity in contrast to larger complement regulators. CTx001 administered by subretinal injection in *in vivo* rodent laser CNV models of disease demonstrated a dose dependent reduction in MAC formation and a reduction in C3b levels in the eyes of treated animals. No adverse findings were reported either systemically or in the eye following subretinal CTx001 delivery. CTx001 was well tolerated in a non-GLP non-human primate (NHP) study at doses up to and inclusive of the maximal feasible dose. A dose response was observed in the levels of mini-CR1 expressed in the vitreous of NHP along with evidence of target engagement with increasing levels of iC3b. All animals progressed to scheduled euthanasia with no gross lesions apparent upon necropsy. There were no test item related clinical observations reported throughout the study. No remarkable findings linked to test item were seen in clinical chemistry, hematology or coagulation parameters. Mild inflammation secondary to the dosing procedure and test item was observed in a dose dependent manner. All ophthalmic findings such as pigment variation, anterior chamber and vitreous cells and retinal hemorrhage were reported as very slight to slight. There were no signs of vasculitis in any dose groups. **Conclusions:** CTx001 is an AAV gene therapy encoding a potent complement regulator that shows promise as a potential treatment for GA. Mini-CR1 effectively suppresses the C3 amplification loop, reducing levels of the opsonins C3b and iC3b. Its ability to traverse Bruch's membrane enables it to address complement overactivation in both retinal and choroidal compartments. *In vivo,* CTx001 produced a significant reduction in MAC formation and C3b levels in rodents and was well tolerated in NHP's. These results support initiation of IND enabling GLP toxicology studies for CTx001.

1625. Preclinical Studies of HG004 Gene Replacement Therapy in Support of Clinical Trial for *RPE65*-Associated Inherited Retinal Dystrophy

Weiwei Wu, Sheng He, Qi Yang, Chenhua Yang, Mengke Zhu, Shaoran Wang, Xuan Yao, Hui Yang, Alvin Luk, Linyu Shi

HuidaGene Therapeutics Co., Ltd., Shanghai, China

Background: Mutations in the *RPE65* gene disrupt the visual cycle, leading to a congenital deficiency in supplying chromophores to rod and cone photoreceptors and resulting in *RPE65*-associated inherited retinal dystrophy (*RPE65*-IRD). Our team has developed an AAV9 gene replacement therapy, HG004, carrying the human *RPE65* gene, and conducted preclinical and IND-enabling studies to support the clinical application of HG004 to treat patients with *RPE65*-IRD. **Methods:** *Rpe65*-deficient mice aged 1 month underwent subretinal injections of either HG004 or a vehicle control. Efficacy was assessed through optical coherence tomography, electroretinography, and histochemical staining. Next, a good laboratory practice (GLP) toxicology study of bilateral injection at three different doses of HG004 was conducted in non-human primates (NHPs), with clinical observation, ophthalmic examinations, vector shedding/biodistribution, and immunogenicity assessments performed at various time points. **Results:** HG004

exhibited a significant amelioration in scotopic b-wave responses across all treatment cohorts compared to the vehicle-injected control group of *Rpe65*$^{-/-}$ mice ($p<0.01$). The rate of restoration surpassed 60% of the wild-type (WT) and was maintained for 34 weeks. The retinal thickness in HG004-treated eyes of 1-month-old *Rpe65*$^{-/-}$ mice resulted in 85% of the WT level while the untreated eyes represented 67% of the WT level at 35 weeks post-injection. In the GLP NHP study, the localization of HG004 predominantly manifested within the retinal tissues, especially in the retina and choroid. No discernible presence in the reproductive system and no manifestations of drug-induced systemic toxicity were observed in NHPs at both the 4- and 13-week post-injection period. Adverse reactions (such as retinal injury at the injection site, vitreous inflammatory cell exudation, and others) were primarily observed within the retinas of all tested NHP groups, suggesting that these non-drug-related events were primarily related to the injection procedure (Figure 1). Immunotoxicity evaluations further demonstrated the absence of drug-induced abnormalities in the whole blood samples of NHPs, and the lack of immune response from AAV or RPE65 antigen-specific T cells in NHPs was also observed. **Conclusions:** Our results across all mouse studies demonstrated dose-responsive restoration of retinal function after the treatment of HG004 with a durable effect in mice for at least 34 weeks after the treatment of HG004. Furthermore, HG004 is safe for use in NHPs. The nonclinical studies supported the evaluation of HG004 in patients with *RPE65*-IRD. The IND has been cleared by the FDA and China CDE, and the Phase I/II clinical trial (NCT05906953) is currently being evaluated in humans.

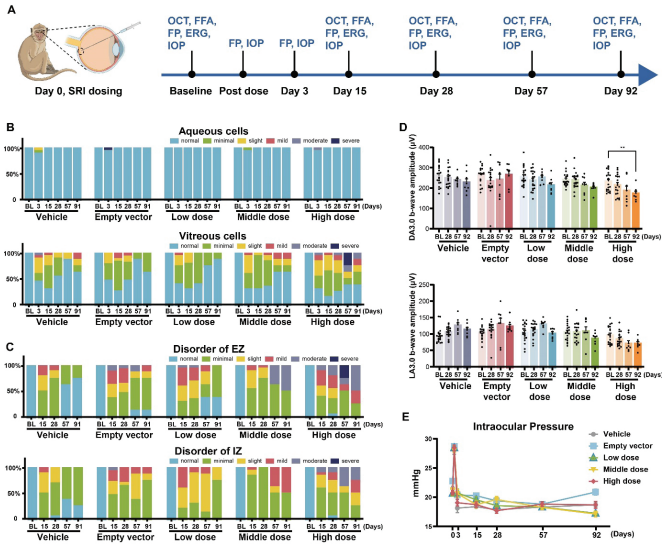


Figure1. Ophthalmological examination and intraocular pressure measurement (IOP)

1626. Preclinical and Phase I Clinical Trial Update of the Safety and Efficacy of SKG0106, a Novel AAV Gene Therapy Product for the Treatment of Neovascular Age-Related Macular Degeneration

Byron Li[1], Yanqun Shu[1], Qiongqiong Ren[1], Peilu Li[1], Huimin Kong[1], Lumei Xiao[1], Bing Wu[1], Sijia Huang[1], Jinxin Deng[1], Zheng Luo[1], Dianyang Chen[1], Youxin Chen[2], Chan Wu[2], Ruoan Han[2], Jay Hou[1]

[1]Skyline Therapeutics, Cambridge, MA,[2]Department of Ophthalmology, Peking Union Medical College Hospital, Beijing, China

SKG0106, which comprises a proprietary capsid and a novel transgene genome encoding an anti-vascular endothelial growth factor (VEGF) protein (Vb24), is a novel recombinant adeno-associated virus (AAV) vector for the treatment of neovascular age-related macular degeneration (nAMD) via intravitreal (IVT) delivery. Upon delivery, the vector transduced retinal cells resulting in effective expression of Vb24, which potently suppressed the action of VEGF in two pharmacologically relevant animal models: a chronic rabbit retinal neovascularization (RNV) model and a monkey choroidal neovascularization (CNV) model. In the rabbit dose range finding (DRF) study, SKG0106 in the dose range of 1E9 to 3E10 vg/eye showed comparable efficacy profiles as Eylea® and lasted until the end of the study (6 weeks) while Eylea® already lost its activity. In the monkey DRF study, SKG0106 showed a dose-dependent effect in reducing CNV grade 4 spot rate, mean spot fluorescein leakage and the thickness of subretinal hyperreflective material (SHRM). The efficacy of SKG0106 at 8E9, 4E10, 2E11 and 1E12 vg/eye was comparable to that of Eylea®, and all dosages were well-tolerated. In a prophylactic long-term study with monkey CNV model, SKG0106 (6E10 vg/eye) exhibited comparable efficacy to that of Eylea® at 6 and 13 weeks following IVT injection. In addition, SKG0106 demonstrated a lasting strong efficacy at the end of 26 weeks. In contrast, Eylea® did not show the inhibitory effect on the lesions. The thickness of SHRM in SKG0106 treated group was significantly thinner than that in both vehicle and Eylea® treated groups. Most importantly, there were no local or systemic adverse events detected in all pharmacological studies and a GLP toxicology study in monkeys. Following the investigational new drug (IND) clearance from both the US FDA and China NMPA, a multicenter, open-label, Phase I trial was conducted in patients with nAMD to evaluate the safety, preliminary efficacy, immunogenicity, and pharmacokinetics characteristics of SKG0106. The study included 3 dose cohorts with 3 patients each at 8E9, 2.4E10 and 7.2E10 vg/eye. Ocular safety, tolerability, best corrected visual acuity (BCVA), retinal central subfield thickness (CST), serum anti-AAV neutralizing antibody titer, and the Vb24 protein level in aqueous humor (AH) were evaluated at baseline, Day-1, Week-2, Week-3, Week-4, and once every 4 weeks after Week-4 up to 48 weeks post dosing. SKG0106 was well-tolerated by all subjects. No dose-limiting toxicities and treatment related serious adverse events were detected up to 30-52 weeks. Asymptomatic trace vitreous cells were observed in a single episode of follow-up 12-16 weeks in patients treated with 7.2E10 vg/eye, which resolved without intervention. More clinical results on BCVA, CST, and Vb24 in AH will be presented at the ASGCT meeting.

1627. Preclinical Evaluation of NG101 for Treatment of Wet Age-Related Macular Degeneration

Juwon Shim, Jeongyun Bak, Youyoung Kim, Hoon Young Kong, Joo Seok Han

Neuracle Genetics Inc., Seoul, Korea, Republic of

Age-related macular degeneration (AMD) is the most common cause of vision loss in individuals over the age of 55, accounting for 6-9% of legal blindness worldwide. Ten to fifteen percent of AMD patients develop choroidal neovascularization (CNV), resulting in wet AMD (wAMD), which accounts for almost 90% of blindness associated with AMD. We developed a novel small promoter, CAT311, derived from the ubiquitous CAG promoter and utilized it in NG101 (AAV8.CAT311AflK), a recombinant adeno-associated virus 8 (rAAV8) encoding Aflibercept, an anti-vascular endothelial growth factor (VEGF) agent, for the treatment of wAMD. To compare transgene expression by the CAG and CAT311 promoters, mice were administered AAV8.CAGGFP or AAV8.CAT311GFP via a single subretinal injection. GFP expression was significantly higher in the retina following the administration of AAV8.CAT311GFP compared to AAV8.CAGGFP throughout 51 weeks. In the laser-induced CNV mouse model, NG101, administered at doses ranging from $1x10^6$ to $1x10^9$ vg/eye, demonstrated a dose-dependent reduction in CNV lesion leakage and size. To evaluate the transgene expression of NG101 in non-human primates (NHPs), cynomolgus monkeys were administered NG101 via a single subretinal injection with doses ranging from $3x10^{10}$ to $1x10^{12}$ vg/eye. The expression of Aflibercept in ocular tissues on day 57 was significantly higher at all doses than the minimum required amount for the treatment of wAMD. Considering the excellent aflibercept expression ability of NG101, we assessed the efficacy of NG101 at doses of $3x10^9$ and $6x10^9$ vg/eye in the NHP CNV model and demonstrated that NG101 effectively inhibited the growth and leakage of new vessels, to the same extent as intravitreal injection of Eylea (2mg). Collectively, these data suggest that NG101, with its efficacy at a low dose, is a promising candidate for wAMD treatment, potentially minimizing toxicities associated with AAV gene therapy.

1628. An Optimized System for AAV Compatible Delivery of Full Length PCDH15 for Treatment of USH1F via RNA End Joining

Claire E. Williams[1], Ryan H. Hsu[2], Kip J. Hermann[1], Lukas C. Bachmann[3], Samuel L. Pfaff[1]

[1]The Salk Institute for Biological Studies, San Diego, CA,[2]The Salk Institute & UC San Diego, La Jolla, CA,[3]Insmed Inc., San Diego, CA

Usher Syndrome 1F (USH1F) is a debilitating disease where patients experience congenital, bilateral, profound sensorineural hearing loss, vestibular areflexia, and adolescent-onset retinitis pigmentosa (RP). USH1F is caused by mutations in one gene, protocadherin-15 (PCDH15) making it an ideal candidate for gene replacement or gene editing strategies for treatment. PCDH15 is a cell-membrane binding protein that is primarily present in stereocilia and the retina and its coding sequence is approximately 5.8 kb, which exceeds the maximum packaging capacity of AAVs. To overcome the size limitations of AAVs for delivery of PCDH15 in a gene replacement strategy, we utilized our novel REJ technology and created five different constructs to optimize the expression of full-length PCDH15 (see abstract: Optimization of RNA End Joining (REJ) to safely and efficiently express large proteins, Bachmann et al.). REJ is a highly efficient mRNA trans-splicing process, which can be used in the delivery of multiple DNA constructs, which post transcription will have the separate RNAs scarlessly recombine in the nucleus, resulting in a full-length mRNA of the desired protein (Figure 1. A). The REJ variants were transfected into HEK293T cells and the expression of the 250kDa PCDH15 dimer was validated by Western blot (Figure 1. B). All screened variants exhibited full-length PCDH15 at varying levels with minimal fragment expression, and an optimal design was found. Proper cell membrane colocalization was confirmed by immunohistochemical visualization (Figure 1. C). Ongoing efforts include functional assays to confirm binding between PCDH15 and its ligand CDH23 (Cadherin-23) and producing AAV for in vivo validation. These findings define an optimized gene replacement system for treating USH1F.

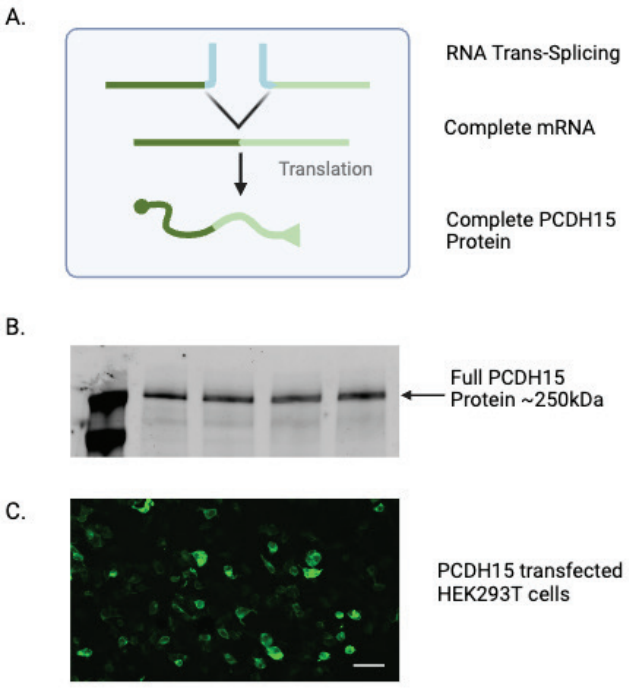


Figure 1. (A) Schematic of REJ-enabled PCDH15 delivery. Binding domains are in blue and the split coding sequence is in green. (B) Western Blot depicting PCDH15 successfully reconstituted in HEK293T cells after transfection with two REJ constructs. (C) Immunohistochemical staining of HEK293T cells after transfection demonstrating cell membrane localization. PCDH15 is visualized in green.

1629. Complehensive Evaluation of Safety and Efficacy of Intravitreal Administration of Three AAV2 Vectors Expressing a Novel Anti-VEGF Molecule in a Nonhuman Primate Model of Choroidal Neovascularization (CNV)

Ethan Li

InnoStellar Biotherapeutic Co., Pudong, China

Inhibition of vascular endothelial growth factor (VEGF) family by intravitreal injections of anti-VEGF molecule(S) for the management of the ocular diseases with pathological neovascularization is a proven treatment. Adeno-associated virus (AAV)-mediated gene therapy has great potential for optimal long-term even life-term treatment of single intravitreal injection (sIVI). In the present study, we investigate efficacy and safety of three intravitreally administered AAV2 vectors expressing a novel anti-VEGF molecule at medium or low doses in a nonhuman primate (NHP) model of choroidal neovascularization (CNV). Persistent expression of anti-VEGF molecule was observed by collecting samples of aqueous humor at different time points from 1 month to 6 months. Laser-induced CNV was inhibited as determined by several ocular tests including imaging techniques of optical coherence tomography (OCT), fundus photos and fluorescein angiography (FA). The level of VEGF in samples of aqueous humor were reduced. Positive expression level of anti-VEGF molecule in different ocular compartments (including retina, choroid, iris/ciliary body, vitreous and aqueous humor) was detected. The biodistrubution of AAV vector DNA in the 16 body tissues as well as in 9 ocular compartments was screened. No immune respones to anti-VEGF molecule were found in ADA assay or IFN-gamma ELISpot assay. Our data suggest that single intravitreal injection of three AAV2 vectors expressing a novel anti-VEGF molecule is safe and may be effective long-term treatment for diseases with ocular neovascularization. The novel anti-VEGF molecule and one vector will be selected for further investigation.

1630. Development of an AAV-Based Gene Therapy for the Ocular Phenotype of Friedreich's Ataxia

Heyu Tang[1], Emily M. Xu[1], Siddhant S. Gupte[1], Kaitlyn R. Calabro[1], Diego S. Fajardo[1], James J. Peterson[1], Hannah L. Friend[1], Sanford L. Boye[2], Shannon E. Boye[3]

[1]Division of Cellular and Molecular Therapy, Department of Pediatrics, University of Florida, Gainesville, FL,[2]Powell Gene Therapy Center, University of Florida, Gainesville, FL,[3]University of Florida, Gainesville, FL

Purpose: Friedreich's Ataxia (FA) is an autosomal recessive neurodegenerative disorder caused by mutations in the *FXN* gene that affects multiple systems. Approximately 30% of FA patients develop ophthalmic symptoms by their second decade of life, which may include nystagmus, saccadic dysmetria, optic nerve atrophy, coupled with the loss of retinal ganglion cells (RGCs) and retinal nerve fiber layer (RNFL) thickness. This study aims to develop and characterize a novel mouse model with *FXN* knocked out specifically in RGCs and to develop an AAV-FXN gene therapy approach that will be evaluated in the model. **Approach:** *Pou4f2*^{Cre} mice (*Pou4f2* promoter driving Cre-recombinase), were bred with *Fxn*$^{+/-}$ mice to generate *Fxn*$^{+/-}$; *Pou4f2*$^{Cre+/-}$ mice. The double heterozygous mice were crossed to *flFxn+/flFxn+* mice to generate *Pou4f2*$^{Cre+/-}$;*flFxn+/Fxn-* (Pou4f2-FXN KO) and *Pou4f2*$^{Cre-/-}$;*flFxn+/Fxn-* (Pou4f2-FXN WT) mice. In-life assessments occurred over 6 months to measure thickness of the RNFL, ganglion cell layer + inner plexiform layer (RGC+IPL), inner nuclear layer (INL), and outer nuclear layer (ONL) via optical coherence tomography (OCT). Full field and pattern ERG (ffERG, pERG) were performed to evaluate retinal function. ffERG measures the response from photoreceptors and bipolar cells, whereas pERG measures specific signals from the RGCs. Pou4f2-FXN WT mice were used as controls. FXN expression will be evaluated by immunohistochemistry (IHC), and immunoblotting. The AAV-P2-V1(Y-F+T-V) capsid, a novel AAV2-based capsid variant developed by directed evolution and rational design, was used to package a construct containing the neuronal specific *Syn1* promoter and FXN-HA (FXN coding sequence plus HA tag) and WPRE element (AAV-FXN). AAV-hSyn1-GFP (AAV-GFP) served as the control. Vectors were intravitreally injected into Pou4f2-FXN KO mice, with one eye receiving AAV-FXN vector and the contralateral eye receiving the AAV-GFP control. Retinal structure and function were/will be assessed by the in-life and post-mortem methods mentioned above. **Results:** Pou4f2-FXN KO mice survived the duration of the study without any health issues and exhibited no ataxia or other symptoms of systematic FXN deficiency. OCT revealed a significant, and progressive decrease in RNFL and GCL+IPL thickness starting at postnatal day 30 (P30). Thinning of the INL was also observed at later time points. pERG data showed a significant difference in P1 waveform amplitudes between the KO and WT as early as P30. By P90, both P1 and N2 waveform amplitudes were significantly reduced in the Pou4f2-FXN KO mice relative to controls. Mice treated with AAV-FXN vector at P30 revealed significant protection of GCL+IPL and INL layers. Significant preservation of pERG was observed by 3 months post-injection. **Conclusions:** We have successfully generated and characterized an RGC-only FXN KO mouse model exhibiting significant and progressive retinal dystrophy. These preliminary data suggest this novel mouse model mimics features of the ocular phenotype observed in FA patients. AAV-medicated FXN expression significantly preserved retinal structure and function in Pou4f2-FXN KO mice. This study has established proof-of-concept for AAV-FXN gene supplementation for the treatment of ocular FA. Future experiments will focus on improvements in vector design and longer-term evaluations of AAV-FXN in the Pou4f2-FXN KO mice.

1631. Delivery to the Suprachoroidal Space of an AAV Vector Encoding a Complement Pathway Inhibitor as a Possible Treatment for Dry Age-Related Macular Degeneration

Mi Shi, April R. Giles, Wei-Hua Lee, Chris Tully, Levi Winter, Ryan Kruse, Jessica Gumerson, Kwi Hye Kim, Ye Liu, Olivier F. Danos, Brendan Lilley

REGENXBIO Inc., Rockville, MD

Dysregulation of the complement pathway plays an important role in the pathophysiology of the dry form of age-related macular degeneration (Dry AMD). Currently approved treatments require frequent injections of complement pathway inhibitors. We examined whether a single ocular delivery of an adeno-associated virus (AAV) vector encoding a complement pathway inhibitor can achieve localized,

durable expression in an effort to identify a potential gene therapy approach for treating Dry AMD. Multiple formats of complement C5 inhibitors were designed for expression from AAV vectors. The affinities toward C5 from human, cynomolgus macaque (NHP) and mouse were examined with a Bio-layer Interferometry assay (BLI), and all inhibitors tested showed sub-nanomolar affinities to their cognate C5 target. Potent inhibitor bioactivity was demonstrated through inhibition of complement activation in hemolysis assays. Additionally, human iPSC-derived RPE cells, when transduced with an AAV vector expressing a C5 inhibitor, displayed a strong inhibition of membrane attack complex (MAC) formation, indicating anti-C5 bioactivity of the secreted transgene product. We further examined ocular expression in mice following subretinal injections of vectors encoding the different inhibitors, and identified a format optimized for ocular biodistribution. We then tested this vector for expression and tolerability in NHP following either subretinal or suprachoroidal injections. The vector was well tolerated as assessed with *in vivo* imaging and ocular tissue histopathology. The bioactivity of *in vivo* expressed C5 inhibitors present in NHP vitreous humor was demonstrated through hemolysis assay. The results here support further preclinical development of a minimally invasive approach to the treatment of Dry AMD that has the potential to reduce the treatment burden and deliver therapeutic molecules directly into the site of pathogenesis.

1632. Beyond AAV, Gene Editing of HSV in Patients with Herpetic Stromal Keratitis

Yujia Cai, Sikai Ling, Shuhui Zhang

Shanghai Center for Systems Biomedicine, Shanghai Jiao Tong University, Shanghai, China

Numerous preclinical studies have shown in vivo CRISPR therapy is an attractive strategy to treat ocular diseases. However, clinical evidences for gene editing in the eyes are still rare. We showed safety and efficacy of applying CRISPR both in preclinical and clinical studies to treat herpes stromal keratitis (HSK). In our study, we used virus-like particle (VLP) to delivery CRISPR/Cas9 in the form of mRNA. In an investigator-initiated clinical study of treating refractory HSK cases with acute corneal perforation, we found neither detectable CRISPR-induced off-target cleavages by GUIDE-seq nor systemic adverse events for 18 months on average in all three patients. The HSV-1 remained undetectable during the study. The same drug has also been under evaluation in a phase I trial. The early results showed that the intrastromal injection of the VLP-delivered CRISPR was well tolerated without seeing major adverse effects. Building on the safety and efficacy from HSK, we also extended VLP-delivered CRISPR for treating glaucoma by targeting mutant gene in the trabecular meshwork (TM), which mediated efficient gene editing in non-human primates. Overall, our studies suggested in vivo CRISPR delivery with VLP holds great potential in treating ocular and infectious diseases.

1633. CRISPR/Cas9-Mediated Gene Editing for Herpes Simplex Virus Reduces Viral Reactivation in a Latent Rabbit Keratitis Model

Nadia Amrani[1], Kevin Luk[1], Pankaj Singh[2], Mason Shipley[2], Meltem Isik[1], Martina Donadoni[3], Anna Bellizzi[3], Ilker K. Sariyer[3], Danna Neumann[2], Jennifer Gordon[1], Guoxiang Ruan[1]

[1]Excision BioTherapeutics, Watertown, MA,[2]University of Wisconsin-Madison, Madison, WI,[3]Temple University, Philadelphia, PA

Herpes simplex keratitis (HSK), caused by HSV-1 infection of the cornea, is a major cause of blindness. In the United States, approximately half million people are afflicted with HSK. HSV-1 is a neurotrophic virus that first enters the axons of sensory neurons, migrates to the trigeminal ganglia (TG) and other ganglia, and establishes lifelong latent infection. Recurrent reactivation of HSV-1 and the infection of the cornea in HSK cause corneal opacity, edema, corneal scarring, and neovascularization, that can lead to irreversible vision impairment and blindness. Currently, there is no vaccine for HSV-1 and treatment with nucleoside analogs, which only inhibit virus replication, cannot cure the infection. Recent studies have shown that genome editing tools can target the HSV-1 and reduce the virus shedding. However, this approach has not been optimized or extensively tested in latent HSV-1 animal models. Therefore, an efficient and safe treatment for HSV-1 keratitis that prevents HSV-1 reactivation from viral latent reservoirs is highly needed.In this study, we utilized CRISPR/SaCas9 to target two critical genes in HSV-1, namely ICP0 and ICP27. The Dual Cut method enables the induction of large deletions in the viral genome, effectively preventing viral escape after DNA repair. Transient transfection of Vero cells with plasmids expressing SaCas9 and paired gRNAs resulted in a significant reduction in HSV-1 viral load and titer. Transduction of Vero cells with all-in-one AAV2 vectors expressing SaCas9 and paired gRNAs nearly eliminated the viral load and titer. To assess the *in vivo* efficacy of CRISPR/Cas9 gene editing, we initially used corneal scarification to deliver all-in-one AAV8 (Y733F) and AAV9 vectors expressing SaCas9 and paired gRNAs to a latent rabbit model of HSV-1 keratitis. This approach led to a remarkable reduction of over 60% in viral shedding from the treated rabbit eyes. Subsequently, we investigated the use of intravenous (IV) administration to deliver the all-in-one AAV9 vector expressing SaCas9 and paired gRNAs in the same rabbit model. Impressively, all but one rabbit treated with SaCas9 vectors exhibited undetectable viral shedding following HSV-1 reactivation. It is important to note that in that one rabbit, we could not detect expression of Cas9, suggesting that there was a technical issue with delivery. Moreover, even at a low AAV dose (6E+12 VG/kg) following IV delivery, we observed substantial levels of AAV vector genomes in the TG where the latent HSV-1 is located. Additionally, we detected a reduction in HSV-1 viral DNA and LAT RNA in the TG of rabbits treated with the AAV9-SaCas9 vector compared to the control group. These results suggest effective targeting of the latent HSV-1 reservoir in the rabbit HSV-1 keratitis model.In summary, our findings demonstrate that the delivery of all-in-one AAV9-SaCas9 vectors can serve as an effective one-time therapeutic strategy for treating HSV-1 keratitis.

1634. Efficacy and Safety of a CasRm-Based Therapy for Choroidal Neovascularization in Non-Human Primates

Hong Zhang[1], Jiawen Luo[1], Cunhua Ji[1], Yan Li[1], Fazhou Zhang[1], Chao Zhu[1], Hui Xu[1], Zhendong Xiao[2], Junjiu Huang[3], Junbin Liang[1]

[1]Reforgene Medicine, Guangzhou, China,[2]Biotherapy Center, The Third Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China,[3]MOE Key Laboratory of Gene Function and Regulation, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou, China

Introduction Age-related macular degeneration (AMD) is one of the leading blindness diseases in the elderly population, affecting nearly 288 million people worldwide by 2040. Despite several anti-vascular endothelial growth factor (anti-VEGF) injections exhibiting excellent efficacy in the initial two-year treatment for neovascular AMD (nAMD), the visual acuity of the majority of patients after two-year frequent administrations chronically drops down due to subretinal fibrosis or retina atrophy. Additionally, parts of patients with nAMD don't respond to anti-VEGF treatments. Therefore, there is still a larger unmet need for novel, long-term effective treatments for patients with nAMD. Here we present a specialized AAV treatment carrying a novel CasRm system (RM-102) which we have discovered and demonstrated its specific and superior editing efficiency on the target RNA. **Methods** The AAV vectors carrying a CasRm-based target RNA editing system were first generated and purified, then were subretinally administrated into non-human primates (NHP). The choroidal neovascularization (CNV) spots were induced by laser photocoagulation at week 4 post-dose. Then follow-up evaluations for safety and efficacy including fluorescence angiograms (FFA), spectral domain optical coherence tomography (SD-OCT), fundus photography, and post-laser-photocoagulation were conducted. **Results** The incidences of grade IV lesions in animals with two versions of RM-102 treatment at week 2 or 4 post-laser were significantly reduced (RM-102-V1: 4% and 0%, respectively, P<0.05) compared to the control group (45% and 48%, respectively), to the same extent as a 2 mg Eylea intravitreally administered at the time of lesioning (Figure 1A and 1B). The RNA and protein levels of VEGFA related to nAMD were remarkably reduced after 23 weeks post-injection. The findings from OCT and other ocular tests showed that there were no drug-related adverse events, except for reversible mild ocular inflammations in the early phase which are common in gene therapies (Figure 1C). Furthermore, the immunogenicity assay showed that there is no detectable immune response to our CasRm in primates treated with RM-102 (Figure 1D). Moreover, RM-102 was predominantly distributed in ocular tissues and was only detected at low levels in the spleen and liver of a few animals. The viral genomes weren't detected in any other tissues of most animals. **Conclusions** The results from the NHP study suggest that RM-102 administered via a single subretinal injection effectively suppressed the laser-induced CNV and was well tolerated. RM-102 is a novel, long-term treatment for patients with nAMD in response to anti-VEGF as well as patients with diabetic macular edema, and also will bring new trials to the non-responders to anti-VEGF.

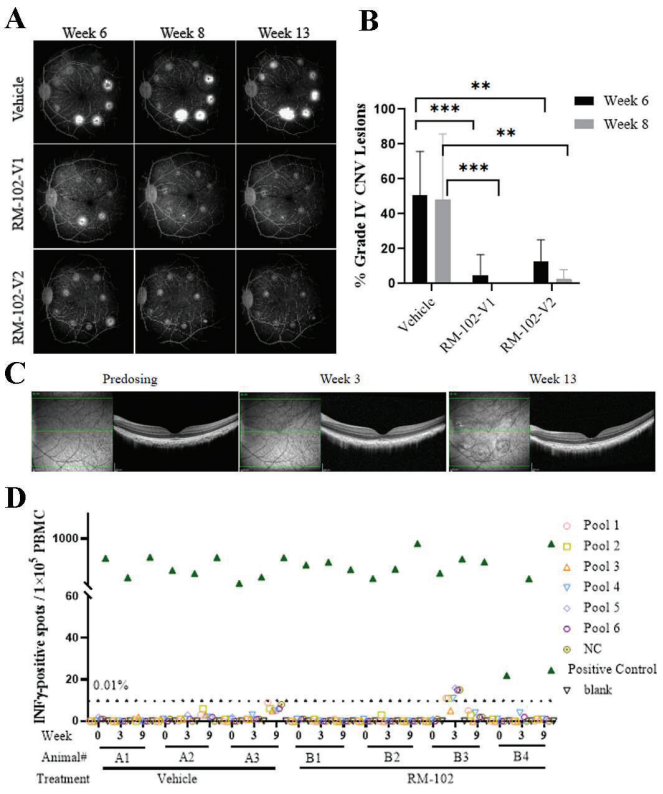


Figure 1. The part of efficacy and safety data of RM-102 in non-human primates

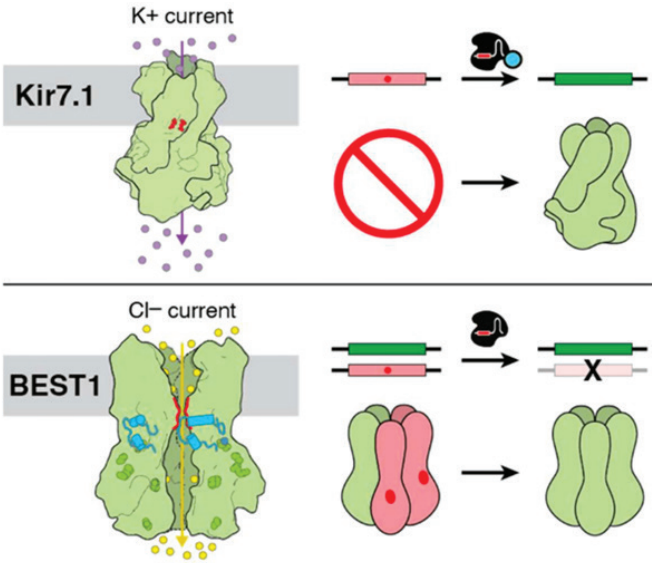
1635. CRISPR Vision Program: Nonviral Genome Editing to Treat Congenital Blindness

Krishanu Saha[1], Mary Haak-Frendscho[2], David M. Gamm[1], Shaoqin Gong[1], William Murphy[1], T. Michael Nork[1], Bikask Ranjan Pattnaik[1], Adam Silverman[2]

[1]University of Wisconsin-Madison, Madison, WI,[2]Spotlight Therapeutics, Hayward, CA

Advances in genetic screening have revolutionized the identification of genetic causes of visual impairment. However, most of these causes are not yet addressable in the clinic. CRISPR genomic medicine is a rapidly evolving area of biomedical research, with its first drug approval in 2023. We present our collaborative CRISPR Vision Program for the nonviral delivery of CRISPR-based genome editors to treat a form of Leber Congenital Amaurosis (LCA) and Best disease as models to facilitate translational research. **Methods:** We established a collaborative academic-industry partnership supported by the US National Institutes of Health. We identified three key projects using genomic editing for a *KCNJ13* mutation causing LCA16 and *BEST1* mutations causing autosomal dominant Best vitelliform macular degeneration (*Figure*). Genomic medicines will be delivered through nonviral nanoparticle or non-packaged delivery formulations. Two cores are evaluating the safety and efficacy of these formulations within models spanning human bioengineered cell culture systems and non-human primates. Finally, a regulatory core aims to share our interactions with the FDA and the larger field to accelerate the development of an investigational new drug application for clinical trials by 2028. **Results:** Synthetic nonviral delivery of adenosine base editor (ABE) corrected *KCNJ13*

disease mutation and restored the encoding inwardly, rectifying potassium (Kir7.1) channel function. Functional restoration was demonstrated in vitro in retinal pigment epithelium (RPE) derived from human induced pluripotent stem cells (hiPSCs). In mouse models, sustained visual function was observed after subretinal injection of the nanoparticles, with normal electroretinogram wave responses and preserved retinal structure. Furthermore, a distinct nonviral formulation of engineered ribonucleoproteins (eRNPs) resulted in robust editing in the RPE after subretinal injection. Finally, evaluation of channel function in Best disease patient-derived hiPSC-RPE cells showed rescue of function upon genome editing, testifying to the efficacy of our strategy. **Conclusions:** We are moving the CRISPR-based medicines into the eye to treat inherited retinal disorders. Our Program could accelerate the development of mutation-specific treatment interventions to match the speed of genetic variant discovery.



1636. Efficient AAV Vector-Mediated Gene Transfer to Mice Inner Hair Cells with High-Concentration of Carbohydrates

Masao Noda[1], Ryota Koshu[1], Mari Shimada[1], Chizu Saito[1], Naomi Takino[2], Mika Ito[2], Makoto Ito[3], Shin-ichi Muramatsu[2]

[1]Otolaryngology, Jichi Medical University, Shimotsuke, Japan,[2]Neurological Gene Therapy, Jichi Medical University, Shimotsuke, Japan,[3]Jichi Medical University, Shimotsuke, Japan

Hearing loss is a common sensory disorder of increasing importance, especially in an aging society where it is also a known risk factor for dementia. Gene therapy targeting the inner ear is a promising approach to treating sensorineural hearing loss. Clinical trials of gene therapy using adeno-associated virus (AAV) vectors are underway in humans and are expected to expand. The efficiency of AAV vector-mediated gene transfer is influenced by serotype, dosage, and animals to be treated. While investigating the effect of hypertonic carbohydrates, an inhibitor of clathrin-mediated endocytosis, on the transduction of engineered AAV vectors, we found that high concentrations of carbohydrates increased transduction efficiency in cultured cells. AAV.GTX is a double tyrosine-mutant form of AAV9 (Y446F and Y731F). AAV.GT5 has triple amino acid substitutions (S472A, S587A, and N706A) in the AAV3B capsid. The transduction efficiency of AAV.GT5 is similar to that of parental AAV3B, but AAV.GT5 has less reactivity to anti-AAV capsid-neutralizing antibodies. In HEK293 and HEI-OC1 cells, a progenitor hair cell line cells, up to 240 mM of sucrose or glucose in the culture medium enhanced the transduction of AAV.GTX and AAV.GT5. We examined whether the addition of high concentrations of sucrose could enhance gene transfer efficiency in the inner ear of adult mice. We made a small incision in the posterior portion of the left auricle under a microscope and drilled a hole into the inner ear bursa of 9-week-old C57BL6/J mice. Cochleostomy was performed to create a small perforation in the basal turn of the cochlea, and the GFP-expressing AAV vector solution (1.3×10^{10} vector genome/ear) was injected with or without 160 or 320mM sucrose. Histological analysis three days after the injection showed that both AAV.GTX and AAV.GT5 showed GFP expression in inner hair cells and spiral ligaments. More than 80% of inner hair cells could be transduced with both AAV vectors. Assessment by auditory brainstem response showed no noticeable hearing loss. This simple and versatile method may be valuable in developing novel gene therapies for adult-onset sensorineural hearing loss.

Friday Posters: Musculo-Skeletal Diseases

1637. Unlocking the Genetic Secrets Behind Familial Recurrent Patellar Dislocation

Liren Wang, Jinzhong Zhao

Shanghai Sixth People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai, China

Background and aims: Familial recurrent patellar dislocation, a notable orthopedic challenge, is characterized by the recurrent dislocation of the kneecap. This condition is often observed within certain families, suggesting a genetic predisposition. Despite its prevalence, research to date has been limited, with no comprehensive genetic studies encompassing more than one affected family. This study aims to bridge this research gap by identifying genetic factors contributing to familial recurrent patellar dislocation, thereby enhancing our understanding of its pathogenesis and impact on joint health and patient quality of life. Methods: In this pioneering study, we utilized whole-exome sequencing, a powerful tool for uncovering genetic variations. We meticulously analyzed the genetic profiles of six distinct families, each with a documented history of familial recurrent patellar dislocation. Our approach was comprehensive, focusing on identifying common genetic denominators and potential pathogenic mutations across these families. Results: Our extensive analysis revealed a significant finding: a consistent defect in the genes associated with extracellular matrix production was present in all six families. This defect was particularly evident in genes responsible for collagen synthesis, a key component for maintaining connective tissue strength and joint stability. The mutations identified in these collagen-producing genes likely

contribute to the compromised structural integrity of the knee joint, predisposing individuals to recurrent dislocations. Conclusion: This study represents a significant leap in the field of genetic orthopedics, marking it as the largest and most comprehensive investigation into familial recurrent patellar dislocation to date. The findings underscore the crucial role of extracellular matrix-related genes in the development of this condition. These insights open up new possibilities for targeted genetic screening and therapeutic interventions, aiming to mitigate the recurrence of patellar dislocations and improve the quality of life for affected individuals and families.

1638. Examination of Caregiver Perspectives on Genetic Testing and Caregiver Burden of Patients with Native American Myopathy

Magnolia Wang[1], Cynthia M. Powell[2]

[1]Human Genetics, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Genetics, University of North Carolina School of Medicine, Chapel Hill, NC

Introduction: Native American Myopathy (NAM, or STAC3 Disorder) is an ultra-rare, autosomal recessive condition originally characterized in the Lumbee Tribe of North Carolina. A founder pathogenic missense variant was identified as the NM_145064.3:c.851G>C mutation of the STAC3 gene, and molecular diagnosis is established by detecting biallelic pathogenic variants. Due to its rarity, NAM and its impact on patients remains poorly characterized. This study examines the perspectives of caregivers on genetic testing, counseling, and caregiver burden, with the aim to provide insight for physicians, scientists, and policy-makers to improve patient care of this ultra-rare disorder. Methods: Patients diagnosed with NAM were identified in the UNC Health System and recruited via email. Either one or both caregivers, or the adult patient themself (aged 26 and over) completed the questionnaire. Responses were evaluated on a 5-point scale, where higher scores were associated with greater outcomes. Scores within one patient cohort were averaged to produce a final score between 0-100. Results: Perspectives on Genetic Testing and Counseling Based on Child Ethnicity: Among the 17 enrolled subjects, 9 (52.9%) were Native American, 5 (29.4%) were Black, and 3 (17.7%) were White and Native American subjects. White and Native American subjects consistently reported greater outcomes compared to their Native American- and Black-identifying counterparts. White and Native American subjects strongly agreed upon the utility of undergoing genetic testing to confirm diagnosis (3, 100%), and that genetic counseling helped manage their diagnosis (3, 100%). This starkly contrasts the large proportion of Black subjects who did not undergo genetic testing (2, 40%), did not find it to be helpful (2, 40.0%), or did not receive genetic counseling (2, 40%). Similarly, a quarter of Native American subjects (2, 22.2%) strongly disagreed with genetic testing or did not find it to be helpful, and more than half (5, 62.5%) either did not receive, strongly disagreed, or remained neutral toward the utility of genetic counseling (Figures 1a and 1b). Perspectives on Caregiver Burden Based on Child Ethnicity: Black and Native American subjects consistently demonstrated greater caregiver burden than their White and Native American counterparts. While White and Native American subjects unanimously reported that they "Never" felt exhausted from child care (3, 100%), around half of Black (2, 40.0%) and Native American subjects (5, 57.1%) described frequent burnout (Figure 1c). All White and Native American subjects concurred that child care did not interfere with work or social activities (3, 100%), but a sizable proportion of Black (1, 20.0%) and Native American (4, 57.1%) did not agree (Figure 1d). Moreover, White and Native American subjects strongly felt that others understood their family situation (3, 100%), yet a majority of Black (4, 80.0%) and Native American subjects (4, 57.1%) lacked this support (Figure 1e).

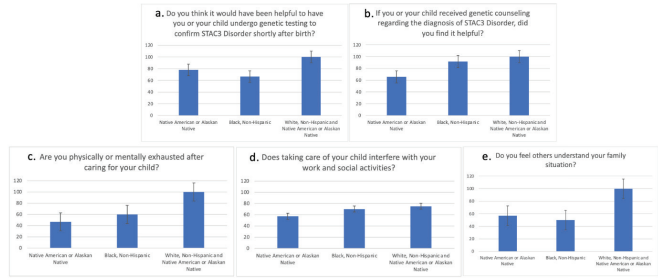


Figure 1.
a: Perspectives on the Utility of Genetic Testing to Confirm STAC3 Disorder Diagnosis
b: Perspectives on the Utility of Genetic Counseling for STAC3 Disorder
c: Perspectives on Physical and Mental Exhaustion Following Child Care
d: Perspectives on Interference of Child Care With Work and Social Activities
e: Perspectives on How Well Others Understood Family Situation

Conclusions: We have characterized the demographics of the 17 enrolled subjects, and identified trends in genetic testing, counseling, and caregiver burden among different ethnic groups. We hope this analysis facilitates research efforts to improve patient care for this ultra-rare genetic disorder.

1639. Development of *In Vitro* Models and Viral Vectors for Targeted Gene Replacement Therapy in Calpainopathy (LGMD2A/R1)

Julienne Dietz[1], Lampros Mavrommatis[2], Wenli Zhang[3], Dirk Grimm[4], Anja Ehrhardt[3], Matthias Vorgerd[5], Eric Ehrke-Schulz[6]

[1]Human Medicine, Center for Biomedical Education and Research (ZBAF), Witten/Herdecke University, Witten, Germany,[2]Ruhr University, Bochum, Germany,[3]Witten/Herdecke University, Witten, Germany,[4]Heidelberg University Hospital, Heidelberg, Germany,[5]Ruhr University, Witten, Germany,[6]human Medicine, Center for Biomedical Education and Research (ZBAF), Witten/Herdecke University, Witten, Germany

Introduction: Calpainopathy, (Limb Girdle Muscular dystrophies 2ALGMD2A/R1) is caused by mutations in the calpain 3 gene (CAPN3), is characterized by progressive, symmetrical weakness of the proximal limb and girdle muscles. Although physical therapy and orthopedic surgery can address symptoms, no disease modifying or curing treatment available. To support the development of targeted gene addition approaches we aimed to develop differentiated human CAPN3 deficient cell culture disease models and explore different muscle specific Adeno associated virus (AAV) derived vectors for CAPN3 gene delivery. **Methods:** We established two genetically different patient derived cell culture disease models with defined CAPN3 mutations. We used immortalized myocytes as well as induced pluripotent stem cells (iPSCs) derived myocytes and applied advanced protocols using electrical pulse stimulation (EPS) to evoke differentiation into Myotubes and sarcomere formation in 2D cell culture. Both models were characterized with regard to differences compared healthy controls. To analyze viral vector mediated CAPN3 gene replacement in the differentiated myotubes, we designed vectors based on muscle

specific capsids of AAV9 and AAVmyo encoding the full length CAPN3 cDNA fused to a GFP gene via a 2A peptide signal under control of the muscle specific MHCK7 promotor. Vector genomes were assembled and vectors were generated and produced to high titers. Subsequently both vectors were utilized to transduce differentiated CAPN3 deficient myotubes and healthy control myotubes. CAPN3 gene transfer and expression was analyzed by RT-qPCR, Western blot and fluorescent microscopy. Systemic *in vivo* delivery of both vectors was performed for biodistribution- and gene expression analysis in mice. **Results:** Both CAPN3 deficient cell culture models were suitable for in vitro differentiation into mature contractile Myotubes and displayed morphological as well as physiological differences compared to healthy controls. AAV mediated gene transfer of the human CAPN3 cDNA resulted in enhanced CAPN3 gene expression *in vitro* and had compensatory effects on mRNA and protein levels in the two different Capn3-disease models. Preliminary *in vivo* experiments showed that systemic delivery resulted in robust CAPN3 gene expression in muscular tissue for both vectors, with AAVmyo showing reduced gene expression in non-target organs. **Discussion:** This study provides novel preclinical *in vitro* disease models that help studying potential treatment options for Calpainopathy and provides insights helping to choose ideal gene transfer vehicle and or transgene expression setup for future translation into *in vivo* studies regarding tissue specificity and expression strength.

1640. Novel, Clinically Relevant Composite Bioassays for Cardiac and Skeletal Muscle Protection as Conferred by Dystrophin-Mimetics

Kyle A. Hubble, Maria P. Corzo, Coral M. Kasden, Alaine I. Castillo, Rufranshell Reyes, Benjamin W. Kozyak, Hansell H. Stedman

University of Pennsylvania, Philadelphia, PA

INTRODUCTION. Despite groundbreaking progress, challenges in interpreting clinical outcomes have complicated the development of effective, durable, and safe gene therapy for Duchenne muscular dystrophy (DMD). DMD results from the lack of functional dystrophin protein, which resides in the costameric lattice linking the extracellular matrix to the intracellular cytoskeleton. Dystrophic muscle is extremely susceptible to eccentric contraction-induced membrane rupture, resulting in myocyte death and muscle wasting. In addition to the skeletal muscle pathobiology, cardiomyopathy is the leading cause of death for DMD patients. The classical primary outcome measure for therapeutic clinical trials for DMD is the North Star Ambulatory Assessment (NSAA), a 17-item functional motor assessment for ambulatory children. Other outcome measures, such as serum markers and muscle biopsies, are highly invasive thus limiting their collection. Here we present clinically relevant composite bioassays to measure cardiac and skeletal muscle protection. One such assay revealed that dystrophin-deficient muscle is ~10,000-fold more susceptible to acute injury than wild type. This assay uniquely revealed the shortcomings of other candidate therapeutics, providing a novel platform for advancing the field. **METHODS.** We established novel assays to measure the extent of acute injury in dystrophic skeletal and cardiac muscle. The susceptibility to contractile-induced skeletal muscle injury was assessed in *mdx* mice. Prompted by our earlier findings of paradoxical changes in serum creatine kinase levels in mdx mice exercised by alternative regimes we have investigated the association of noninvasive urinary N-terminal fragment of titin (NTFT) levels with several provocative muscle contraction regimes. Mice were subjected to downhill treadmill running and urine collection. Following case reports of young DMD patients experiencing myocardial injury due to acute bouts of extreme hypertension and increased afterload, we questioned whether this episodic injury could be modeled in the *mdx* mouse, generally disregarded as a cardiac model for DMD due to its mild phenotype. Increased afterload was chemically induced in *mdx* mice, and circulating cardiac troponin I (cTNI) was measured following a 48-hour drug infusion. **RESULTS.** We developed and validated novel, clinically relevant skeletal muscle and cardiac protection assays to test and resolve investigational therapeutics for DMD. We found that the urinary NTFT to recent downhill distance traveled index was highly consistent in wild type and *mdx* mice as young as 4 weeks old to up to 9 months old. While untreated *mdx* mice exhibited a 10,000-fold increase in NTFT accumulation over WT mice (150.98 vs 0.14 NTFT/distance, $p<0.0001$, respectively), *mdx* mice treated with next generation dystrophin mimetic gene therapies were well protected (1.71 NTFT/distance, p vs untreated *mdx* <0.0001). From our cardiac protection assays, preliminary studies indicate a 25-fold serum cTNI spike in *mdx* mice over WT (9.48 vs 0.38 ng/mL, $p=0.0077$, respectively), while dystrophin mimetic-treated *mdx* mice were approaching WT-like (0.85 ng/mL, p vs untreated *mdx* = 0.1783). **CONCLUSIONS.** Here we present physiologically relevant assays for skeletal and cardiac muscle protection in preclinical DMD animal models. Unlike conventional measures, these assays provide the necessary resolving power to differentiate related treatment groups in blinded studies, with the potential to more confidently predict clinical outcomes of patients in clinical trials. We predict these measures, particularly normalizing urinary NTFT to recent cycles of eccentric contraction, to be an essential component of next generation clinical studies of DMD therapeutics.

1641. Suppression-Replacement Design for Reducing Body Myopathy Using U7 snRNA-Mediated Exon Skipping

Michael Leeson[1], Jachinta Rooney[2], Yaqun Zou[2], Veronique Bolduc[2], Joseph Beljan[3], Kevin M. Flanigan[4,5,6], Carsten G. Bonnemann[2]

[1]NHGRI/NIH, Bethesda, MD,[2]NINDS/NIH, Bethesda, MD,[3]The Ohio State University, Columbus, OH,[4]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[5]Departments of Pediatrics, The Ohio State University, Columbus, OH,[6]Department of Neurology, The Ohio State University, Columbus, OH

Reducing body myopathy with infantile or early childhood onset is a rapidly progressive, X-linked dominant disorder caused by specific *FHL1* variants in exons 4 and 5 coding for the LIM-2 domain of the Four and a Half LIM domain 1 protein. Intracytoplasmic inclusion bodies containing variant and wild-type FHL1 protein as well as other trapped proteins are forming in skeletal muscle and initially result in a scapuloperoneal distribution of weakness, but rapidly progresses to diffuse hypotonia, contractures, and respiratory failure. Loss of FHL1 causes a milder, X-linked recessive myopathy. This project aims to treat this dominant disorder with a suppression-replacement gene therapy

strategy. The suppression will be achieved by U7 snRNA-mediated exon skipping of *FHL1* exon 3 to result in transcripts that lack the typical translation initiation codon and are predicted to undergo nonsense mediated decay, which is expected to prevent production of both variant and wild-type FHL1. The replacement will contain silent variants resistant to the sequence-dependent U7 snRNA exon skipping, while retaining the functional wild-type amino acid sequence. The current work is focused on determining optimized target sequences to induce exon skipping, which were generated by both rational design (targeting splice donor, splice acceptor, and putative exon splice enhancer sites) and using eSkip-Finder (a machine learning-based web application developed to predict exon skipping efficacy of novel antisense oligonucleotides). To help selection, sequences were initially synthesized as fully PS-2'OMe antisense oligonucleotides and cotransfected into HEK293 cells with a splicing reporter plasmid containing *FHL1* exon 3 with flanking intronic sequence. Skipping efficacy was detected using qualitative RT-PCR. Promising candidates will be tested in cell lines with high FHL1 expression using U7 snRNA cassettes introduced by AAV transduction.

1642. Discovery and Characterization of Novel AAV Capsids with Enhanced Skeletal Muscle Tropism Using Directed Evolution

Adelaida Palla[1], Quan Jin[2], Haley Heusey[1], Frank Li[1], Jimmy Tran[3], Amit Kumar[3], Dora Bodnar[1], Chris Hinckley[4], Ka Ying Hung[2], Samneet Kashyap[2], Aissatou Diallo[2], Emily Rouse[2], Margaret Kelemen[2], Lucia Xiang[2], Aditya Utturkar[2], Misha Chittoda[2], Harini Dhamodaran Sampath[2], Shawn Kyle Bourque[2], Samantha Desmarais[2], Kate Henry[2], Shuyun Deng[2], Wei Tong Chen[1], Davide Gianni[2], Jun Shi[1], Junghae Suh[2], Yuan Yuan[2]

[1]Neuromuscular and Muscle Diseases Research Unit, Biogen, Cambridge, MA,[2]Gene Therapy Accelerator Unit, Biotherapeutic and Medicinal Sciences, Biogen, Cambridge, MA,[3]Nonclinical Safety, Biogen, Cambridge, MA,[4]Translational Cellular Sciences, Biogen, Cambridge, MA

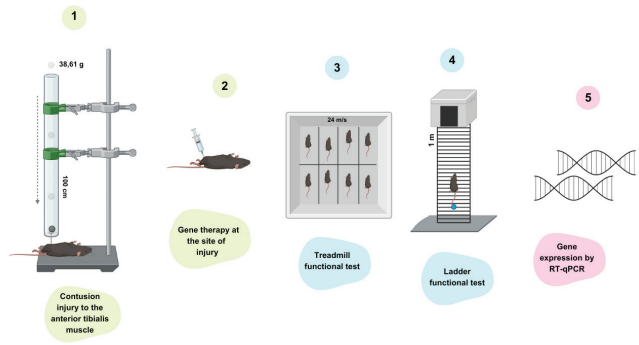
Gene therapy holds considerable promise for treatment of musculoskeletal and neuromuscular disorders. However, low muscle affinity in existing AAV vectors requires high viral doses for systemic delivery, which has been associated with adverse events across clinical trials. Thus, there is a need to identify novel vectors with enhanced muscle targeting to improve gene delivery efficiency with lower doses. By conducting iterative rounds of selection of a RGD peptide insertion AAV library in cynomolgus macaques, we identified AAV capsid variants with enhanced muscle transduction. One such variant, was used to treat cynomolgus macaques with immunosuppressants. This AAV variant led to enhanced muscle transduction compared to AAV9, with minimal safety or pathology findings. In addition, the AAV variant demonstrates acceptable productivity and manufacturability. Importantly, several AAV variants show human and murine cross-reactivity. They mediated efficient skeletal muscle and heart transduction in mice at lower doses compared to AAV9. They also demonstrated enhanced transduction compared to AAV9 in human muscle cells and 3D myobundles. Here we demonstrate these novel muscle-tropic capsids have higher potency than AAV9, which could potentially drive the development of safer gene therapies targeting muscle diseases.

1643. Evaluating the Impact of Soluble TGF-β1 Receptor Expression Controlled by an NF-κB-Inducible Promoter on *In Vivo* Muscle Fibrosis Reduction

Luana De Mambro[1], Leonardo Martins[2], Roberta S. Stilhano[3]

[1]Universidade Federal de Sao Paulo, Sao Paulo, Brazil,[2]Department of Medicine, Institute of Medical Biology of Polish Academy of Sciences, Lódz, Poland,[3]Faculdade de Ciencias Medicas da Santa Casa (FCMSCSP), Sao Paulo, Brazil

One of the main challenges in regenerative medicine is the regeneration of skeletal muscle tissue after volumetric loss (VML), in which a persistent inflammatory environment, marked by the activation of fibrotic genes and muscle atrophy induced by the cytokine TGF-β1, leads to extensive fibrotic tissue development. Thus, this study aimed to produce two lentiviral vectors: the therapeutic one, carrying the soluble receptor of TGF-β1 (Lv_NF-κB_sTβRII) and the control (Lv_NF-κB_Luci), both under control of the NF-κB inducible promoter. For this purpose, the vectors were designed and cloned, and subsequently *in vitro* studies were conducted, where C2C12 cells were cultured in cDMEM (supplemented with 10% of fetal bovine serum and 1% penicilin/streptomycin), transduced, and injured with $BaCl_2$, having gene expression evaluated by RT-qPCR. For myogenic differentiation, the cells were cultured in differentiation medium and analyzed by RT-qPCR and immunocytochemistry. In the *in vivo* tests, mice were divided into control groups, injury, and injury with treatment. They were injured in the anterior tibialis muscle by contusion, and Lv_NF-κB_sTβRII was injected to the site of the lesion.



Functional analysis was performed on a treadmill and ladder. Molecular analysis was made by plasma TGF-β1 ELISA and RT-qPCR to assess inflammation, fibrosis, myogenic differentiation, and expression of the soluble TGF-β1 receptor. For statistical analysis, independent t-test, one-way ANOVA, and non-parametric Friedman, Kruskal-Wallis, and Mann-Whitney tests were used in GraphPad Prism 9.0 software. A 95% confidence interval was used for all analyses. In the *in vitro* results, there was an increase in the number of sTβRII copies of 442 times in 24 hours and 67 times in 48 hours among C2C12_NF-κB_sTβRII compared to the control. In addition, there was an increase of 10% in the density of the area marked by MyHC-emb in the cell overexpressing sTβRII and

2.55 times decrease in Pax7 expression. In the *in vivo* results, the injury group showed the lowest performance on the treadmill over 30 days and the lowest average load supported on the ladder, where only 20% of the animals were able to support the group's maximum load (31g), which had the longest average time climbing. Meanwhile, the injury with treatment group showed the best performance on the treadmill and the highest average load supported on the ladder (36g), with 40% of the animals adhering, followed by the control group with 60% of the animals adhering at the same weight. Plasma ELISA showed a reduction of TGF-β1 in the injury and injury with treatment groups after 16 days compared to the control. In the analysis of gene expression by RT-qPCR of muscle tissue, lower expression of NF-κB (0.0477 ± 0.01475) and IL-6 (0.04292 ± 0.02553) was found in the injury group and higher expression of Smad7 (0.07426 (± 0.01778); Pax7 (0.3471 ± 0.05433); MyoD (1.103 ± 0.1209); MyoG (0.9891 ± 0.1932) and sTβRII (1.096 ± 1.049) in the injury with treatment group. Therefore, modulation of the expression of sTβRII by the NF-κB inducible promoter and improvement in muscle regeneration by sequestration of TGF-β1 were found. In addition, the Lv_NF-κB_sTβRII vector demonstrated improvement in muscle function and functional performance of the mice, as well as an increase in the expression of myogenic differentiation markers.

1644. Overexpressing Murine Prg4 and or NFκB-Regulated Il-1ra with High Capacity Adenoviral Vectors (HCAd) by Intra-Articular Injection Improves Motor and Pain Endpoints and Articular Cartilage Maintenance in a Murine Model of Post-Traumatic Osteoarthritis (PTOA)

Zelong Dou[1], Racel Cela[1], Philip Ng[1], Jiansen Yan[1], Ming-Ming Jiang[1], Carolina Leynes[1], Camilla Fernanda Majano[1], Heidi Reesink[2], Matthew Grol[3], Nele A.T. Haelterman[1], Yangjin Bae[1], Brendan H. L. Lee[1]

[1]Department of Molecular & Human Genetics, Baylor College of Medicine, Houston, TX,[2]Cornell University, Houston, NY,[3]Department of Physiology and Pharmacology, Western University, London, ON, Canada

Osteoarthritis is mainly characterized by loss of articular cartilage, subchondral bone remodeling, inflammation at intra-articular space, synovitis, and pain. The precise molecular mechanism of OA is complex and combinatorial approaches to treatment are likely required. One of the most extensively studied targets for OA therapy is IL1 which can be regulated through modulating IL1 receptor antagonist (IL1RA). Besides, Prg4 loss of function mutation in human patients lead to the camptodactyly-arthropathy-coxa vara-pericarditis syndrome, and the Prg4 knockout mice showed OA-like phenotype in an early stage, suggesting the chondroprotection of Prg4. Local intra-articular injection is believed to be a promising OA treatment option as it can bypass the challenges of systematic delivery. However, even locally being delivered, recombinant proteins and small molecules can be quickly filtered by synovial capillaries and lymphatics, which will significantly decrease the treatment efficacy. In this regard, a greater promise is held by intra-articular delivery of therapeutic transgenes with their sustained local expression after a single injection. We have shown that individually expressed Prg4 and Il1-ra after intra-articular HCAd gene therapy could delay OA progression in small and large preclinical models. This has led to a current phase I clinical trial of intra-articular HCAd5 Il1-RA gene therapy in OA patients (NCT04119687). Given though they target different contributors to OA, we hypothesized that intra-articular delivery of single high capacity adenovirus (HCAd) synergistically overexpressing both chondroprotective murine Prg4 and inflammation inducible Il1-ra would improve joint preservation, compared to their single delivery alone. Using a murine PTOA model generated by destabilization of medial meniscal tibial ligament (DMM) surgery bilaterally we performed knee intra-articular injection of HCAd overexpressing EF1-mPrg4 and NFkB-mIl-1ra with either original or reverse oriented target genes, and the combined NFkB-mIl1ra;EF1-mPrg4, and HCAd0 (control virus without transgenes) at postoperative week 2. At postoperative week 12 behavioral tests including Catwalk and Hot plate were applied for functional readouts, followed by harvesting knee joints for histological, 3-dimensional phase contrast micro-CT analysis, and immunohistochemistry analysis for expression levels of target genes and related inflammation markers. We found that the paw print intensity in HCAd-EF1-mPrg4 treated DMM surgery mice was significantly higher compared to those treated with HCAd0. The hotplate result showed that DMM surgery mice treated with HCAd-NFkB-mIl-1ra had significantly delayed time of response compared to HCAd0 control. Histological and phase contrast micro-CT results revealed comparable treatment efficacy of NFkB-mIl-1ra;EF1-mPrg4 and EF1-mPrg4. In addition, as shown by the undergoing murine PRG4 immunohistochemistry, compared to controls similar amount or more PRG4 expressing chondrocytes with wider distribution were observed in both NFkB-mIl-1ra;EF1-mPrg4 and EF1-mPrg4 treated mice knee articular cartilage, meanwhile we will measure the expression levels of proinflammatory markers. These findings suggested that overexpressing murine Prg4 and Il-1ra in superficial articular chondrocytes and synoviocytes with HCAd, similar to monotherapy, can delay signs of PTOA progression in a surgical mouse model of OA.

1645. AAV-Encoded Soluble PDL1 for Autoimmune Disease Gene Therapy

Wenjun Li[1], Junjiang Sun[2], Susi Feng[1], Chengwen Li[3]

[1]UNC Gene Therapy Center - Chapel Hill, NC, Chapel Hill, NC,[2]University of North Carolina at Chapel Hill, Chapel Hill, NC,[3]UNC at Chapel Hill, Chapel Hill, NC

Autoimmune diseases are a broad range of diseases involving multiple organs that profoundly affects patients' daily activities, mental health, and significantly impacting their life quality and life expectancy. Current treatments for autoimmune disease have limitations in terms of duration, range of relief, as well as affected population. PD-L1, as a checkpoint protein, plays important roles in immune regulation, and has been reported to be highly corelated with the initiation and progression of multiple autoimmune diseases. In this study, we used rheumatoid arthritis (RA) as a platform to investigate the efficiency of PD-L1 in treating autoimmune disease. We have demonstrated that intra-articular injection with wild type PD-L1 improved local inflammation in the RA joints. To optimize the transgene expression and increase therapeutic effect, we have designed an optimized soluble PD-L1(shPD-L1) that was more potent than wild type PD-L1 via AAV vector mediated intra-articular delivery, with a lower dose

needed for arthritis improvement in a collagen-induced arthritis (CIA) mouse model. Since autoimmune diseases involve multiple tissues, to extend the application of PD-L1 for systemic treatment, we further explored the systemic therapeutic effects of adeno associated virus (AAV) encoded shPD-L1 gene therapy in RA. Two weeks prior to CIA induction, AAV8/shPD-L1 were administered intravenously to mice. Notably, CIA mice treated intravenously with AAV8/shPD-L1 exhibited significant improvements in joint inflammation (1.8 ± 1.4 vs 9.9 ±1.3) and paw swelling (2.4±1.4 vs 10.7±2.2) compared to CIA untreated mice, similar to that in naïve mice. Protein levels of shPDL1 remained stable over 7 weeks. Moreover, these mice displayed a slight increase in body weight after AAV8/shPD-L1 treatment when compared to untreated CIA mice. Both total immunoglobulin levels and anti-collagen antibody levels were remarkedly lower in treated CIA mice at day 49 post AAV injection. Serum cytokine analysis revealed reduced levels of pro-inflammatory cytokines IL-1a (65.3±17.1 vs257±49.1), IL-6 (14.3 ±10.6 vs93.5± 51.0), IL-17 (1.8±1.1vs 7.1±5.5), and TNF-a (5.6±2.9vs15.9±6.5) in AAV8/shPDL1-treated mice when compared to untreated mice. The intravenous injection of AAV8/shPDL1 also altered the immune cell profile in the spleen, with treated mice showing reduced spleen sizes, decreased Th2 and Th17 levels, and increased T cell apoptosis when compared to untreated CIA mice, indicating a potential immune regulatory effect of shPDL1. Since AAV liver targeted gene therapy in clinical trials have demonstrated the liver toxicity in 30-50% patients and the expression of PD-L1 in the liver may also impact local immunity. Next, we carried out intramuscular delivery of AAV6 vectors encoding shPDL1. Consistent to AAV8/shPD-L1 liver targeted gene therapy, intra-muscular administration of AAV6/shPD-L1 vectors led to decreased paw swelling, reduced joint inflammation, and lower level of pro-inflammatory cytokines with neglectable levels of viral genome detected in liver. The therapeutic effect from AAV6/shPD-L1 muscular injection was dose dependent. In conclusion, our findings suggest that systemic expression of shPD-L1 from AAV-mediated delivery holds potential as a treatment for RA and other autoimmune disorders. Intramuscular administration of AAV/shPD-L1, in particular, offers an advantageous approach without the concern of liver toxicities.

1646. Development of a Chitosan Methacrylate Hydrogel Platform for Localized and Sustained Gene Delivery to Enhance Cartilage Regeneration in Osteoarthritis

Eunji Lee, Eunhyun Ji, Youjin Kim, Kye Il Joo
Ewha Womans University, Seoul, Korea, Republic of

Osteoarthritis (OA) is a prevalent degenerative joint disease, impacting a significant portion of the global population. Despite its high prevalence, current treatments for osteoarthritis remain inadequate, predominantly providing only symptomatic pain alleviation. Controllable and sustained local gene therapy offers a promising approach to treat osteoarthritis through the direct administration of therapeutic genes to the specific cells. In this study, we designed an injectable chitosan methacrylate (CHIMA) hydrogel platform that enables localized and continuous gene delivery without the need for invasive bone surgeries or implant insertions, aiming to enhance cartilage regeneration. Conventional approaches involving direct injection of gene modalities for local gene therapy are impeded by challenges such as suboptimal transfer efficiency, and short half-life. To overcome these challenges, our approach involves delivering a recombinant adeno-associated virus (AAV) vector which encodes a chondrogenic regeneration factor, through CHIMA hydrogel. CHIMA, a light-inducible mechanically reinforced chitosan, showed low toxicity and facilitated prolonged bioactivity of regeneration factors administrated via CHIMA-mediated AAV. Furthermore, incorporation of AAV into the CHIMA hydrogel encoding genes promoting regeneration, not only reduced the expression of inflammation and pain-related factors but also improved cartilage cell regeneration. The developed CHIMA hydrogel platform holds promise as an innovative approach for treating osteoarthritis by providing a stable and controlled environment for sustained gene delivery, ultimately enhancing the prospects of cartilage regeneration.

1647. Intravenous Delivery of AAV Gene Therapy for the Treatment of SOD1-ALS Provides Broad SOD1 Lowering in NHP

Michael D. Grannan, Tyler C. Moyer, Margaret R. Dalrymple, Ambreen Sayed-Zahid, Xiao Ren, Chiso Egbuchulam, Anupriya Kulkarni, Wenya Yang, Joseph Clement, Jeffrey Thompson, Nilesh Pande, Elizabeth Mellen, Naomi Muse, Crystal Taveras-Cui, Vikram Arora, Chanchal Sadhu, Dinah W. Y. Sah, Mathieu E. Nonnenmacher, Rajeev Sivasankaran, Todd A. Carter
Voyager Therapeutics, Inc., Lexington, MA

Mutations in superoxide dismutase 1 (SOD1) result in progressive motor neuron loss through toxic gain-of-function properties and are responsible for up to 20% of familial amyotrophic lateral sclerosis (ALS), or approximately 2% of all ALS patients in the U.S. Studies evaluating different strategies for SOD1 reduction have demonstrated reduced neuropathology, improved motor behavior, and extension of survival in transgenic mice expressing mutant SOD1. The recent FDA approval of an antisense oligonucleotide targeting SOD1 has further validated SOD1 as a therapeutic target. While approaches to reduce SOD1 have demonstrated varying degrees of efficacy, they are suboptimal in relying on direct CNS administration that fails to achieve the broad, CNS-wide SOD1 reduction thought to be necessary for maximal therapeutic benefit, or systemic administration that requires high doses with associated significant safety considerations. We previously reported the results from a series of in vitro and in vivo studies demonstrating SOD1 reduction following IV delivery of an AAV gene therapy targeting SOD1. In the G93A mouse model of disease, we demonstrated robust SOD1 knockdown throughout the rostral-caudal extent of the spinal cord, significant improvements in motor performance, and survival extension beyond what has previously been reported with intraparenchymal, intrathecal, or intracisternal delivery. In the current studies, we combined a highly potent siRNA against SOD1 with an IV-delivered, blood-brain barrier-penetrant TRACER™ capsid for evaluation in NHPs. Following a 2-month in-life period, we observed favorable biodistribution to the spinal cord and motor cortex which resulted in significant reduction of SOD1 mRNA. The enhanced BBB-penetrance and natural peripheral tissue detargeting inherent to the novel capsid enabled efficacy at a lower dose than typically used in intravenous AAV delivery, resulting in a favorable safety profile. These results demonstrate that the combination of a potent SOD1 RNAi transgene with a novel TRACER™ capsid produces

significant reduction of SOD1 mRNA in critical spinal cord and brain regions impacted in ALS and support its continued development and advancement into the clinic.

1648. High Potency MyoAAV Capsids Enhanced Skeletal Muscle Correction in GSD IIIa Mice

Baodong Sun

Pediatrics, Duke University School of Medicine, Durham, NC

Background: Glycogen storage disease type III (GSD III) is caused by glycogen debranching enzyme deficiency, which leads to pathological accumulation of cytoplasmic glycogen in muscle and liver. We recently reported a gene therapy study using an AAV9 vector to express a bacterial debranching enzyme (pullulanase) from a novel dual promoter, which consists of a liver-specific promoter (LSP) and a ubiquitous CMV enhancer/chicken β-actin (CB) promoter. We demonstrated that the LSP-CB dual promoter effectively suppressed transgene-specific cytotoxic T lymphocyte responses in GSD IIIa mice while allowing sustained pullulanase expression and glycogen reduction in liver, heart, and skeletal muscles. Tabebordbar and colleagues recently developed a class of RGD-motif-containing MyoAAV capsids that transduce muscle with superior efficiency and selectivity over the parental AAV9 capsid in mice and nonhuman primates. In this experiment, we compared two lead MyoAAV capsids with AAV9 for transduction efficiency and therapeutic efficacy in GSDS IIIa mice. **Methods:** An AAV vector carrying the LSP-CB dual promoter and a codon optimized CpG-free pullulanase cDNA (AAV-Dual-Pull$^{CpG\text{-}free}$) was packaged in AAV9, MyoAAV4A, and MyoAAV4E capsids and the resulting viral vectors were intravenously injected into 3-month-old GSD IIIa mice at the same dose ($1x10^{13}$ vg/kg). Six weeks later, muscle functional improvement was evaluated by treadmill and wire-hang tests. Tissues were collected to assess AAV vector biodistribution, pullulanase expression, and glycogen content. Urine samples were collected to analyze disease biomarker glucose tetrasaccharide Glc4 by mass spectrometry. **Results:** In liver, all the three AAV vectors led to a similar level of pullulanase expression and comparable potency in glycogen clearance (**Table 1**). In heart and skeletal muscles, both the MyoAAV4A and MyoAAV4E vectors demonstrated significantly higher treatment efficacy than the AAV9 vector, as determined by enhanced vector transduction efficiencies (AAV genome copy numbers, data not shown), increased pullulanase activities, decreased glycogen contents, improved muscle functions (treadmill and wire-hang performance), and reduced levels of disease biomarker urinary Glc4. The MyoAAV4A vector showed better therapeutic outcomes than the MyoAAV4E vector in general (**Table 1**). **Conclusion:** Our data demonstrated that the two MyoAAV capsids had remarkably enhanced transduction efficiencies in the cardiac and skeletal muscles of GSD IIIa mice, in comparison with AAV9. Follow-up experiments to test effective vector doses and long-term therapeutic efficacy are being conducted. These novel muscle-tropic capsids can also be broadly used for treating other inherited conditions that affect cardiac and skeletal muscles.

Table 1. Biochemical correction and muscle functional improvement in GSD III mice by AAV treatment

		UT	MyoAAV4A	MyoAAV4E	AAV9	WT
Pullulanase activity (mU/mg protein)	Liver	2.11 ± 0.41	39.39 ± 15.23	42.05 ± 7.28	52.6 ± 23.53	1.85 ± 0.88
	Heart	3.18 ± 0.79	238.7 ± 33.5	128.5 ± 40.8	20.53 ± 10.28	2.44 ± 0.30
	Diaph	2.99 ± 1.48	110.6 ± 22.5	107.5 ± 36.1	4.19 ± 1.18	3.64 ± 2.70
	Quad	0.43 ± 0.30	71.39 ± 30.7	47.31 ± 42.25	-0.11 ± 0.43	0.05 ± 0.42
	Gast	0.06 ± 0.20	73.83 ± 24.36	45.77 ± 22.57	0.22 ± 0.13	-0.22 ± 0.33
Glycogen content (μmol glucose/g tissue)	Liver	259.90 ± 47.96	85.25 ± 22.53	85.00± 54.90	74.33 ± 32.19	8.23 ±5.68
	Heart	77.30 ± 10.62	7.75 ± 0.94	11.92 ± 1.43	35.42 ± 7.32	4.10 ± 1.52
	Diaph	236.60 ± 78.38	9.25 ± 2.16	12.83 ± 5.04	100.92 ± 28.29	5.10 ± 2.99
	Quad	210.60 ± 69.74	25.42 ± 6.51	25.67 ± 7.20	156.42 ± 55.51	6.60 ± 1.56
	Gast	204.80 ± 37.44	29.58 ± 8.49	30.08 ± 7.32	160.3 ± 22.1	10.6 ± 6.46
Wire-hang (seconds)		11.80 ± 2.29	172.11 ± 65.53	61.83 ± 18.07	22.28 ± 6.72	333.22 ± 130.93
Treadmill (meters)		24.68 ± 5.03	351.32 ± 106.85	375.18 ± 71.25	106.93 ± 29.68	>500
Urinary Glc4 (mmol/ mol creatinine)		199.51 ± 66.96	10.03 ± 5.86	40.62 ± 47.33	141.50 ± 93.61	4.43 ± 1.71

Data shown are mean ± standard deviation. n= 6 for AAV-treated mice each group, n=5 mice for untreated (UT) and wild-type (WT) control mice. Diaph, diaphragm; Quad, quadriceps; Gast, gastrocnemius.

1649. CRISPR/Cas9-Based Precision B Cell Gene Engineering Coupled with Artificial Intelligence-Guided Protein Design Produces Active and Sustained Levels of Tissue Nonspecific Alkaline Phosphatase for the Treatment of Hypophosphatasia

Monika Musial-Siwek, Sarah Leach, Wayne Bainter, Madison Clements, Swati Singh, Sakshisingh Thakur, Glory Adekanye, Amy Lundberg, Megan L. Brophy, Priyanka Vaishampayan, Timothy Mullen, Connor Malloy, Anja F. Hohmann, Sean Keegan, Sean Arlauckas, Adam Lazorchak, Toshiro Ohsumi, Hanlan Liu, Richard A. Morgan

Be Biopharma, Cambridge, MA

Hypophosphatasia (HPP) is an inherited disorder characterized by defective bone mineralization, caused by deficiency of tissue nonspecific alkaline phosphatase (ALP). ALP is a dimeric zinc-containing metalloenzyme which hydrolyses inorganic pyrophosphate (PPi) and a variety of organic phosphates. Lack of functional ALP leads to elevated levels of PPi, which in turn inhibits bone mineralization, leading to osteomalacia/rickets (soft bones) and can be lethal without treatment. Currently, the only treatment for HPP is enzyme replacement therapy requiring subcutaneous injections 3-6 times per week which is accompanied by injection site reactions including lipodystrophy. B Cells can be engineered to produce a therapeutic protein, and then differentiated into terminally differentiated plasma cells, that secreted the target protein as a B Cell Medicine (BCM). BCMs have many of the attributes of plasma cells; natural longevity (potentially persisting for decades), capacity for high levels of protein secretion (thousands of molecules/cell/sec), the ability to engraft without host preconditioning, and the ability to re-dose, making them an attractive platform for the sustained supply of biologics. We sought to harness the power of BCMs by engineering them to produce sustained levels of ALP, thus offering a potential new treatment option for HPP. In this study, primary human B cells were expanded, precision engineered by CRISPR/Cas9 genome editing with AAV-mediated homology directed repair (HDR) to insert an ALP gene expression cassette into the various loci, including *CCR5*

(a safe harbor locus) and *JCHAIN* (a gene highly expressed in plasma cells). Guided by an artificial intelligence-based ALP protein structure design engine (i.e., AlphaFold2, an optimized version developed for dimeric proteins), protein constructs were optimized for activity and stability by fusing ALP with different Fc fragments (ALP-Fc) coupled with variety of linkers. Engineered BCMs secreted active ALP or ALP-Fc proteins up to 200 ng/1e6 cells/24hr as measured by Legendplex. *In vitro* phenotypic correction with supernatant from ALP or ALP-Fc secreting BCMs (ALP-BCMs) was demonstrated using the MC3T3 cell line mineralization model with robust mineralization observed. To validate our candidates *in vivo*, we have used immunodeficient transgenic mice that express human IL-6 (NOG-hIL6) and have been previously demonstrated to facilitate engraftment of BCMs. Following a single IV injection of ALP-BCM, ALP protein and enzyme activity was detected in mouse plasma from multiple ALP constructs. Importantly, ALP-BCM was well tolerated over the course of the *in vivo* study, with long-term follow-up in progress. In summary, we demonstrated successful production of active ALP *in vivo* coupled with artificial intelligence-guided protein design highlights the therapeutic utility of our BCM platform. The potential therapeutic application of this unique biologic delivery system could afford a novel treatment modality for HPP.

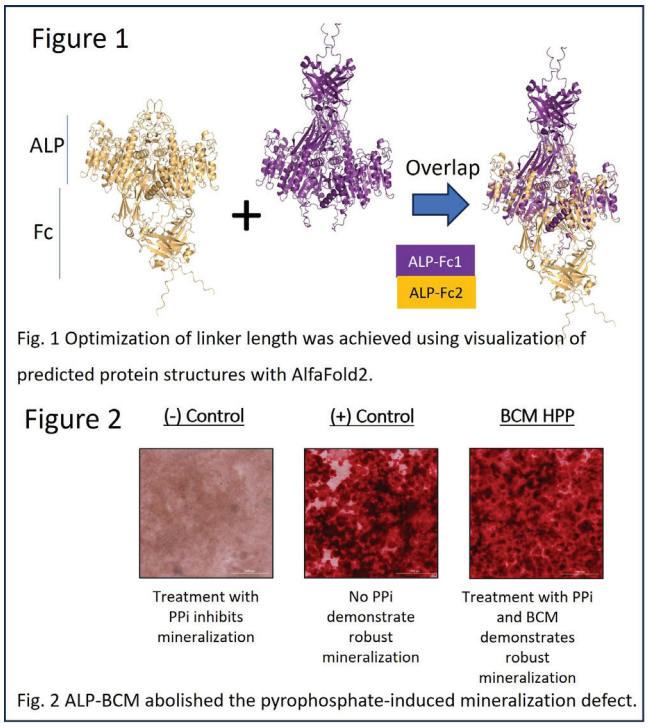


Fig. 1 Optimization of linker length was achieved using visualization of predicted protein structures with AlfaFold2.

Fig. 2 ALP-BCM abolished the pyrophosphate-induced mineralization defect.

Friday Posters: Genetic Disorders of the Blood and Immune System

1650. Novel Lentiviral Gene Therapy for Purine Nucleoside Phosphorylase Immune Deficiency

Robyn Loves[1,2], Weixian Min[1], Matilde Leon-Ponte[1], Arundhati Nair[1], Santhosh Dhanraj[3], Chetan Tailor[3], Eyal Grunebaum[1,2,4]

[1]Developmental and Stem Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada,[2]Department of Immunology, University of Toronto, Toronto, ON, Canada,[3]Tailored Genes, Toronto, ON, Canada,[4]Division of Immunology and Allergy, The Hospital for Sick Children, Toronto, ON, Canada

Background: Purine nucleoside phosphorylase (PNP) deficiency results in impaired purine metabolism and severe T cell lymphopenia. We developed a novel third-generation self-inactivating lentiviral vector (LVV)-based autologous gene therapy, referred to as LVV EFS hPNP. The objective is to test the ability of LVV EFS hPNP to correct metabolic and immune abnormalities in PNP-deficient (PNP-/-) mice. **Methods:** Bone marrow-derived lineage-negative (lin-) cells from PNP-/- mice were transduced with LVV EFS hPNP at an MOI of 50. PNP-/- mice received 6Gy total body irradiation, and were transplanted with 2.0x10^6 LVV EFS hPNP-transuced lin- cells or lin- cells from PNP+/- proficient littermates and followed for 12 or 24 weeks. **Results:** Donor chimerism in the bone marrow and thymus of mice treated with LVV EFS hPNP were 41.8±18.0% and 49.0±35.6%, respectively, which were significantly lower than chimerism following transplants with PNP+/- cells (91.8±7.54% and 98.5±1.48%). Vector copy number was detectable post-gene therapy in the spleen (2.82±5.06), thymus (3.22±5.54), and bone marrow (1.57±2.10). Despite showing lower levels of donor chimerism and large variability in VCN, which may suggest poorer survival and engraftment of cells originating from PNP-/- mice, restoration of PNP enzyme activity was successfully achieved in mice treated with LVV EFS hPNP. PNP enzyme activity measured in the spleen, thymus, bone marrow, and peripheral blood was 79.5±88.5%, 99.5±147.3%, 52.8±55.7%, and 56.1±61.3% respectively, of levels found in PNP+/- mice. Variability in tissue-specific enzyme activity correlated with VCN In the thymus and bone marrow. Further indication for rescue of systemic PNP activity was performed by measurement of urinary uric acid, which was normalized in PNP-/- mice out to 24 weeks post-transplant. Disease correction was also reflected in lymphocyte subpopulations in the thymus of mice treated with LVV EFS hPNP. Notably, the CD4+CD8+ double positive lymphocytes were normalized to percentages observed in PNP+/- mice at 24 weeks post-transplantation (p=0.99), and were significantly improved when compared to PNP-/- untreated mice (p<0.0001). **Conclusions:** This study demonstrates the potential of LVV EFS hPNP to correct enzyme activity in PNP-/- mice. Subsequent testing will investigate functional immune reconstitution and genotoxicity following gene therapy with LVV EFS hPNP.

1651. Development and Characterization of NCG-X Mouse Model for Gene Therapy in Erythroid Disorders

Huiyi Wang[1], Lu Yang[2], Meirong Wu[2], Mengting Wang[2], Xueya Ma[3], Juan Liang[4], Jing Zhao[2], Cuxiang Ju[2]

[1]GemPharmatech Co.Ltd., Nanjing, China,[2]GemPharmatech Co. Ltd, Nanjing, China,[3]GemPharmatech o. Ltd, Nanjing, China,[4]GemPharmate Co. Ltd, Nanjing, China

The NCG-X mouse model, a highly immunodeficient rodent model, has been engineered to address genetic therapies for erythroid-related diseases. With its c-Kit gene mutation, the NCG-X mice exhibit compromised hematopoietic stem cell (HSC) development, creating a bone marrow microenvironment more favorable for engraftment and development of human HSCs. We conducted extensive characterization and demonstrated that compared to its HSC-NCG counterpart, the NCG-X model exhibited human erythrocyte and platelet development in the mouse bone marrow and peripheral blood following human HSC engraftment. Moreover, targeted clearance of host-derived macrophages through Clo-lip treatment facilitated the reconstruction of human erythrocytes and platelets in the peripheral blood of NCG-X mice. Therefore, we believe the NCG-X model offers a unique and advantageous platform for evaluating the efficacy of gene therapies targeting disorders within the erythroid lineage. Its distinctive immune-deficient features, coupled with the observed improvements in erythroid and platelet development, make it suitable for pharmacological exploration and assessment of gene therapy efficacy in the context of erythroid-related diseases. This novel model holds promise for advancing our understanding of gene therapies and pharmacological interventions.

1652. Introducing CRISPR/Cas9 T Cell Pipeline for Highly Efficient Correction of Finnish Founder Mutations in *ADA2*, *AIRE* and *RMRP*

Katariina Mamia[1], Solrun Kolbeinsdottir[2], Anna Komisarczuk[1], Frida Frida Høsøien Haugen[1], Salla Keskitalo[3], Carolina Wieczorek-Ervik[1], Pavel Kopcil[1], Britt Olaug-Lindestad[1], Monika Szymanska[1], Kornel Labun[4], Markku Varjosalo[3], Martin Enge[2], Shiva Dahal-Koirala[1], Emma Haapaniemi[1]

[1]University of Oslo, Oslo, Norway,[2]Karolinska Institute, Stockholm, Sweden,[3]University of Helsinki, Helsinki, Finland,[4]University of Bergen, Bergen, Norway

Emerging CRISPR/Cas9 gene therapy trials demonstrate the therapeutic potential of correcting mutations in defected patient cells. However, as these trials are largely focused on targeting common hematologic diseases, little is known about how to successfully optimize CRISPR for rare primary immunodeficiency disorders (PIDDs). To address this challenge, we developed a CRISPR/Cas9-based T cell editing pipeline that streamlines CRISPR tool discovery for highly efficient and safe correction of mutations in patient T cells. In this study, we focus on correcting mutations in the *ADA2*, *AIRE* and *RMRP* genes to establish, improve and validate the safety of the pipeline, which can now be applied for other relevant PIDDs. Our data demonstrate a rapid and selective expansion of CD3+ T cells from patient and healthy donor peripheral blood mononuclear cells (PBMC) after 8-day cytokine stimulation. CRISPR guide-RNAs (gRNA) for *ADA2*, *AIRE* and *RMRP* were screened in the stimulated patient T cells, and off-targets of selected gRNAs were assessed using genome-wide, unbiased identification of double-strand breaks enabled by sequencing (GUIDE-seq), where no off-targets were identified. To enhance translational potential of the pipeline, we tested multiple strategies to increase homology-directed repair (HDR) and developed an HDR-optimized T cell pipeline that can achieve up to 80% mutation correction levels in patient T cells. Next, we assessed the safety of the pipeline and studied the impact of CRISPR editing at genomic, transcriptomic and proteomic level. First, we performed whole-genome sequencing (PacBio) on corrected cells, and only identified editing at the target site (*ADA2*). Then, we sorted CD3+ patient (ADA2) and HD T cells for Sc-seq analysis. These data demonstrate that nearly all (<90%) of the edits were biallelic and that apart from the target site, CRISPR did not induce significant transcriptomic changes in the cells. Finally, to study protein expression after mutation correction, we performed mass spectrometry analysis on corrected patient (ADA2) cells. We show that corrected patient T cells express ADA2 at levels which correlate with genomic correction. In conclusion, our work demonstrates that the CRISPR/Cas9 T cell pipeline is both efficient and safe and allows for precise custom editing in patient T cells. Furthermore, it has the potential to be converted into a GMP-compatible workflow and scaled up to fit clinical gene therapy needs for PIDD patients.

1653. Advancing X-Linked Agammaglobulinemia Therapy: A Universal Gene Targeting-Based Correction

Hana Y. Ghanim[1], Mara Pavel-Dinu[1], William Feist[1], Robel Fesseha[1], Sofia Elena Luna[1], Matthew H. Porteus[2]

[1]Stanford University, Stanford, CA,[2]Stanford University School of Medicine, Stanford, CA

Primary immunodeficiencies constitute a group of monogenic disorders marked by recurrent infections, autoimmunity, and inflammation. Among these, X-linked Agammaglobulinemia (XLA), predominantly affecting males, results from the inactivation of the Bruton tyrosine kinase (BTK) gene. This condition, if untreated, leads to childhood fatality. The current mainstream treatment, immunoglobulin replacement therapy, while life-sustaining, falls short of being curative, leaving patients vulnerable to respiratory infections and chronic lung disease. In addressing this unmet need, our research focuses on the development of a novel therapeutic approach: a universal gene targeting-based correction. This strategy involves the precise correction of the underlying genetic mutation in a patient's hematopoietic stem and progenitor cells (HSPCs), aligning with endogenous BTK gene regulation and protein expression. Our optimization and implementation of highly efficient gene-targeting tools and methods have yielded over 50% allelic modification at the BTK locus. Importantly, our approach has successfully restored expression levels to near-unmodified levels, establishing the feasibility and efficacy of this cutting-edge therapeutic strategy. Furthermore, our preliminary xenotransplantation studies provide compelling evidence of the successful engraftment of the integrated allele in the bone marrow. Impressively, this engraftment was not only maintained

but exhibited enrichment in the spleen. This observation is particularly intriguing considering existing literature, which suggests a selective advantage for B cells expressing a healthy BTK gene, underscoring the potential therapeutic benefits and the encouraging prospects of our gene-targeting approach. In upcoming studies, we plan to comprehensively characterize BTK-modified cells using highly-sensitive methods, including CyTOF-cytometry by time of flight, single-cell RNA sequencing, and Assay for Transposase-Accessible Chromatin. Beyond advancing XLA therapy, our research holds the potential to unravel novel insights into the pathophysiology of XLA disease.

1654. A Gene-Editing Approach to Study Variants of Unknown Significance (VUS) and Repair Patient Mutations in the Rare Disease Hemophagocytic Lymphohistiocytosis (HLH)

Dror Assa[1,2,3], Elise W. Wolf[1,2,3], Allison J. Bien[1], Srishti U. Sahu[1,3], Alžběta Ressnerová[1,4,5], Ross C. Wilson[1,3], David N. Nguyen[1,2,3]

[1]Innovative Genomics Institute, Berkeley, CA,[2]Department of Medicine, University of California San Francisco, San Francisco, CA,[3]Department of Molecular and Cell Biology, University of California Berkeley, Berkeley, CA,[4]Central European Institute of Technology, Brno, Czech Republic,[5]Mendel University in Brno, Brno, Czech Republic

Hemophagocytic lymphohistiocytosis (HLH) is a rare immunological disease characterized by fevers, cytopenia, and increased inflammatory cytokines' release leading to a life threatening condition. Familial HLH is caused by loss-of-function mutations in genes involved in cytotoxic granule release from immune cells such as $CD8^+$ T cells and Natural Killer (NK) cells. Mutations in the gene *PRF1*, which encodes for the protein perforin, is among the leading causes for familial HLH; however, in many cases gene variants have an unknown effect on its function, and therefore on the disease. Here, we demonstrate using CRISPR/Cas9 combined with homologous-directed repair templates (HDRTs) as a platform to investigate the effects of variants of unknown significance (VUS) on cytotoxic cells' function. We show that introduction of VUS from patients into healthy donor cells followed by enrichment of edited cells leads to a reduction of perforin levels, which is associated with decreased efficiency in a target cell killing assay. Finally, we used base editing in primary human T cells to revert a simulated patient mutation. In conclusion, our study introduces a novel platform for evaluating the impact of VUS on genetic diseases, offering valuable insights into the functional consequences of these variants. Additionally, we present a promising strategy for correcting disease-causing mutations using base-editing techniques.

1655. Targeted Base Editing Models Hematopoiesis and Pathophysiology of VEXAS Syndrome

Martina Fiumara[1], Raffaella Molteni[2], Corrado Campochiaro[3], Guido Pacini[1], Alessandro Tomelleri[3], Elisa Diral[4], Angelica Varesi[1], Alessandra Weber[1], Davide Stefanoni[2], Roberta Alfieri[5], Luca Basso-Ricci[1], Pamela Quaranta[1], Luisa Albano[1], Angelo D'Alessandro[6], Marco Matucci-Cerinic[3], Raffaella Di Micco[1], Alessandro Aiuti[1], Fabio Ciceri[4], Ivan Merelli[1], Lorenzo Dagna[3], Serena Scala[1], Simone Cenci[2], Giulio Cavalli[2], Luigi Naldini[1], Samuele Ferrari[1]

[1]SR-TIGET, Ospedale San Raffaele, Milan, Italy,[2]Division of Genetics and Cell Biology, Ospedale San Raffaele, Milan, Italy,[3]Unit of Immunology, Rheumatology, Allergy and Rare Diseases, Ospedale San Raffaele, Milan, Italy,[4]Unit of Hematology and Stem Cell Transplantation, Ospedale San Raffaele, Milan, Italy,[5]Institute for Biomedical Technologies, National Research Council, Milan, Italy,[6]Department of Biochemistry and Molecular Genetics, University of Colorado, Denver, CO

VEXAS syndrome is a newly described, treatment-refractory, severe, highly prevalent disease caused by somatic mutations in the *UBA1* gene in hematopoietic stem/progenitor cells (HSPCs). VEXAS is hallmarked by vacuoles in myeloid/erythroid precursors and presents with systemic inflammation. About 50% of patients develop hematologic malignancies or myelodysplasia, contributing to the poor prognosis. The pathophysiology and impact of VEXAS syndrome on human hematopoiesis remain elusive. Moreover, the lack of suitable disease models hampers investigation of disease development and preclinical drug testing. Here, we develop *in vitro* and *in vivo* models of VEXAS syndrome by targeted base editing strategies and validate them by multiparametric flow cytometry and multi-omic analyses on VEXAS patients. UBA1 base editing in wild-type human HSPCs installed with >90% efficiency the most common VEXAS mutation. Clonogenic assays of *UBA1*-edited HSPCs revealed an exclusive myeloid output *in vitro* and a significant loss of the erythroid HSPC differentiation potential compared to mock-edited controls. Xenotransplantation of edited HSPCs in immunodeficient mice resulted in a severe reduction in circulating B cells, while NK and myeloid compartments were preserved. Bone marrow (BM) HSPCs were 5-fold lower than in control mice and largely myeloid-biased. Concordantly, while myeloid cells and HSPCs were mainly (>80%) *UBA1* mutated, the lymphoid compartment showed predominance of wild-type alleles. Strikingly, the distribution of *UBA1*-mutant cells across lineages in humanized mice mirrored the composition of our VEXAS patient cohort, where mutant cells accounted for 90% of total myeloid cells/HSPCs and for less than 5% of lymphoid lineages. VEXAS patients' marrow showed unbalanced composition of the HSPC compartment, lower abundance of stem cells, multipotent and lymphoid progenitors, and expanded myeloid populations. This was paralleled by a significant expansion of circulating myeloid-biased HSPCs and immature myeloid cells. Single-cell transcriptomics on the hematopoietic compartment of VEXAS patients harboring different *UBA1*mutations identified specific HSPC populations of VEXAS patients and revealed upregulated stress response and immune activation pathways across VEXAS cell clusters compared with healthy controls. Overall, our data show that base editing-mediated insertion of somatic mutations causing VEXAS

syndrome in wild-type HSPCs phenocopies most features of patients' hematopoietic system. These models will support the dissection of mechanistic bases underlying VEXAS pathogenesis and enable preclinical testing of novel therapies.

1656. Creating High-Quality RNA-Seq Reference Data from ATCC's Verified Cell Lines

Ajeet P. Singh, Rula Khairi, Amy L. Reese, Jade Kirkland, Corina Tabron, Noah Wax, James Duncan, Robert Marlow, Steve King, Ana Fernandes, John Bagnoli, Briana Benton, Jonathan L. Jacobs
Sequencing & Bioinformatics Center, ATCC, Manassas, VA

ATCC®'s authenticated cell lines are vital for biomedical research. Despite the widespread use of RNA sequencing in many studies, a comprehensive dataset that captures the full RNA profiles of these cell lines is not readily available in the public domain. Such a dataset would serve as a reference control for experimental design and guide the selection of appropriate cell line models for specific use cases. Our rigorous RNA sequencing, backed by stringent quality control measures, has led to the development of the ATCC Cell Line Land tool. This tool provides valuable insights into the intrinsic transcriptional patterns and molecular identities of ATCC®'s cell lines, establishing it as a gold-standard reference for researchers.Our sequencing revealed previously unidentified RNA transcripts and regulatory elements, illuminating the cell lines' molecular complexities. A comparative analysis highlighted unique RNA expression patterns, distinguishing between various cell lines, and revealing molecular differences. Notably, this analysis identified variations between derivative and parental cell lines, genetically modified immortalized primary cells and native ones, and healthy primary and cancerous cells. RNA sequencing datasets can integrate with existing genomic and proteomic databases, enabling comprehensive multi-omics analyses and cross-validation of molecular insights. Researchers can utilize the RNAseq data from ATCC Cell Line Land upon receiving cells, saving time, and enhancing experimental clarity. The reference dataset facilitates informed cell line selection and establishes a reliable molecular baseline for experimental controls. Such foundational resources drive advancements in cellular biology, disease modeling, and therapeutic research. Our RNA sequencing framework sets the stage for integrating multi-omics data, exploring cellular responses, and refining molecular characterizations. Collaborative efforts are encouraged to continually update and refine these reference-quality databases, ensuring their relevance and applicability in evolving research landscapes.

1657. The Preclinical Mouse Model for Evaluating Therapeutic Approaches to Hemophilia A

Lingyu Song, Yunlong Jiang, BingZhou Han, Jianming Xu, Hongyu Wang, Xiang Gao
GemPharmatech Co. Ltd, Nanjing, China

Hemophilia A, a hereditary bleeding disorder characterized by deficiency or dysfunction of coagulation factor VIII (FVIII), poses substantial challenges in clinical management. Creating preclinical animal models that accurately mimic the pathological process of human disease is essential for advancing our comprehension of hemophilia A and investigating potential therapeutic approaches. We used the CRISPR-Cas9 system to knock out the region spans exon 16 and 17 of the mouse F8 gene in the C57BL/6J strain, creating the B6-F8-KO mouse model. The reduced expression level of F8 in the mouse liver is confirmed by using primers targeting three different mRNA regions. Quantification of FVIII activity in plasma revealed a significant reduction to approximately 3% of the levels observed in wild-type mice, closely resembling the clinical phenotype of moderate hemophilia A. Additionally, B6-F8-KO mice showed prolonged activated partial thromboplastin time, and increased bleeding volume in the tail-cut experiment. The F8 recombinant protein therapy in B6-F8-KO mice showed improved APTT, indicating promise for the model in evaluating new therapies for hemophilia A.In summary, the B6-F8-KO mouse model serves as a valuable resource for understanding hemophilia A and evaluating innovative therapeutic interventions.

1658. Evolution Directed Gene Engineering (EDGE) Identifies Factor VIII Variants Augmented for Gene Therapy in Hemophilia A Mice

Yani Suber[1], Lucas Van Gorder[2], Julia Chau[1], Kyumin Lee[1], Riley Peng[1], Benjamin Samelson-Jones[1]
[1]Center for Cellular & Molecular Therapies at Children's Hospital of Philadelphia, Philadephia, PA,[2]University of Pennsylvania, Philadelphia, PA

Introduction Emerging clinical trial results across multiple rAAV products for hemophilia A (HA) have questioned the long-term durability of high transgene-produced factor VIII (FVIII) levels. In order to address this limitation, we aimed to identify high activity variants FVIII variants which could provide enhanced hemostatic activity at low stable expression levels. To this end, we developed a novel screening approach Evolution Directed Gene Engineering (EDGE). Our previous studies suggested with coagulation factor IX has not evolved with optimized specific activity (Samelson-Jones et al. Blood Advances 2021). Based on this work, we hypothesized that FVIII similarly has not been optimized for high activity. EGDE utilizes sequence alignment of mammalian orthologues as well as the HA database to identify amino acid positions that have been evolutionary conserved but without missense variants that cause loss-of-function. *Results* EGDE identified FVIII variants with single amino acid substitutions at 12 position which resulted in ≥2-fold enhanced activity compared to FVIII-WT. Multiple amino acid substitutions at K659 (K659M, K659V and K659C) resulted in FVIII variants with up to 6 fold increase in activity. To evaluate if these EDGE-identified variants can enhance HA gene therapy, we administered rAAV8 vectors with B-domain deleted FVIII variants under the control of a liver-specific promoter to CD4/HA$^{-/-}$ mice at a dose of 4 x 10^{12} vg/kg. The transgenes were not codon optimized. After 8 weeks, mice treated with AAV8-FVIII-K569M (n=6) and AAV8-FVIII-K659V (n=4) had a median FVIII activity of 85 U/dL and 63 U/dL, respectively, which was significantly higher than AAV8-FVIII-WT (n=4) activity of 0.5 U/dL ($p < 0.01$) (**Figure**). Transgene expressed K659M and K659V variants also had a higher FVIII specific activity of 84,000 U/mg and 50,000 U/mg respectively when compared to the FVIII-WT of 15,000 U/mg. In a separate experiment, mice treated with 4 x 10^{12} vg/kg of

AAV8-FVIII-K659C also had enhanced FVIII activity levels of 30 U/dL after 14 weeks (n=6) compared to FVIII-WT activity of 5 U/dL (n=5). Further, the HA mice expressing FVIII-K658C normalized their bleeding with a median blood loss of 5 uL/g after tail clip injury (bleeding was not significantly different p=0.5 than WT mice, blood loss was 4 uL/g), while mice expressing FVIII-WT had higher blood loss of 10 uL/g (p=0.03). These results indicate the EDGE identified FVIII variants that enhance rAAV gene therapy for HA. *Conclusions* The EDGE screen can be used to identify single missense mutations in FVIII that result in enhanced specific activity. FVIII variants FVIII-K659M, FVIII-K659V and FVIII-K659C exhibit hyperactivity and improved hemostasis after rAAV gene therapy in mice compared to FVIII-WT. These novel variants have potential to address the current limitations of rAAV gene therapy for HA and EDGE may be helpful for other disease targets.

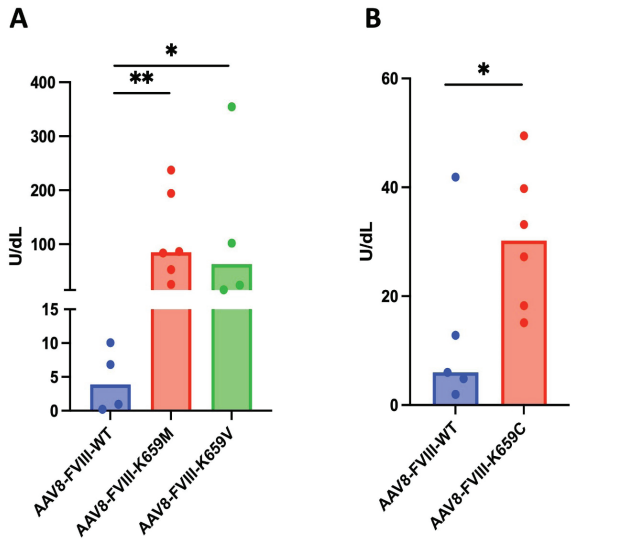


Figure 1. FVIII activity after liver-directed gene therapy of K659 variants in mice. (A) FVIII activity 8 weeks after injection with 4 x 10^{12} vg/kg AAV8-FVIII-WT (n=4), AAV8-FVIII-K659M (n=6) or AAV8-FVIII-K659V (n=4). (B) FVIII activity 14 weeks after injection with 4 x 10^{12} vg/kg with AAV8-FVIII-WT (n=5) or FAAV8-FVIII-K659C (n=6). Bars represent the median and statistical significance was determined by one-tailed Mann-Whitney with p-values ≥ 0.5 considered not significant (ns). 1-4 mice per experiment were removed from analysis because of assumed vector administration challenges based on no detectable FVIII activity or antigen.

1659. Intraosseous Delivery of a Platelet-Specific Factor VIII-Lentiviral Vector to Treat Hemophilia A in a Canine Model

Cameron W. Rementer[1], Timothy C. Nichols[2], Chong Li[3], Xiaohe Cai[1], Ting-Yen Chao[4], Yuanyi Xu[4], Elizabeth Merricks[5], Carol H. Miao[6]

[1]Center for Immunity & Immunotherapies, Seattle Children's Research Institute, Seattle, WA,[2]UNC at Chapel Hill Francis Owen Blood Rsch. Lab., Chapel Hill, NC,[3]Seattle Childrens Hospital, Seattle, WA,[4]Seattle Children's Research Institute, Seattle, WA,[5]University of North Carolina, Chapel Hill, Chapel Hill, NC,[6]Seattle Children's Research Institute, Seattle, WA

Hemophilia A (HemA) is a genetic disease resulting from a factor VIII (FVIII) deficiency. Traditional treatments for HemA are costly and require repeated dosing of replacement protein. Additionally, current AAV-mediated gene therapy generated decreased levels of FVIII over time. We demonstrated previously that intraosseous (IO) gene therapy via delivery of lentiviral vectors (LVs) into bone marrow targeting FVIII expression in platelets successfully treated HemA mice. To facilitate clinical translation, we investigated the treatment of HemA dogs using this approach. An LV vector containing the cFVIII gene was injected into the tibia/iliac bones of 6 HemA dogs (4 in Cohort #1, 2 in Cohort #2). Blood samples were taken at various timepoints post-procedure. For the dogs in Cohort #2, we incorporated several improvements, including performing injections in both the tibia and the iliac bone. Additionally, perioperative hemostasis was performed using bypassing agents to avoid inhibitor formation. All dogs recovered well from the procedure and had blood chemistry values within normal ranges. Expression of cFVIII was examined in platelets and plasma isolated from LV-treated dogs by ELISA and could be detected in platelets with the highest expression at 5-10 mU/10^8 platelets around 1-2 months post-procedure. The improvement of blood coagulation was evaluated by whole blood clotting time (WBCT) and was found to be shortened in multiple time points shortly after IO gene therapy, indicating improved hemostasis. Encouragingly, the dogs experienced fewer bleeding events per year after gene therapy treatment compared to baseline, indicating a partial phenotypic correction. We have established an IO-LV gene therapy protocol to treat HemA dogs successfully with persistent effects of treatment over 2-5 years, including a phenotypic improvement. This represents a potential strategy for gene therapy in HemA patients.

Friday Posters: Epigenetic Editing and RNA Editing

1660. Tunable Transgene Expression Using an ADAR-Mediated Gene Switch

Joseph Silverberg[1], Laura Pereira[1], Daniel M. Cohen[1], Federico Mingozzi[2], Sean M. Armour[1]

[1]Discovery, Spark Therapeutics, Inc., Philadelphia, PA,[2]Former Employee of Spark Therapeutics, Inc., Philadelphia, PA

Adeno-associated viruses (AAVs) are a powerful tool to deliver gene therapies, especially for gene augmentation purposes. However, a limitation of classical expression cassettes is their inability to tune the level of transgene expression post-transduction. The field of gene therapy could benefit from technologies that enable tunable expression of transgenes. One approach to overcome this challenge is to take advantage of gene editing technologies to control the expression of the episomal gene by introducing precise point mutations in the AAV genomes or AAV-derived transcripts. Adenosine deaminase acting on RNA (ADAR) is an enzyme that allows for post-transcriptional editing in both coding and non-coding regions of double-stranded RNA (dsRNA) by editing adenosine-to-inosine (A-to-I). Inosine is interpreted by the ribosome as guanosine (G) during translation, functionally introducing an A-to-G edit. Here, we explore the potential for ADAR to mediate tunable expression of Human Factor IX, hFIX, in the context of AAVs. Within the hFIX transgene sequence, we identified tryptophan codons (TGG) that could be mutated to premature terminating codons (PTC) encoded by an amber stop codon (TAG). One codon, W118STOP, completely inhibited hFIX expression. To edit W118STOP PTC (TAG) back to the native tryptophan codon (TGG) we

designed a trigger RNA for the recruitment of the endogenous ADAR enzyme to enable hFIX expression via an A-to-G point mutation. Huh7 cells transiently co-transfected with W118STOP and trigger RNA achieved expression levels ~40% of wildtype hFIX, whereas those transfected with W118STOP alone had no detectable levels of FIX protein. Sanger sequencing results indicated that ~75% of the mRNA achieved the desired A-to-G edit changing the PTC back to the native tryptophan codon. Incomplete rescue of hFIX expression following editing prompted evaluation of hFIX transcript abundance. The amount of hFIX mRNA in W118STOP was statistically lower than wildtype p=0.0001 and was unaffected by the addition of the trigger RNA. These results demonstrated that our ADAR-based strategy could successfully turn on transgene expression *in vitro*. The ability of an ADAR gene switch to control FIX expression *in vivo* is being investigated using Spark100-ApoE-hAAT FIX AAV vectors. Our work demonstrates the potential to achieve tunable expression of an AAV-delivered transgene using an ADAR-responsive premature termination codon.

1661. Epigenetic Reactivation of γ-Globin Expression as a Novel Curative Option for β-Hemoglobinopathies

Simone Amistadi[1], Letizia Fontana[1], Chiara Magnoni[1], Panagiotis Antoniou[1], Ali Faiq[2,3], Melissa Whitehead[2,3], Claudio Mussolino[2,3], Annarita Miccio[1]

[1]Imagine Institute, Paris, France,[2]Institute for Transfusion Medicine and Gene Therapy, Freiburg, Germany,[3]Medical Center - University of Freiburg, Freiburg, Germany

β-hemoglobinopathies are severe genetic diseases caused by mutations affecting the production of the β-globin chain. Their clinical severity is mitigated by the co-inheritance of mutations that reactivate γ-globin (*HBG*) genes expression and result in elevated production of fetal hemoglobin (HbF) in adults, a condition named hereditary persistence of HbF (HPFH). While the transplantation of allogenic hematopoietic stem and progenitor cell (HSPC) provides a definitive therapy for patients with β-hemoglobinopathies, the frequent absence of a compatible donors demands for alternative strategies such as the transplantation of autologous HSPCs upon gene correction. Targeted nucleases and base editors have been exploited to recreate HPFH mutations and reactivate HbF. However, the unwanted off-target cleavage activity and the genotoxicity associated with these genome editing strategies raise safety concerns for clinical applications. Therefore, novel and safer therapeutic strategies are still needed for the treatment of β-hemoglobinopathies.Here, we investigated the epigenetic regulation of the *HBG* genes to develop novel therapies based on programmable epigenome editing. We aimed at modulating gene expression without altering the underlying DNA sequence. Analysis of epigenetic marks at the *HBG* promoters revealed a distinct pattern of modifications in adult versus fetal erythroid cells. Primary fetal erythroblasts, which express γ-globin, showed enrichment of H3K27ac and H3K4me3 and low DNA methylation, marks typically associated with active transcription. To recreate this epigenetic configuration at the *HBG* promoters in adult cells, we tested CRISPR-based epigenome editors (capable of depositing histone acetylation and removing DNA methylation) in HEK293T, a cell line that expresses low γ-globin levels. These editors reached up to 10^4-fold increase in *HBG* expression with the most effective combination of single guide RNAs spanning the promoter region. We further validated the strategy in HSPCs derived from patients with sickle cell disease, by RNA delivery of the editing components. We observed no alterations between treated and non-treated HSPCs during their differentiation towards the erythroid lineage. In HSPC-derived erythroblasts we observed an increased transcription of *HBG* genes and higher amount of HbF positive cells, as assessed by flow cytometry. Importantly, this correlated with high levels of HbF production (~50%), measured by cation-exchange HPLC, and a substantial correction of the sickling phenotype in mature erythrocytes. Altogether, these results confirmed the crucial role of epigenetic modifications in regulating the β-globin locus and the possibility to reprogram the epigenome to reactivate *HBG* expression and HbF production. In conclusion, this strategy overcomes the genotoxicity risks associated with the use of DNA-cleaving enzymes and offers a safe and effective alternative for the treatment of β-hemoglobinopathies.

1662. Simultaneous Five-Gene Epigenetic Editing to Generate TGF-b and Adenosine-Resistant Allogeneic CAR T Cells

McKensie A. Collins, Laura Kehoe, Avery Houston, Kunza Ahmad, Thijs Udo, Moe Zaker, Scott Clarkson, Benjamin Hallisey, Kendra Bayne, Melissa Pannone, Mary Morrison, Ricardo N. Ramirez, Ari E. Friedland, Pietro Spinelli, Vic Myer, Aron Jaffe, Jamie Schafer

Chroma Medicine, Boston, MA

CAR T cell therapy has generated meaningful clinical responses in patients with CD19+ and BCMA+ hematologic malignancies but cures are limited and patients with solid tumors continue to have poor responses to CAR T. One contributing factor is immunosuppressive molecules in the tumor microenvironment (TME) that limit anti-tumor responses. Soluble factors like adenosine and TGF-b—both well-characterized as drivers of profound immunosuppression—may inhibit the efficacy and durability of CAR T responses. Additionally, the use of autologous cells requires individualized manufacturing which increases cost and restricts availability, with many patients succumbing to progressive disease before receiving treatment. Allogeneic CAR T cells have the potential to address these bottlenecks, but durable responses are limited. Editing of multiple genes (multiplex editing) is necessary to overcome the above limitations but approaches that nick or cut DNA often cause undesired genomic rearrangements. However, epigenetic editing has the potential to durably regulate multiple genes in a single step without cutting or nicking the DNA. This may enable efficient generation of complex cell products such as allogeneic CAR T cells that evade TME-mediated immunosuppressive mechanisms. In this study, activated T cells were engineered via nucleofection with a dCas9-based epigenetic editor and gRNAs to silence the receptors for TGF-b and adenosine: TGFBR2 and ADORA2A. Target expression was durably repressed, as assessed by flow cytometry and RT-qPCR respectively, in single- and dual-target conditions for 35 days through repeat anti-CD3/CD28 stimulations. Next, multiplex-silenced BCMA-targeting CAR T cells were generated and downstream signaling in response to TGF-b or adenosine, cytotoxicity, proliferation, and cytokine production were evaluated following antigen challenge via a BCMA+ cell line. Downstream signaling as assayed via flow cytometry for phospho-SMAD and phospho-CREB showed an ~80% (pSMAD) and ~60% (pCREB) reduction in response to TGF-b

or 2-chloro-adenosine respectively, equivalent to Cas9 knockout controls. Multiplex-edited CAR T cells also had improved anti-tumor potency at multiple effector:target ratios in the presence of TGF-b, and comparable cytotoxic function to unedited CAR T cells under standard culture conditions. Silenced cells were also more proliferative than unedited CAR T cells in the presence of TGF-b following tumor stimulation. IFNg production by silenced cells was uninhibited by TGF-b, while unedited CAR T cells showed a nearly 70% reduction in IFNg production following tumor re-stimulation. As duration of response is an issue for allogeneic products, we believe the inclusion of survival- or potency-enhancing edits with allogeneic modifications addresses a significant unmet need. We combined TME and allogeneic edits, producing cells in which five targets—TGFBR2, ADORA2A, B2M (to reduce MHC class I), RFXAP (to reduce MHC class II), and CD3D/CD3G (to reduce TCR)—were durably silenced in a single step. Expression was repressed at >90% for all five targets for 35 days through two re-stimulations and a 95% reduction in mRNA expression of ADORA2A, highlighting the efficiency of generating a multi-edited cell without genotoxic risk. Simultaneous silencing of five genes demonstrates the multiplexing capability of epigenetic editing and shows the potential to improve allogeneic CAR T cell therapies by rendering the cells resistant to TGF-b and adenosine while maintaining genomic integrity. Chroma's epigenetic editing technology may enable development of the complex next-generation CAR T cell therapies required to improve patient access and overcome biological barriers to success.

1663. Durable *HTT* Silencing Using Non-Evolved dCas9 Epigenome Editors in Patient-Derived Cells

Jennifer Waldo[1,2], Julian A. N. M. Halmai[1,2], Ankita Singh[1,2], Maria N. Florendo[1,2], Jan A. Nolta[2], Kyle D. Fink[1,2]

[1]Ctr. for Interventional Genetics, MIND Institute, Department of Neurology, UC Davis Medical Center, Sacramento, CA,[2]Stem Cell Program, Gene Therapy Center, Institute for Regenerative Cures, UC Davis Medical Center, Sacramento, CA

Huntington's Disease (HD) is an autosomal dominant neurodegenerative disorder caused by a trinucleotide repeat in exon 1 of the Huntingtin (*HTT*) gene. This expansion leads to protein misfolding that causes widespread cellular dysfunction and ultimately leads to neuronal cell death. The advent of nuclease-deficient CRISPR-Cas9 (CRISPR-dCas9) gene regulation technologies allows targeting of the causative gene and subsequent downregulation via fused effector domains that induce heterochromatin at the epigenetic level through DNA methylation (DNMT3A/L) and H3K9me3 deposition (KRAB), blocking transcription. Therefore, we propose using dCas9 epigenetic editing to downregulate *HTT* as a therapeutic approach for HD. *HTT* has large haplotype blocks that allow for allele-specific targeting based upon the presence of heterozygous single nucleotide polymorphisms (SNPs). These SNPs are in genomic regions devoid of NGG PAM sites, a requirement for *sp*dCas9 binding. To address this bottleneck, a screen was conducted of multiple dCas9 variants fused to KRAB and DNMT3A/L with increasingly expanded PAM targeting to initially assess the ability to downregulate total *HTT*. Surprisingly, only *sp*dCas9 was able to significantly knockdown *HTT*, while expanded PAM site variants dxCas9 and dCas9-VQR were less efficient in reducing *HTT* expression. ChIP-qPCR of the *HTT* promoter showed a decrease in the binding efficiency of dCas9 variants, likely leading to the decreased efficiency of *HTT* downregulation. We further investigated DNA methylation changes through reduced representation bisulfite sequencing, showing high on-target increases in DNA methylation and few off-targets. In addition, we demonstrate mitotically stable *HTT* silencing of up to 6 weeks *in vitro* in a rapidly dividing cell line. We then assessed total *HTT* knockdown in HD patient-derived fibroblasts and neuronal stem cells. We identified significant downregulation of *HTT* in our treatment group compared to an unguided control. RNAseq was used to identify differential gene expression between treatment and controls, and gene ontology analysis was performed to identify the rescue of biological processes involved in *HTT* molecular pathogenesis. Off-targets were assessed through overlaying genome-wide changes in H3K9me3 enrichment, dCas9 binding, and differential gene expression. An additional bottleneck is the delivery of large dCas9 epigenome editors to the CNS. Current studies are addressing novel dual AAV and viral-like particle delivery for our identified epigenome editors *in vivo*. This approach holds great promise for those suffering from HD.

1664. Endogenous ADAR Based RNA Editing Therapy for Usher's Syndrome Type II

Pengfei Yuan[1], Huixian Tang[2], Zexuan Yi[1], Gangbin Tang[1], Yongjian Zhang[1], David Gamm[3,4], Divya Sinha[3], Yanxia Zhao[1]

[1]EdiGene Inc., Beijing, China,[2]Peking University, Beijing, China,[3]Waisman Center, University of Wisconsin– Madison, Madison, WI,[4]Department of Ophthalmology and Visual Sciences, McPherson Eye Research Institute, Madison, WI

Usher Syndrome type II (USH2) is a hereditary condition marked by retinitis pigmentosa and moderate to severe hearing loss, primarily resulting from mutations in the USH2A gene. This gene, notable for its substantial size, produces Usherin, a protein essential for the structural and functional integrity of both the retina and inner ear. A prominent mutation in USH2A, c.11864G>A (p.Trp3955*), introduces a premature stop codon, significantly impairing Usherin function. This mutation exhibits diverse geographical prevalence across Europe, with exceptionally high incidence in Slovenia, where 82.5% of USH2A patients possess it. It also accounts for 20% of USH2A patients in Germany. Programmable A to G base editing offers a theoretical solution for correcting this mutation. Beyond CRISPR-based ABE, various ADAR-dependent RNA base editing tools have been developed. These tools leverage adenosine deaminases acting on RNA (ADAR) mediated RNA editing, a widespread eukaryotic cell process where adenosine is deaminated to inosine, which is subsequently recognized as guanosine in splicing or translation, enabling genetic message rewrites. Among these, LEAPER and its advanced version, LEAPER 2.0, facilitate precise, efficient, and enduring RNA transcript editing using engineered circular arRNAs (circ-arRNAs) and endogenous ADAR. Compared to CRISPR-based ABE, LEAPER avoids permanent DNA damage, desirable or otherwise, and potential in vivo immunogenicity. Based on LEAPER RNA editing, we tried to develop therapy for the USH2A c.11864G>A mutation. In this study, we first refined arRNA for high editing efficacy and minimal bystander impact using a mini-reporter system in 293T cells. We then incorporated this arRNA into

AAV and tested it in retinal organoids differentiated from CRISPR-edited iPSCs carrying the homologous c.11864G>A mutation and patient-derived iPSCs with compound heterozygous c.11864G>A. The editing rate reached up to 50% in CRISPR-edited and 35% in patient-derived cases, with minimal bystander effects observed. Since no functional animal disease model exists for the USH2A c.11864G>A mutation, we substituted mouse USH2A gene exon 60 with human Exon 61 harboring the c.11864G>A mutation. This model showed clear hearing loss at high frequency range without affecting vision. LEAPER delivered by AAV effectively alleviated hearing impairment in this humanized mouse model. To further evaluate the editing efficiency and safety in non-human primates (NHPs), we targeted an adjacent adenosine in the NHP USH2A 11864 site as a proxy, administering AAV8-arRNA through sub-retinal injections. Editing at the targeted site achieved up to 40% efficiency in the infected retina, with no safety issues observed over a six-month monitoring period. Collating our findings from the retinal organoid studies, the humanized mouse model, and NHP experiments, we posit that our approach shows significant potential as a therapeutic intervention for the USH2A c.11864G>A mutation.

1665. Evaluating CRISPR Activation Therapy for Pitt-Hopkins Syndrome

Renee Napoliello[1], Katherine L. Lucot[1], Julian Halmai[2], Kyle D. Fink[2]

[1]UC Davis School of Medicine, Sacramento, CA,[2]UC Davis, Sacramento, CA

Introduction: Pitt-Hopkins Syndrome (PTHS) is a genetic intellectual disability with no FDA-approved treatments. PTHS is caused by *de novo* mutations in *Transcription Factor 4* (*TCF4*), which result in haploinsufficiency (expression of a single wild type allele is insufficient for cellular function). *In vitro*/*vivo* studies of rodent and human PTHS models identified *TCF4* as critical for both neuronal development and postnatal function. *Kim et al. 2022* postnatally reinstated *Tcf4* expression in a mouse model of PTHS to fully rescue behavior associated with learning and memory, and partially normalize electrophysiological and molecular markers of neuronal function. It is plausible that restoration of *TCF4* expression could ameliorate symptoms in human cases of PTHS. CRISPR activation (CRISPRa) is a fusion of a nuclease-deficient Cas protein with a transcriptional activator; when targeted to regulatory genomic regions this tool can promote expression of target genes. CRISPRa can be employed to regulate *TCF4* expression by targeting the promoter of *TCF4*. However, the most common and well-characterized iteration of CRISPRa (based on spCas9) is too large to package into a single AAV (Adeno-Associated Virus). This is of concern as AAVs are a favored delivery method for DNA-based therapeutics. AAVs have low immunogenicity, can target specific cell types, and deliver their cargo as a stably expressed nuclear episome. For translational relevance of evaluating CRISPRa to treat PTHS, three methods to circumvent AAV packaging limits were evaluated. These methods were: a dual-plasmid system using the standard spCas9 with a protein intein split fusion, a single plasmid system with the small ortholog cjCas9, and a single plasmid system with the small ortholog cas12f. *Methods:* 23 gRNAs designed for spCas9-based CRISPRa targeting *TCF4* were screened in HEK293 cells. Nine guides were from a dataset based on a predictive algorithm (Horlbeck, et al., 2016) and 14 from the ChopChop software. The gRNA expression plasmid was co-transfected with two plasmids encoding half each of the split spCas9 CRISPRa. qPCR was used to assess changes in *TCF4* expression. Lead gRNAs were selected and the same experiment performed on neural stem cells (NSCs). Twenty gRNAs for cjCas9 and for cas12f (orthologs with AAV-packaging competent CRISPRa constructs) were designed with the CCTop software to target *TCF4*. Transfections of gRNA-expressing and effector domain-expressing plasmid were performed in HEK293s, followed by qPCR. The same transfection with lead gRNAs was performed in neural stem cells (NSCs). *Results:* From HEK293 transfection several spCas9 gRNAs exhibited significant upregulation of *TCF4* using the dual-plasmid method. The lead spCas9 guide was chosen as the gRNA with the greatest TCF4 expression fold change in NSCs. In addition, from HEK293 transfection, several cjCas9 and cas12f gRNAs exhibited significant *TCF4* upregulation, and the lead guide for each ortholog was chosen based on transfection in NSCs, similar to the spCas9 experiments. *Conclusion:* CRISPR activation can significantly upregulate *TCF4* across multiple cell lines. Further, multiple translationally relevant iterations of CRISPRa are effective at targeting TCF4 for activation. In future studies, the three CRISPRa constructs paired with their most potent gRNAs will be further tested via AAV transduction. Multiple PTHS patient-derived neuronal cultures will be treated with the CRISPRa-containing capsids to evaluate potency of *TCF4* upregulation and extent of functional rescue of disease phenotype.

1666. dCasMINI Mediated *ZC4H2* Reactivation in Human Cells

Yomira Palacios, Julian Halmai, Kyle D. Fink

Neurology, UC Davis, Davis, CA

ZC4H2 Associated Rare Disorders (ZARD) is an ultra-rare X-linked intellectual disability caused by a spectrum of inherited and de novo mutations in the *ZC4H2* gene. *ZC4H2* encodes a zinc finger protein that is highly expressed in the brain during early neurodevelopment, yet its direct role in regulating neurodevelopment remains elusive. Female somatic cells undergo random X chromosome inactivation to regulate gene dosage. In X-linked intellectual disability, affected regions such as the brain, result in mosaic cells that express either the wildtype or mutant allele. In mutant-expressing cells, the presence of the second protective X chromosome can be utilized to reintroduce the expression of the healthy silenced gene. Our group was the first to successfully demonstrate the feasibility of reactivating the wildtype allele in mutant loss of function cells of an X-linked neurodevelopmental disorder in female somatic cells. This approach relied on using CRISPR/dCas9 fused to the catalytic domain of DNA methylcytosine dioxygenase TET1 which reactivates expression by active removal of promoter DNA methylation. Although, this tool offers a promising therapeutic approach for X-linked disorders primarily affecting females, packing limitation remains a current problem for therapeutic delivery. In addition, while about 60% of human genes regulate expression through CpG islands in their promoter regions, the *ZC4H2* promoter lacks CpG islands. This highlights the need to explore alternative pathways for gene reactivation, potentially providing a simpler mechanism without the generation of differentially methylated epi-alleles. Recently, a newly engineered miniature CRISPR-Cas system was developed that demonstrated robust gene upregulation of silenced autosomal genes in HEK293T cells when fused to a transcriptional activator

domain (VPR). These results demonstrate the feasibility of gene upregulation from a miniature CRISPR system. Our experiments utilize this improved CRISPR ortholog (dCasMINI) to demonstrate the feasibility of reactivating an X-linked gene in clinically relevant cells in a methylation-independent manner. Electroporation-mediated delivery of dCasMINI fused to VPR has demonstrated gene reactivation of *ZC4H2* in patient-derived B-lymphocytes. Ongoing studies address gene reactivation of *ZC4H2* in iPSC derived Neural Stem Cells (NSC). In addition, RNA collection for mutant and treated NSC have been done to perform RNA sequencing to address rescue of differential expressed genes associated with ZARD. This study can serve as proof-of-principle for other X-linked genes and broaden the therapeutic implication of smaller Cas-mediated programmable transcription.

1667. Gene Therapy Approach for Neurofibromatosis Type 1 via Cell-Specific Epigenetic Editing in Schwann Cells

Adele Guglielmi[1], Max Miller[1], Niraj R. Punjya[1], Ulrika Beitnere[2], Tomas Salter-Cid[1], Claire Caputo[1], Kenneth Sims[3], Kyle D. Fink[4], Henriette O'Geen[1], David J. Segal[5]

[1]Genome Center, UC Davis, Davis, CA,[2]Molecular Biology, Max Delbrück Center for Molecular Medicine, Berlin, Germany,[3]Battelle's Advanced Materials and Energetics Group, Battelle Memorial Institute, Columbus, OH,[4]Institute for Regenerative Cures, UC Davis, Sacramento, CA,[5]Genome Center, UC Davis Genome Center, Davis, CA

Neurofibromatosis type 1 (NF1) is a genetic tumor predisposition syndrome arising from mutations in neurofibromin (NF), a negative regulator of RAS activity. The bi-allelic inactivation of neurofibromin in a subpopulation of Schwann cells (SCs) leads to the occurrence of neurofibromas, the primary cause of morbidity in NF1 patients. The clinical and genetic heterogeneity, the cell and tissue specific symptoms of NF1, together with the complexity of the ubiquitously expressed RAS pathway, require the development of a versatile therapeutic approach to selectively target both the peripheral and central nervous system. Our strategy aims to obtain the gene repression of the downstream mitogen-activated protein kinases MAP2K1-2 in the RAS signaling through a persistent, cell specific epigenetic editing. A synergistic effort has been made to develop, on one hand, a non-viral, cell-type targeted vector carrying a CRISPR-based tool to counteract the effects of the mutated NF, to be tested *in vitro*, and, on the other, a polymer nanoparticle (PNP) delivery system able to vehicle large DNA payloads *in vivo*. We are cloning a single vector housing the SCs specific promoter MPZ(P0), together with the inactivated (dead) form of CRISPR-associated nuclease (dCas9), the DNA methyltransferase (DNMT3A-L) and the repressor KRAB domains, the TdTomato reporter and two MAP2K1-2 guide RNAs, previously screened in Neuro2A cells, for their ability to successfully downregulate the mRNA targets. The generated vector will be initially tested *in vitro* via nucleofection in the murine Schwann cell line. The TdTomato positive cells will be sorted and replated to monitor in time the repression of the two targets MAP2K1 and 2 by qPCR and WB analysis. In parallel, more than 500 PNPs have been synthetized and screened *in vitro* for their DNA loading capability and transfection efficiency. The PNPs biodistribution will be evaluated also *in vivo* in murine models. Ultimately, the final vector will be loaded in the pre-screened top PMPs and delivered *in vivo* to identify the best delivery candidates for their ability to downregulate MAP2K1 and 2 expression in wild type mice.

1669. Accelerating the Discovery of Novel Hypercompact Transcriptional Activators with Machine Learning

M. Zaki Jawaid, T. Blair Gainous, Kavita Jadhav, Aayushma Gautam, Chris Still, Timothy P. Daley, Alexandra Collin de l'Hortet, Dan O. Hart, Robin W. Yeo

Epic Bio, South San Francisco, CA

Due to the programmability and versatility of CRISPR-Cas systems, gene therapy research is emerging as an increasingly promising strategy to treat genetic disease. However, many genetic diseases, such as those caused by haploinsufficiency, cannot be treated with traditional knock-out based CRISPR gene therapies, since they are caused by allelic loss-of-function. Due to its ability to both activate as well as suppress gene expression, epigenetic editing using CRISPR-dCas systems has the potential to address many genetic diseases that are unsuitable for traditional gene therapies. However, the versatility of epigenetic editing is constrained, especially in the context of gene activation, by the limited number and large size of most modulator peptides, severely limiting their therapeutic utility. To address these limitations, we have built a generative AI platform capable of designing de novo hypercompact modulator peptides with the ability to transcriptionally upregulate a genetic locus. We first collected a large corpus of training data by performing high-throughput screens to discover novel transcriptional activators among peptides derived from human, viral, and archaeal proteomes. We then trained a machine learning ensemble model, composed of a decision tree model and a convolutional neural network, which leverages transfer learning (via large protein language model embeddings) to predict transcriptional activators based on peptide sequence alone. By exploiting a novel sampling algorithm, which we call evolutionary Monte Carlo search, to more efficiently traverse the predicted activator fitness landscape, we used this machine learning platform to generate a library of several thousand hypercompact peptides predicted to be transcriptional activators. We experimentally screened these peptides and validated that our generative AI approach dramatically increased discovery rate (up to 45-fold) resulting in the discovery of hundreds of novel transcriptional activators sharing little sequence similarity with known naturally occurring peptides. We next investigated the evolutionary, biochemical, and biophysical properties of the synthetic activator library, revealing that validated activators consistently lack conserved functional domains but do share certain biochemical features, such as strong negative electrostatic potential. We subsequently selected 10 of our top synthetic activators for further characterization, and assessed their activation strength by screening them at an artificial GFP locus as well as at an endogenous human locus (CD45), comparing their potency to gold standard activators (e.g. VPR, VP64, ···). Additionally, using RNA-seq, we assessed off-target gene expression changes to evaluate the specificity (and thus safety) of on-target transcriptional upregulation using a synthetic activator. These results demonstrate the capability of machine learning to accelerate the discovery of novel functional peptides to expand our toolbox of epigenetic modulators for future therapeutic applications.

1670. Comprehensive Engineering of a CasX-Based Repressor to Create Highly Potent Epigenetic Editors

Jason D. Fernandes, Emeric J. Charles, Ross White, Kirsten A. Reimer, Christopher Duncan-Lewis, Ludmila Lokteva, TC Steven Keller, Shyam Sundhar Bale, Raniel Alcantara-Lee, Ashley T. Wong, Benjamin L. Oakes, Sarah K. Denny

Scribe Therapeutics, Alameda, CA

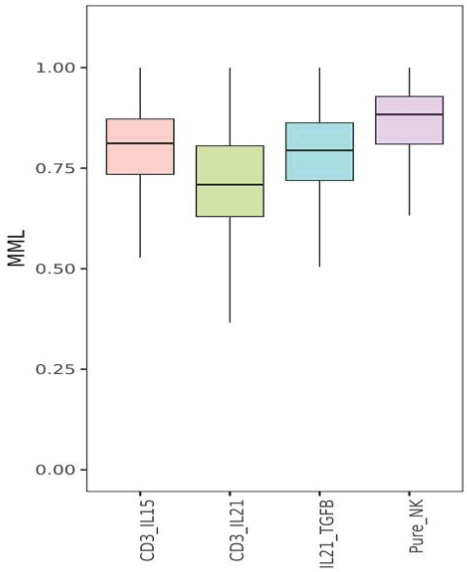
Epigenetic editing is a powerful and innovative approach to control the expression of disease-associated genes without permanently changing the underlying DNA sequence. Current systems for epigenetic modulation are limited by their large sizes that hinder effective delivery and potency issues that require high doses for efficacious knockdown. Improving these characteristics would require effectively harnessing multifactorial epigenetic machinery to achieve potent and sustained gene perturbation. Here, we leveraged comprehensive engineering approaches to query from tens of thousands of novel compositions to create unique epigenetic repressors based on catalytically-inactive versions of compact, highly engineered CasX (Cas12e) that enable the precise targeting and repression of therapeutic targets in vitro and in vivo. These molecules and subsequent learnings for building epigenetic silencing tools were discovered by testing comprehensive libraries containing tens of thousands of distinct transcriptional repressor domains mined from genomes across the tree of Life. By assessing target knockdown in pooled human cell screens, we were able to identify compact transcriptional repressor domains that were significantly more potent than the widely used repressor domains in CRISPRi. Many of our newly identified domains were significantly diverged from natural human repressor domain sequences and substantially enhanced repression. Tethering the resulting CasX-based repressors (XR) to engineered DNA methyltransferase domains produced optimized Epigenetic Long-Term X-Repressors (ELXR) that achieved sustained and heritable silencing of a therapeutically relevant locus in vivo when delivered via mRNA in a single dose of LNP. Our findings demonstrate that holistic and extensive engineering of CRISPR epigenetic repressors can provide significant benefits in achieving potent and sustained gene repression across multiple in vitro and in vivo systems.

1671. Defining the Epigenetic Signatures of *In Vitro* Expanded NK Cells Used for Cancer Therapy

Megan Zinsky[1], Natalie Holl[2], Ruyan Rahnama[1], Jun Choe[2], Danielle Grace Jones[2], Oscar Martinez[2], Michael Koldobskiy[1], Challice Bonifant[1]

[1]Pediatric Oncology, Johns Hopkins University School of Medicine, Baltimore, MD,[2]Johns Hopkins University School of Medicine, Baltimore, MD

Background: Natural Killer (NK) cells have powerful anti-tumor effect as innate lymphoid cells, especially for acute myeloid leukemia (AML), since myeloid blasts express NK cell activating ligands. NK cells in AML patients have been shown to be dysfunctional; therefore, treatment with adoptive transfer of expanded NK cells is promising. Since large NK cell doses are required for treatment efficacy, NK cells must be activated and expanded after isolation through cell-cell contact and cytokine stimulation. Therefore, NK cells expanded for clinical trials are commonly co-cultured with irradiated K562 "feeder" cells expressing membrane bound IL-15 or IL-21 and 41BB ligand, and then maintained in culture with supplemental IL-2, to sustain an activated phenotype. Recent studies have described improved NK cell anti-tumor functionality by also treating cells *in vitro* with the immunosuppressive cytokine TGFβ. Our goal is to describe the epigenetic signature of NK cells expanded *in vitro* using these techniques. **Hypothesis**: Whole genome profiling of NK cells expanded using clinically relevant manufacturing methods will elucidate global DNA methylation patterns that correlate to optimal NK cell activation and *in vivo* anti-tumor cytotoxicity. **Methods**: To expand human NK cells, CD3+ cells were depleted from human donor peripheral blood mononuclear cells. Remaining cells were co-cultured with: (1) K562.mbIL15.41BBL + IL-2; (2) K562.mbIL21.41BBL + IL-2; (3) K562.mbIL21.41BBL + IL-2 + TGFβ and maintained in culture for 10 days. Each expansion was performed three times. NK cell purity was tested before isolating genomic DNA for whole genome bisulfite sequencing (WGBS). DNA isolated from freshly isolated, non-activated NK cells were included as a control. WGBS libraries were sequenced using 150-bp paired end indexed sequencing and differentially methylated regions were detected using BSmooth and InformME packages. **Results**: For NK cell isolations, mean starting and final NK cell purity percentages were 8 ± 1.6% and 94.3 ± 0.5%, respectively. CD3 depletions were also effective, with mean starting and final CD3+ percentages as 69.9 ± 0.9% and 0.26 ± 0.1%, respectively. The mean fold NK cell expansion measured at day 7 and 10 were not significantly different between activation methods (K562.mbIL15.41BBL: 46.69 ± 15.33, 352.2 ± 204.1; K562.mbIL21.41BBL: 33.65 ± 4.63, 191.1 ± 48.8; K562.mbIL21.41BBL+TGFβ: 41.9 ± 0.77, 200.9 ± 25.96 respectively at day 7 and 10). All data are presented as mean ± SD. Initial data from WGBS and PCA analysis demonstrated global differences in mean methylation level between the four groups. **Conclusions**: We have identified clear differences in global methylation patterns between our NK cells expanded using clinically relevant methodology. We are currently analyzing the gene-specific methylation differences between these cells and seek to identify an epigenomic signature that correlates with optimal anti-tumor NK cell functionality. We plan parallel analysis of transcriptional phenotype using RNAseq and surface protein and receptor expression using multi-parameter flow cytometry. We expect to further identify unique characteristics that define our expanded NK cell products to inform manufacturing plans for future clinical trials.



Friday Posters: Targeted Gene Insertion

1672. From Promoter to Transgene Efficiency: Insights from Promoter Screening in Induced Pluripotent Stem Cells

Kiavash Kiaee, Michael Nehil, Shawdee Eshghi
Ginkgo Bioworks, Inc., Boston, MA

Generating therapeutic cells from iPSCs is an attractive approach to address challenges in precision genome engineering and manufacturing scalability in current cell therapy approaches. Differentiation from a pluripotent state to the desired therapeutic cell involves large scale epigenetic changes and genome rearrangements that can often lead to transgene silencing. In this study we sought to optimize transgene expression in iPSCs by systematically screening promoter in a site-specific manner. We conducted an arrayed screening of commonly used parts of promoters ranging in size between 60 bp to 1200 bp integrated in a site-specific manner into therapeutically relevant loci like TRAC in iPSCs. Surprisingly, widely reported promoters such as EF1a and CMV exhibited minimal expression, in stark contrast to the robust and sustained transgene expression observed with PGK and UbC promoters. Notably, the investigation revealed a discrepancy in transgene expression from the EF1a promoter between plasmid-based and TRAC locus-integrated constructs, suggesting locus-specific silencing of certain promoters.Motivated by the need to enhance our understanding of locus-specific silencing and the differential expression patterns exhibited by widely used promoters, our findings offer key insights for enhancing gene integration strategies and the optimal choice of HDR templates for reliable transgene expression for biotechnology and cell therapy applications.

1673. Efficacy & Safety of AAV9-Mediated *GDAP1* Gene Replacement Therapy in CMT4A Mice

Merve Emecen Sanli, Thomas Dong, Anjala Jiji, Yuhui Hu, Steven J. Gray, Xin Chen
University of Texas Southwestern, Dallas, TX

Charcot Marie Tooth (CMT) manifests as a diverse group of neuropathies with both clinical and genetic variations, encompassing various Mendelian inheritance patterns. Over 1,000 mutations in 80 disease-associated genes have been identified. A small subgroup of hereditary motor and sensory neuropathies in CMT is attributed to mutations in the ganglioside-induced-differentiation-associated protein 1 gene (*GDAP1*). GDAP1-related mutations can be inherited in either an autosomal recessive (AR, more common) or an autosomal dominant (AD) pattern. AR mutations, known as CMT4A, exhibit demyelinating, axonal, or intermediate disease forms. CMT4A leads to an early-onset, severe neuropathy affecting all extremities, often resulting in loss of ambulation in early adulthood (second-third decade). In advanced stages, many of these patients develop vocal cord paresis (unilateral or bilateral) and diaphragmatic weakness. In contrast, AD mutations, termed CMT2K, present with axonal disease and tend to have a more benign course. Since there is no specific treatment and it is a single gene disease, we hypothesized that spinal cord and peripheral nerve delivery of the *GDAP1* gene through lumbar intrathecal (IT) injection during early life can improve disease symptoms in *Gdap1*^{R191X} mice. A codon optimized human *GDAP1* gene driven by a weak ubiquitous promoter was synthesized and cloned into a self-complement adeno-associated viral serotype 9 (scAAV9) vector (AAV9/*GDAP1*). The construct was validated *in vitro* for gene expression following transfection into HEK293 and Lec2 cells. To test the safety of AAV9/*GDAP1* gene therapy, an in vivo safety study was conducted using IT administration of AAV9/*GDAP1* to wild type (WT) mice at postnatal p7-10 with low or high dose. The results showed a significant dose dependent increase in human *GDAP1* mRNA expression in the sciatic nerve. No adverse changes of body weight or survival rate were observed with the treatment. In parallel, to test the efficacy of AAV9/*GDAP1* gene therapy, an *in vivo* efficacy study is ongoing using IT administration of AAV9/*GDAP1* to *Gdap1*^{R191X} mice at postnatal day 7-10 or 120 with low (1.25E11vg), middle (2.5E11vg), or high (5E11vg) dose. h*GDAP1* mRNA expression was dose-dependently increased in the sciatic nerve. All mice undergo behavioral phenotyping involving hindlimb clasping, wire hang, beam walk, digigate, grip strength, and rotarod, as well as nerve conduction velocity (NCV) testing. No significant behavioral phenotypes were observed in the *Gdap1*^{R191X} mice up to 15 months of age, so although no adverse effects on the phenotypes occurred, we could not evaluate the effect of the treatment to alleviate disease-relevant symptoms. NCV test on the *Gdap1*^{R191X} mice demonstrated a decrease in motor nerve conduction velocities (MNCV), which was rescued with the AAV9/*GDAP1* treatment in a dose-dependent fashion at 13 and 19 months of age. Currently, behavioral phenotyping at older timepoints, biomarker analyses using collected sera, and histopathological examination on harvested organs and peripheral nerves are underway to further explore treatment efficacy. In summary, the cumulated data demonstrate that a single dose of IT administration of AAV9/*GDAP1* appears to be

safe and effective in correcting NCV due to GDAP1 deficiency. These results indicate a potential applicability of GDAP1 gene replacement therapy for CMT4A patients.

1674. Integrase-Deficient All-in-One Lentivector Carrying Cas9 Protein, sgRNA & Donor DNA for Directed Chromosomal Knock-Ins

Stanislav Indik, Alex Roser, Dominic Lichtscheidl, Iris Hoger

University of Veterinary Medicine, Vienna, Austria

Here we describe an integrase-deficient lentivector that contains all components necessary for site-directed insertion of viral DNA into mammalian genomes. The vector particles carry the Cas9 protein, together with the viral RNA that serves as a template for the synthesis of the donor DNA containing the transgene(s), the sgRNA-coding region and the sgRNA target site. The DNA produced from the viral RNA in the transduced cells is cleaved together with the chromosomal target site by the co-delivered Cas9/sgRNA. The cleaved DNA is then inserted into the target locus in a homology-independent manner. To prevent cleavage of the vector plasmid (containing the sgRNA target site) during lentivector production, we used an inducible sgRNAi system to control Cas9/sgRNA activity so that sgRNA is present only in the transduced cells. We show that after transduction, the lentivector DNA can be inserted into a predetermined locus in the genome of HEK293 cells. In the most extreme case, we knocked in an 8.6 kb donor containing three transgenes (mCherry, puroR, lacZ). Cultivation of the cells in a medium containing puromycin allowed the enrichment of mCherry-positive cells to >80%. We also used splice acceptor (SA)-containing promoterless donors for insertion into the intronic sites. For example, we inserted a lentivector donor DNA into the last intron of the hspa8 gene to express the HSPA8-mCherry fusion protein. The characteristic cytoplasmic localization of the red fluorescent signal in the transduced cells indicated that splicing was efficiently redirected to the SA site in the insert. The specificity of the insertions and the length of the inserts were confirmed by junction PCR and long template PCR, respectively.

1675. New *In Vitro* Models System for Treat Pitt Hopkins Syndrome

Yanina Tkachenko, Cassandra Dennys, Abuzar Kaleem, Babatunde Ogunlade, Meysam Ganjibaksh, Vera Iakovleva, Shibi Likhite, Kathrin Meyer

Gene Therapy, Nationwide Children's Hospital, Columbus, OH

Introduction: Autosomal dominant mutations in TCF4 cause Pitt-Hopkins syndrome. Pitt-Hopkins syndrome (PTHS) is characterized by intellectual disability, a marked delay in motor development, lack of functional speech and disordered breathing. Understanding upstream regulation of TCF4 expression and transcription is critical to developing therapeutic strategies. TCF4 is a basic helix-loop-helix protein that is broadly expressed especially in the nervous system. It works to regulate gene expression by interacting with other basic helix-loop-helix proteins and either allowing or repressing transcription. With TCF4 being a large gene encoding with many known protein isoforms, is a challenging target for classic gene replacement approaches. Since patients still harbor one healthy copy of the gene, upregulation of TCF4 expression is an attractive alternative approach to gene replacement. Here, we developed a novel gene therapy to enhance the TCF4 expression using an AAV-based targeted U7 small RNA to modulate TCF4 promoter activity. **Methods:** We designed multiple U7 small RNA cassettes targeting different regions of the TCF4 promoter that we predicted to be able to modulate promoter activity. We tested efficacy of the U7 cassettes via transient transfection in HEK293 cells for expression and ability to upregulate TCF4 expression. Next, we generated AAV9 viral particles and verified efficacy in an *in vitro* model system using human PTHS patient cell lines by RT-PCR and immunofluorescent imaging. Functional assays were used to determine the effect of the treatment on cellular health as well. In addition, we used mouse embryonic fibroblasts (MEFs) from a mouse model that expresses the green fluorescent protein under the human TCF4 promoter as an additional model system to further validate our approach. This mouse model will then be used to validate the technology in vivo as a next step. **Results:** We developed a U7 small RNA based technology that was designed to upregulate endogenous TCF4 expression via promoter regulation. We were able to demonstrate increased expression of TCF4 in multiple model systems including HEK293 cells and a novel human patient in vitro model. Importantly, we found upregulated TCF4 levels via RT-PCR as well as immunofluorescence staining. The increased expression of TCF4 also improved the otherwise hampered survival of co-cultured neurons in contact with patient derived induced astrocytes, providing functional evidence of treatment efficacy. Moreover, using mouse embryonic fibroblasts expressing GFP under the control of the human TCF4 promoter, we showed upregulation of GFP expression following AAV9. U7-TCF4 treatment as well in yet another model system. **Conclusion:** We developed a novel U7 small RNA based technology allowing modulating TCF4 promoter activity. Our data indicates efficacy of the approach in HEK293 cells as well as multiple reprogrammed patient cell lines and mouse embryonic fibroblasts expressing GFP under the human TCF4 promoter. Our novel therapeutic strategy is highly promising and could be advantageous over existing gene replacement strategies as it would allow to use the endogenous gene to generate all naturally occurring TCF4 isoforms instead of delivery a single transcript. Our approach could be tailored to delivery via AAV or non-viral systems. As a next step, we will verify efficacy of promoter regulation in vivo. This novel technology allows upregulation of gene expression and could become a promising novel approach for treatment of haploinsufficiencies in general.

1676. Low RT-Based Fidelity in Mouse Hepatocytes: Challenges and Solutions

Maike Thamsen Dunyak, Patrick Hanna, Angela Nan, Mollie O'Hara, Rejina Pokharel, Jenny Xie, Jonathan D. Finn

Tome Biosciences, Watertown, MA

Integrase-mediated programmable genomic integration (I-PGI) utilizes the DNA targeting properties of Cas9 and couples it with the efficient, size-independent DNA integration abilities of large serine integrases (LSIs). This method enables the integration of large pieces of DNA in a site-specific, directional manner that is not dependent on double stranded breaks (DSBs). I-PGI technology can be used to develop gene

therapies for monogenic liver diseases with the potential of a single drug per disease for diseases with a heterogeneous genetic landscape. I-PGI uses a Cas9 nickase (nCas9) with a writing enzyme, such as reverse transcriptase (RT), to write an integrase target site (attB, here called a 'beacon') in a programmed location. Beacon placement (BP) requires a minimum insert length of 38bp which is significantly longer than the majority of edits currently being explored with base and prime editing (<3 bp). After multiple rounds of optimization, we were able to efficiently place beacons with good beacon fidelity (BF) in human cycling cells (HEK293), non-dividing primary human hepatocytes (PHH), and primary cynomolgus hepatocytes (PCH) using transient mRNA delivery and chemically modified attachment guide RNA (atgRNA) pairs.When translating our technology *in vivo* to mice, we observed a surprising result, that despite being able to achieve a high level of editing in mouse hepatocytes following lipid nanoparticle (LNP) delivery of the editing components, that nearly all observed edits were incorrect, leading to very low BF rates. We found that this fidelity issue was also observed *in vitro* in non-dividing primary mouse hepatocytes, was independent of mouse strain, and was also observed in primary rat hepatocytes.To address the mouse beacon fidelity issue, we took two approaches. First, we engineered transgenic mice which had intact beacons in specific locations, allowing us to optimize integrase and DNA template dosing and kinetics. Second, we hypothesized that beacon fidelity in mice is impacted by the non-dividing nature of hepatocytes and can be improved by utilizing cycling hepatocytes. Therefore, we utilized neonatal mice which have a much higher proportion of proliferating hepatocytes than adult mice. Using LNP delivery in neonatal mice, we were able to significantly increase BF. We subsequently have demonstrated that these beacons are functional and have demonstrated functional gene expression after I-PGI mediated targeted integration in a therapeutically relevant target site.In summary, we have identified a previously undescribed challenge when using RT-based editing to write long (~40 bp) sequences in non-dividing mouse hepatocytes. This issue is likely due to DNA repair pathway differences between cycling and non-cycling mouse cells and appears to be limited to rodents, as we can routinely achieve high levels of beacon placement and fidelity in both human and non-human primate hepatocytes.

1677. A Programmable Mammalian Transposon Achieves Accurate Integration of a *CFTR*$_{2\text{-}27}$ Superexon in Human Bronchial Epithelial Cells

Subhrangshu Guhathakurta, Jaclyn M. Kubala, Daniel Pers, Sharmishtha Musalgaonkar, Rupesh Naidu, Lillian Campos, Oleg Iartchouk, Joseph J. Senn, Nancy L. Craig, Joseph J. Higgins

SalioGen Therapeutics, Lexington, MA

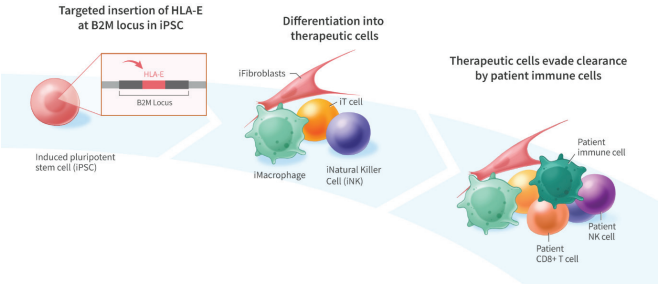
Introduction: Introducing large genes to targeted sites under endogenous regulatory control is ideal in diseases such as Cystic Fibrosis (CF). Recombination systems using DNA DSB-induced HDR have safety limitations. Partial replacement of the CF transmembrane regulator gene (*CFTR*$_{2\text{-}27}$, 4.4 kb) in intron 1 may provide a one-time, curative treatment that restores *CFTR* expression and corrects >97% of downstream mutations. We showed restoration of function using non-viral delivery of our first-generation bioengineered mammalian transposase derived from *Myotis lucifugus (bMLT)* to integrate genes in rodent livers and in retinas of mice and NHPs. The current data uses a second-generation *bMLT* designed to target *CFTR* intron 1 sequences and insert a *CFTR*$_{2\text{-}27}$ cDNA superexon. This process, called Gene Coding, achieves on-target integration, proper splicing, and adequate expression levels without the risk of adverse genomic events. **Methods:** We modified the amino acid sequence of *bMLT* using AlphaFold prediction models and cryoEM structural analyses of *bMLT* bound to DNA. We covalently fused zinc fingers (ZnF) to modified *bMLT* to interact with a recognition sequence near a preferred target site in *CFTR* intron 1 (*ZnF-bMLT*). DNA donor elements contained a splice acceptor site followed by either a GFP reporter or tagged *CFTR*$_{2\text{-}27}$, and a polyadenylation sequence. These sequences were flanked by insulators and *bMLT* terminal inverted repeats. HEK293T cells were lipofected with *ZnF-bMLT*, a *GFP* donor, and an optimized *CFTR* intron 1 landing pad plasmid (*pCFTR*) using the published "find and cut-and-transfer (FiCAT)" reporter assay to analyze targeting efficiency of three *bMLT* mutants. *pCFTR* targeting was confirmed by PCR and Illumina NGS. To assess *CFTR* intron 1-specific targeting efficiency in the human genome, published self-reporting transposon (SRT) methods were used to identify on- and off-target integrations in HEK293T cells after lipofection with an SRT donor and *ZnF-bMLT* (n=8 per group). A tagged promoterless donor (*GFP* and *CFTR-GFP* donor conditions) and *ZnF-bMLT* (n=6) was used to measure targeting efficiency. GFP expression was expected if the promoterless donor was controlled by endogenous *CFTR* regulatory elements. Targeting efficiency was estimated by the percentage of GFP positive cells using FACS and NGS. Wild-type and CF mutant (G542X) human bronchial epithelial cells (16HBE) were transfected and assessed by PCR and ddPCR for proper splicing and genomic integration. **Results:** The FiCAT reporter assay showed >60% integration at the *pCFTR* target sequence and was confirmed in HEK293T cells engineered with multiple artificial *CFTR* intron 1 genomic landing pads containing a specific target sequence flanked by *ZnF* recognition sites. Various *bMLT* mutants reduced off-target insertions by 3-to-4-fold while maintaining on-target insertion efficiency. Importantly, insertions increased in the immediate region of the *CFTR* intron 1 target site by ~100-fold in 16HBE G542X cells using *ZnF-bMLT*. Total off-target integrations decreased by >10-fold when compared to *bMLT* without ZnF fusions. These results confirmed integration in *CFTR* intron 1 of a G542X CF patient cell line using our second-generation *bMLT*. Finally, on-target integration was shown in up to 10% of transfected HEK293T cells when *bMLT* fused to two *ZnF* flanking the *CFTR* intron 1 target site was used as the targeting enzyme. **Conclusions:** The simple and efficient, single enzyme-driven Gene Coding technology presented achieves on-target insertion of the *CFTR*$_{2\text{-}27}$ superexon into the endogenous *CFTR* intron 1 with minimal off-target integrations. This technology has broad therapeutic applications in many genetic disorders that current gene therapies do not address.

1678. Targeted Insertion of HLA-E at the B2M Locus of mRNA-Reprogrammed iPSCs Facilitates the Development of Allogeneic Cell Therapies with Enhanced Safety Features

Elizabeth Belcher[1], Raven Dance Hinkel[1], Christopher B. Rohde[2], Matthew Angel[2], Kyle M. Garland[1]

[1]Eterna Therapeutics Inc., Cambridge, MA,[2]Factor Bioscience Inc., Cambridge, MA

Allogeneic cell therapies derived from induced pluripotent stem cells (iPSCs) are an exceptionally promising class of cellular therapeutic, as they can greatly reduce the manufacturing complexities of autologous and donor-derived allogeneic cell therapies such as scalability, batch-to-batch consistency, and cost. However, host immune cell recognition and clearance of exogenous cells can lead to iatrogenic toxicities and ineffective therapeutic responses. This immune rejection is often caused by CD8+ T cells recognizing foreign HLA-I molecules, while a complete lack of HLA-I expression can result in NK-mediated immune rejection (the "missing self" response). Modulation of HLA class I elements for evasion of clearance by both CD8+ T cells and NK cells has been explored as a strategy to improve *in vivo* persistence. We previously reported generation of iPSCs using an mRNA-based process that avoids the genomic integration and instability risks of DNA and viral reprogramming methods. We also previously reported a novel mRNA-encoded dimerizing gene-editing endonuclease, UltraSlice™ for high-specificity gene editing and demonstrated its use in inactivating β2 microglobulin (B2M), a key component of HLA class I molecules. Here, we report the development of an mRNA-engineered iPSC line developed using UltraSlice™ to express a B2M-HLA-E fusion transgene in lieu of the endogenous B2M gene product to mimic native B2M expression (i.e., upregulation when exposed to proinflammatory stimuli). Using UltraSlice™, we optimized the conditions for insertion of a flexible linker-containing *HLA-E* transgene upstream of the stop codon of the native *B2M* gene in mRNA-reprogrammed iPSCs. Specifically, a single-stranded DNA template encoding a $(GGGGS)_4$ flexible linker and *HLA-E* flanked by 125-nt sequences homologous to the *B2M* locus was co-delivered to iPSCs along with UltraSlice™ mRNA targeting exon 3 of the B2M gene. We found that the sequence of the flexible linker was susceptible to hairpin formation, and initial experiments resulted in insertion of a truncated sequence. We then designed and tested two new linkers with GC content reduced from 83.3% to 71.7% and 61.7% , which increased the theoretical Gibb's free energy from -6.0 to -3.92 and 0.64, respectively. We then analyzed insertion, finding no significant difference in the rate of insertion but observing successful insertion of the full repair template when using the two templates with reduced GC content. These edited populations were plated as single cells to obtain clonal mRNA-reprogrammed iPSC lines that express B2M-$(GGGGS)_4$-HLA-E fusion protein. These cells may prove useful for the rapid development of therapeutics with the potential for increased efficacy and safety owing to the immune-evasive nature of the cells.



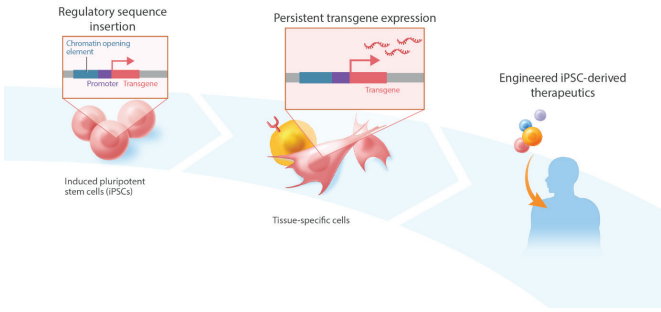
1679. Sustained Transgene Expression in Engineered iPSC-Derived Tissue-Specific Cells

Taeyun Kim[1], Raven D. Klee[2], Ismet Caglar Tanrikulu[1], Christopher B. Rohde[1], Matthew Angel[1]

[1]Factor Bioscience Inc., Cambridge, MA,[2]Eterna Therapeutics Inc., Cambridge, MA

Induced pluripotent stem cells (iPSCs) provide an ideal starting point for the generation of gene-edited tissue-specific cells. In particular, engineered iPSC-derived mesenchymal stromal cells (iMSCs) combine the advantages of tissue-derived MSCs (immunomodulation, clinical safety, low immunogenicity, tissue penetration, etc.) with gene editing (e.g., overexpression or knock-out of immunomodulatory factors), and thus, presents a new pathway for the development of cell therapies with higher efficacy and defined modes of action. iMSCs readily overcome the clonality, viability and expandability challenges associated with tissue-derived MSCs and display greater immunomodulation even in their naive form. Engineered iMSCs (EiMSCs) that overexpress transgenic immunomodulatory cytokines could offer further therapeutic potential. Although the overexpression of transgenic cytokines in EiMSCs presents an exciting therapeutic opportunity, transgenic expression can be unstable or silenced during the differentiation of iPSCs to EiMSCs. To enable sustained transgene expression upon EiMSC differentiation, we explored the use of universal chromatin-opening elements (UCOEs) in transgenic cassettes that express green fluorescent protein (GFP) under the EF1α promoter. Cassettes with and without a UCOE were inserted into the adeno-associated virus integration site 1 (AAVS1) safe-harbor locus in iPSCs through the use of mRNA encoding UltraSlice™ gene-editing endonucleases and single-stranded DNA (ssDNA) repair templates. Clonal iPSC lines were isolated by the single-cell deposition of GFP-sorted cells, biallelic insertion into AAVS1 was verified by amplicon sequencing, and GFP expression during differentiation toward EiMSCs was monitored. EiMSC lines all displayed traditional MSC surface markers upon differentiation, but they showed differences in GFP expression: While only ~30% of EiMSCs engineered without a UCOE 6-3 were GFP-positive, use of a UCOE yielded in uniform GFP expression (>99%) that persisted after 10 passages and 2 freeze-thaw cycles. Inflammatory (e.g., IL7 and IL15) and anti-inflammatory cytokines (e.g., IL4 and IL10) are being explored for their opposing, but potentially crucial roles in MSC therapy by directing and enhancing immunomodulation and survival of immune cells to alleviate conditions such as cancer and inflammatory disease, respectively. We therefore applied our established workflow to knock-in expression cassettes encoding either IL7-IL15 or IL4-IL10 fusion proteins into

iPSCs and differentiate them to clonal EiMSC lines. Taking advantage of the high efficiencies obtained for UltraSlice™-mediated knock-ins, clonal iPSC lines were successfully established via single-cell deposition without the use of a reporter system. MSC markers and transgene expression were confirmed in each line upon differentiation, yielding two EiMSC lines that stably express IL7-IL15 or IL4-IL10 fusion proteins. These lines may prove promising in the development of therapies to treat a wide range of diseases.



1680. Site-Directed Integration of Large DNA Sequences into Endogenous Sites in the Human Genome Using Engineered Modular Integrases (MInts)

Friedrich Fauser, Sebastian Arangundy-Franklin, Jessica E. Davis, Lifeng Liu, Nicholas A. Scarlott, Nicola J. Schmidt, Rakshaa Mureli, Danny F. Xia, Luis Rodriguez, Mohammad Qasim, Vishvesha Vaidya, Sarah J. Hinkley, Bhakti N. Kadam, Nga Nguyen, Stephen Lam, Bryan Bourgeois, Adeline Chen, Andrew Nguyen, Patrick Li, David E. Paschon, Gregory D. Davis, Jeffrey C. Miller

Sangamo Therapeutics, Richmond, CA

Introduction: Programmable targeted integration (TI) of large DNA sequences into genomic loci is a highly desirable genome editing modality for development of genomic medicines, agricultural applications, synthetic biology, and basic research. Most methods rely on the induction of a double-stranded DNA break and subsequent integration of the donor via cellular DNA repair pathways such as non-homologous end joining or homology-directed repair. These methods have various limitations associated with the induction of the DNA break itself, as well as their dependency on competing DNA repair pathways. Site-specific large serine recombinases such as Bxb1 have the potential to overcome these limitations. Bxb1 catalyzes precise directional DNA recombination between its attP and attB recognition sites, without the induction of free DNA ends and therefore not relying on any cellular DNA repair processes. However, reprogramming of Bxb1, or other serine recombinases, has not yet been shown yet. To avoid the various therapeutic liabilities of oversized RNA-guiding cargo, we have taken a protein-guided approach and engineered the Bxb1 amino acid sequence directly against new genomic target sites. Here, we demonstrate that engineered Modular Integrases (MInts), derived from Bxb1, can be efficiently retargeted to endogenous sites in the human genome. **Results and Conclusions:** We first integrated the natural Bxb1 attB recognition site into the AAVS1 locus of human K562 cells and demonstrated >80% TI with wildtype Bxb1. An unbiased genome-wide specificity evaluation showed high specificity, but also identified a small number of attB pseudo-sites with detectable levels of TI (<1%). We then developed a directed evolution system to change the molecular specificity of Bxb1 in bacteria. We also established a high throughput system to validate and characterize artificial attB sequences in K562 cells. This allowed us to identify Bxb1 variants that achieved up to ~40% TI at one of the attB pseudo-sites. Encouraged by these results, we evaluated the genome-wide specificity of the most active Bxb1 variants and demonstrated improved specificity compared to wildtype Bxb1. Next, we attempted retargeting of Bxb1 to the first intron of the human AAVS1 gene, a known safe harbor site. We computationally searched for suitable target sites, and then tested them against selected Bxb1 variants. This effort resulted in >50% TI into the AAVS1 locus with minimal to no indels. We also established alternative donor delivery possibilities, such as minicircles or packaging in AAV. Taken together, we demonstrated reprogramming of Bxb1 to a gene-sized locus in the human genome without relying on other gene editing tools to pre-install a natural Bxb1 recombination site at the desired locus. We further de-risked our MInts platform by evaluating its genome-wide specificity, and establishing therapeutically relevant donor delivery strategies, thus showing potential promise for clinical applications.

1681. Metagenomic Discovery and Screening of Novel Recombinase Proteins for Targeted Integration

Joseph Neumeyer, Meng Wu, Shreya Jambardi, Amy Hengyi Zhao, Bradley Taylor, Georgia Giannoukos, Eugenio Marco, Hui Li

Editas Medicine, Cambridge, MA

Current technologies to integrate multi-kilobase (kb) DNA sequences *in vivo* are limited. Some technologies, including retroviruses or transposases, integrate into the host genome through a random manner, raising potential oncogenesis risk and other safety concerns. Other methods mediated by homology directed repair (HDR) are restricted by low integration efficiency *in vivo*, strict dependence on endogenous DNA repair machinery, and lack of feasibility in post-mitotic cells. Here, we explored site-specific large serine recombinases (LSRs) used by phages to integrate large transgenes into bacterial genomes without relying on endogenous host DNA repair machinery. First, through metagenomic data mining, we discovered thousands of potential LSR candidates and reconstructed their cognate DNA recognition sites. Subsequently, we evaluated thousands of LSRs for their efficacy and specificity for targeted integration of large transgenes into the human genome *in vivo* using high throughput functional screening, and discovered hundreds of highly potent LSRs that can integrate into the human genome with a variety of specificity and efficiency. These LSRs could build the foundation to develop novel gene editing technologies capable of knocking in large transgenes *in vivo*, therefore greatly expanding the potential of genetic medicine for more therapeutic indications.

Friday Posters: On- and Off-Target Method Development

1682. INDUCE-seq: Ensuring the Safe Development of Cell and Gene Therapies by Gene Editing

Simon H. Reed, Patrick van Eijk, Felix Dobbs
Broken String Biosciences Ltd, Cambridge, United Kingdom

Advances in genome editing are making it increasingly possible to develop new cell and gene therapies. Synthetic genes and modifications to existing ones can be made in a site-specific manner, while immune-compatible cells created for use in allogeneic patients enable production of 'off-the-shelf' cellular therapies. However, gene editing is a new way of treating disease, requiring new tools to ensure their safe and efficacious design and use in patients. Genome editing comes with risk, including malignant transformation of target cells caused by culturing cells outside the body. Gene editing can directly cause genomic instability resulting in selection for outgrowth of clones with oncogenic mutations. Furthermore, the gene editing tools themselves can cause genomic instability in the form of DNA breaks at sites other than the intended target. Such 'off-target' editing can activate proto-oncogenes, or disrupt tumor suppressors thus driving carcinogenesis. 'Off-target' mutagenesis may also generate neoantigens, triggering autoimmunity, or other types of cellular dysfunction. Furthermore, rare penetrant mutations are now known to confer severe risk of common disease, underscoring the importance of identifying 'off-target' gene editing to assess risk. More precise methods are needed for testing 'off-target' editing during all phases of therapeutic development, including post-treatment follow-up. At present standardised assays to accurately assess the precision of gene editing in cell and gene therapies are lacking. Here, we describe the development and characterization of INDUCE-seq to address this. CRISPR-Cas9-based gene editing of five well-studied genetic targets, including two that have recently been evaluated by regulators, was conducted by two independent industry partners using two different cell-types. On and off-target gene editing was assessed by measuring DNA breaks in the genome using INDUCE-seq and the genetic mutations caused by editing at these locations were subsequently measured using Duplex-sequencing, which sensitively detects rare mutations. The results of this HESI-sponsored validation study will be presented to demonstrate the reproducibility of the assays for measuring intra and inter laboratory 'on-' and 'off-target' editing, the importance of conducting this analysis in the intended cell/tissue targets and the discovery of confirmed off-target gene editing in cells at genomic locations not predictable by *in silico* analysis alone.

1683. High Throughput Single Cell Analysis Workflow for Accurate Measurement of Genotoxicity Arising from Gene Editing Experiments

Saurabh Gulati, Alex Li, Saurabh Parikh, Qawer Ayaz, Indira Krishnan, Charlie Murphy, Lubna Nousheen, Benjamin Miltz, Ben Schroeder, Shu Wang
Mission Bio, South San Francisco, CA

Conflicts of interest: S.G., C.L., S.P., Q.A., I.K., C.M., L.N., B.M., B.S. and S.W. are employees and shareholders of Mission Bio, Inc. Advances in cell and gene therapy (CGT) are transforming the way we treat and potentially cure certain diseases including cancer and rare genetic disorders. Precise gene editing technologies such as CRISPR-Cas9, TALENs, and ZFNs have transcended traditional boundaries, offering novel approaches to rectify genetic anomalies at their source. Despite these advancements, the editing process yields heterogeneous populations where some cells may have undesired outcomes that bear the risk of genome toxicity. Notably, these adverse outcomes include the introduction of structural variants, copy number alterations, or chromosomal translocations. Therefore, the development of efficacious gene therapies hinges on the ability to accurately measure and understand these events. Furthermore, since "cells" are the functional units of gene editing products, it is prudent to measure the co-occurrences of editing results and potential genotoxicity events in a single-cell context.Here, we demonstrate a microfluidics and multiplex PCR based single-cell technology that simultaneously measures the co-occurrence and zygosity of on-target edit, off-target edits, translocations between predicted edit sites, as well as the genomic CNV landscape in over 10,000 cells in parallel. Due to the single-cell resolution, the technology offers a comprehensive view of the heterogeneous editing profile of gene edited products for a proper and fast evaluation of editing outcome and potential malignant events.To evaluate the robustness of this workflow we used a variety of samples with varying levels of adverse effects occurring from multiple gene editing experiments. These samples all had orthogonal results cross validation using bulk NGS (DNA-seq) and rhAMPseq. Using a targeted sequencing panel we measured on and off target editing activity, presence of structural variations, gene editing induced translocation events and genome wide CNVs at a single cell level in these samples. The resulting data was analyzed using a novel bioinformatics pipeline which was developed to accurately measure all of these modalities simultaneously. Our high-throughput single-cell NGS assay represents a significant advancement in gene editing analysis, measuring off-targets, translocations, and genome stability in over 10,000 cells per assay. This technology has proven effective in accurately detecting a diverse range of INDELs from small (1bp) to large genomic alterations (114kbp) in as few as 0.1% of cells. Additionally, we were able to identify translocation events, pinpointing exact breakpoint coordinates even when they occur in less than 0.5% of the cell population.Alongside these capabilities, we were able to simultaneously measure allelic on-target CRISPR editing activity in each cell, providing a comprehensive view of both intended and unintended genetic modifications. This dual functionality not only aids in optimizing gene editing protocols but also offers critical insights for understanding genotoxicity caused due to gene editing experiments and the development of safe, efficacious cell-based therapeutics.

1684. End-to-End Tools and Services for Interrogation of CRISPR-Cas Associated Genotoxicity

Ellen Schmaljohn, Gavin Kurgan, Kyle Kinney, Morgan Sturgeon, He Zhang, Rolf Turk, Garrett Rettig, Ashley Jacobi
Integrated DNA Technologies, Coralville, IA

Identifying and verifying low frequencies of genomic alterations resulting from off-target editing, gRNA synthesis errors, cross-contamination, or other unintended gRNA activity is critical to preventing unexpected genotoxic effects for the gene editing therapeutic community. However, assembling the necessary components and expertise for genotoxicity characterization studies is expensive and labor intensive. To better enable the gene editing community, we demonstrate a series of tools, workflows, and services that can be leveraged to perform genotoxicity characterization of CRISPR reagents. First, we demonstrate an empirical *in vivo* tag-based off-target nomination service rooted in our RNaseH2-dependent amplification technology as a method to generate an off-target list for a target gRNA. Then, we demonstrate how outputs of this service can be fed into rhAmpSeq to identify and classify indels and chromosomal re-arrangements at on/off-target loci with increased effectivity compared to existing methods. Finally, we demonstrate a method to sensitively identify gRNA cross-contamination down to sub-0.1% gRNA contamination levels by leveraging unique NHEJ DNA repair fingerprint information of select gRNAs. Using this method, we demonstrate that editing attributable to a characterized gRNA activity can be identified at frequencies far below detection limits of canonically utilized tools, and we further use this method to characterize the ability of IDT's synthesis platform to prevent cross-contamination reproducibly. From this work, we demonstrate the importance of manufacturing processes in mitigating unintended genomic alterations and present new tooling/services to improve genotoxicity characterization in gene therapy workflows. For Research Use Only. Not for use in diagnostic procedures. RUO23-2134_001 06/23

1685. Limiting Off-Target Effects of CRISPR-Based Products- Guide RNA Sequencing by NGS

Rebecca A. Bova, Christina Kalinyak, Maggie Luce, Brianna McManus, Servane Orozco, Bradley Hasson
BioReliance Corporation (MilliporeSigma/Merk KGaA), Rockville, MD

Gene editing technologies have come a long way in the past several years and with the first product utilizing CRISPR-Cas9 being approved by the FDA in late 2023, this is likely to be an ever-growing market. These highly specific gene editors utilize a CRISPR complex which is shuttled, or guided, to the intended target sequence by a single-guide RNA (sgRNA) molecule. These molecules ensure that the endonuclease makes it to the correct location, this is done by a complementary base-pair sequence on the sgRNA to where the edit should take place (crRNA) followed by a short DNA sequence that is compatible with the specific endonuclease (tracrRNA). These complexes rely on the specificity of the guide RNA to make it to the correct location in the genome for the correct edit to occur. If the specific "on-target" base pair region is not specific and has any inconsistencies like substitutions in nucleic acid sequence, the incorrect part of the genome may be edited creating what is known as an "off-target" event with an unintended effect. These off-target events are closely monitored by additional quality control (QC) assays. To ensure that the guide RNAs and the endonuclease are shuttled to the correct place in the genome, sequence fidelity of the sgRNA is critical. Currently, sgRNA's are QC'ed using mass spec technology which can miss subtle variations in the sequence, especially at low levels. Therefore, a more sensitive means of sequence order identification is required to verify the fidelity of the sequence. Next Generation Sequencing (NGS) using short-read Illumina technology represents a method that can be used to identify sequence variation to a suitable level within the guide RNA, adding to the safety profile and reduction of off-target effects of a gene editor. NGS provides a more sensitive analysis than currently available methods as to the detection of any variants in the sequence which may be missed by analytical methods. By having a more sensitive method this could help product developers reduce the amount of off-target events helping the safety profile of these types of products which are becoming more common in the industry. In this presentation, we will discuss the development of a method using short read NGS technology for sequencing sgRNAs. Method development includes testing of multiple kits as well as sgRNAs from multiple manufacturers. Our testing includes a comparison of two different library kits to overcome the challenges of sequencing short RNA molecules- the first utilizes sequential ligation that adds a pre-adenylated adapter to the 3' end and another adapter onto the 5' end. The other kit is ligation free but incorporates a 3' poly-A tail and extra bases onto the 5' end followed by "template switching". We have seen that both of these kits work well with sgRNAs from multiple different vendors encompassing different sequences as well as different modifications. Successful sequencing of the libraries has generated complete datasets that have not detected variants due to the purity of the nature of the material used for development. During *in silico* testing, we were able to introduce a single base variant and detect it through our validated variant detection bioinformatics algorithm that can detect indels and various variants. Additional testing demonstrates that we have been able to confirm variants present within the sequences down to as little as 1% in the population. We believe that implementing the use of NGS for sequence identity along with other analytical methods would ensure high quality products with minimal off-target editing.

1686. Potency and Off-Target Evaluation of Targeted AID-Mediated Mutagenesis (TAM) Base Editor *In Vitro*

Chunyan He[1], Li Chen[1], Tiangang Yuan[1], Jingsheng Li[1], Guangyuan Xiao[1], Fengpeng Li[1], Yi Dai[2]
[1]SuZhou GenAssist Therapeutics Co., Ltd, Suzhou, China,[2]Peking Union Medical Hospital, Beijing, China

Background DMD is a progressive muscle-wasting disease caused by mutations in the gene encoding the dystrophin protein. Exon 50 skipping can target 4% of DMD patients with large deletions. Until now, there is no drug for those patients. GEN6050 is an intravenously administered DMD exon 50 skipping base editing drug. The exon 50 skipping mechanism of GEN6050 is to modify the IVS50 5'SS in the human DMD gene and result in exon 50 skipping at mRNA level. For exon 50 skipping amenable DMD patients, exon 50 skipping generate a truncated yet in-framed dystrophin protein (**Figure 1**). **Method** Normal iPSCs derived myotubes/cardiomyocytes were treated with GEN6050 for 24hrs. After 7 days drug treatment, the cells were collected for editing window or off-target evaluation. Untreated samples were used as control. Similarly, GEN6050 treated DMD iPSCs derived myotubes or purified cardiomyocytes were collected for efficacy study. **Results** The editing window of GEN6050 was defined as 10 bp upstream to 30 bp downstream of hE50-sgRNA (from +10 to -30 of hE50-sgRNA) corresponding to -24 to +36 bp of dystrophin gene IVS50 5'SS

according to the efficient editing of GEN6050 (MOI=3E5) in normal iPSCs derived myotubes. Dose dependent target site editing, and exon 50 skipping were observed. The exon skipping efficiency is highly correlated with the editing efficiency, confirmed that the exon skipping is caused by efficient DNA modification at IVS50 5'SS. Similarly, dose dependent transgene transcription, target site editing, exon 50 skipping and dystrophin protein restoration were observed in both iPSC-DMD-del51-53 (exon 50 skipping amenable) derived cardiomyocytes and myotubes (**Figure 2**). High exon skipping were observed at the MOI of 1E6 in iPSC-DMD-del51-53 derived cardiomyocytes (80%) or myotubes (90%). Consistent with high exon skipping efficiency, up to 40% (MOI=1E6) dystrophin protein immunofluorescence restoration were observed in both iPSC-DMD-del51-53 derived cardiomyocytes and myotubes, indicating that GEN6050 has the therapeutical potential for exon 50 skipping amenable DMD patients. To assess off-target of GEN6050, we identified less than 5,000 SNV or 1,000 indel sites through WGS data analysis in GEN6050-treated cardiomyocytes (only 3 overlapped indel sites among 3 replicates) with more than 30% on-target editing efficiency using non-treated sample as reference. In parallel, in silicon prediction by Cas-Offinder identified 134,849 (mismatch <= 4, <=3 DNA and RNA bulge) potential off-target sites. There are 22 overlapped sites for the WGS identified SNVs/indel and in silicon predicted sites. Then, partial (top 9 that ranked by significance with carcinogenesis) of the potential off-target sites were validated by target-NGS. No potential off-targets were found in treated normal iPSC derived cardiomyocytes. **Conclusion** In summary, GEN6050 leverages base editing activities to restore the Dystrophin protein in DMD iPSC derived cardiomyocytes/myotubes with low to non-detectable off-target risk. Our results indicate that GEN6050 can provide a potential cure for DMD exon 50 skipping amenable patients.

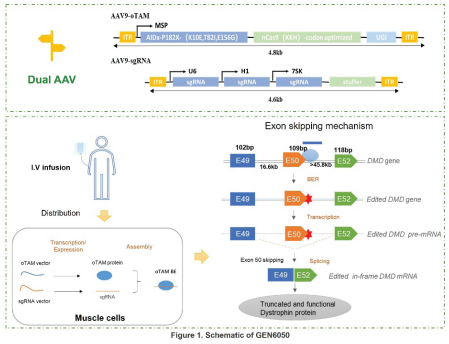


Figure 1. Schematic of GEN6050

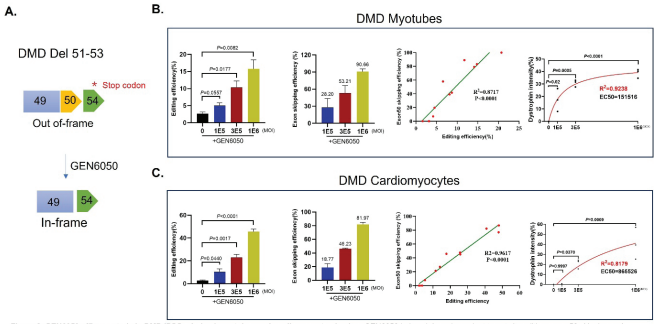


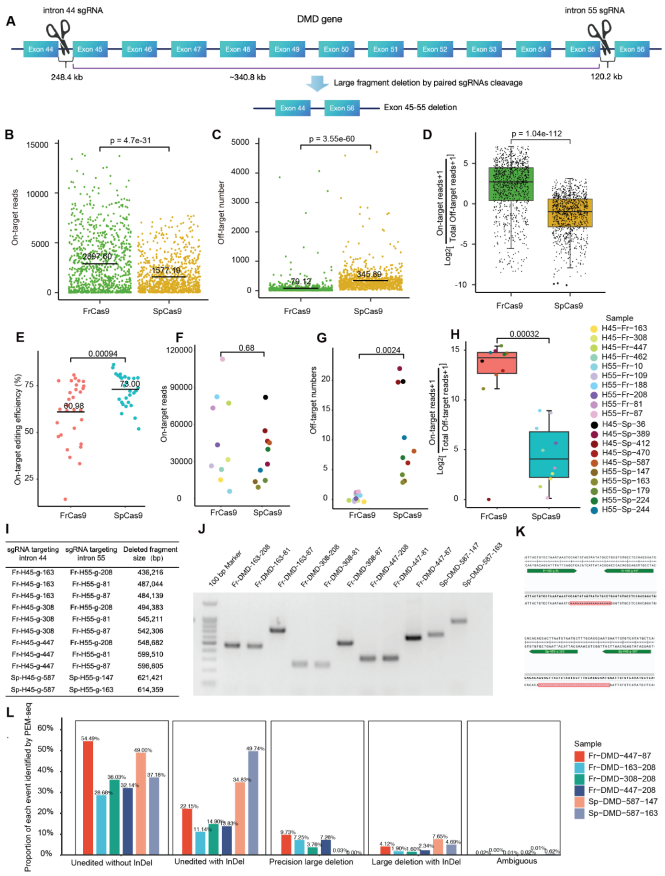
Figure 2. GEN6050 efficacy study in DMD iPSCs derived myotubes and cardiomyocytes *in vitro*. GEN6050 induced dose dependent target site editing, exon 50 skipping and dystrophin immunofluorescence intensity restoration in DMD iPSCs derived cardiomyocytes and myotubes after 7 days drug treatment. The correlation of editing and exon skipping was analyzed with Pearson correlation analysis. The dose dependent dystrophin immunofluorescence intensity restoration was analyzed with non-liner regression model. Data was presented as mean+-SEM, t-test was used for two groups comparison. *P*<0.05 is considered statistically significant. MOI indicates vg/cell.

1687. AID-Seq High-Throughput sgRNA Screening Facilitates FrCas9-Mediated Large Fragment Deletion of DMD Exons 45-55

Rui Tian[1], Huabin Lin[1], Zheng Hu[2]

[1]Generulor, Zhuhai, China,[2]Women and Children's Hospital Affiliated to Zhongnan Hospital of Wuhan University, Wuhan, China

Background: Large deletions of exons 45-55 in the DMD gene treat approximately 60% of Duchenne muscular dystrophy (DMD). Current major challenge with this approach is the inefficiency of large fragment deletion and off-target effects. This problem needs to be solved from two dimensions. The first is to use a new efficient CRISPR/Cas that has lower off-target effects. The second is high-throughput sgRNA screening technology to effectively select the optimal sgRNAs that target the DMD intron 44 and 55. **Result: The cleavage efficiency of FrCas9 is equivalent to that of SpCas9, but with extremely low off-target.** We used pooled AID-seq to evaluate the cleavage efficiency and off-targets of our newly identified FrCas9 and the commonly used SpCas9 among 940 DMD sgRNAs targeting DMD intron 44 and 55. Of note, at the same sequencing depth, FrCas9 showed an average on-target read count of 2897.60, surpassing SpCas9's 1577.19 (Fig 1B). Importantly, the average number of off-target sites per sgRNA for FrCas9 was 79.12, almost an order of magnitude lower than that of SpCas9 (345.89, Fig 1C). Moreover, FrCas9 exhibited a significantly higher on:off ratio than SpCas9 (Fig 1D), confirming its enhanced specificity as well efficacy. **Further screen sgRNAs at the cellular level and confirm the high specificity of FrCas9.** We further employed amplicon sequencing and GUIDE-seq to assess on-target cleavage efficiency in vivo. The results showed that the average editing efficiency of FrCas9 in 30 sgRNAs was comparable to that of SpCas9 (Fig 1E, F). However, FrCas9 exhibited significantly fewer off-target effects compared to SpCas9 at cellular level (Fig 1F-H). No FrCas9 off-target was detected at 5 sites, and only one off-target was detected at the remaining 5 sites (Fig 2G). In contrast, SpCas9 showed significantly higher numbers of off-target sites and reads, with multiple off-targets detected per sgRNA (Fig 1F-H). **The screened FrCas9 sgRNA can efficiently and accurately delete the DMD exon 45-55 large fragment.** We further selected six FrCas9 sgRNAs and three SpCas9 sgRNAs for large fragment deletion of DMD exon 45-55. The fragment sizes deleted by paired sgRNAs are detailed in Fig 1I. The presence of small target bands resulting from large deletions of all paired sgRNAs were confirmed by the PCR (Fig 1J) and Sanger sequencing (Fig 1K). PEM-seq was further used to identify editing events by paired sgRNAs. The precise large fragment deletion efficiency of FrCas9 is as high as 9.73%, while that of SpCas9 is only 0.03% (Fig 3L). Additionally, we also calculated the proportion of large deletion accompanied by InDel, which are also useful and safe for DMD exon 45-55 deletion. FrCas9 achieved a 4.12% large fragment deletion efficiency with InDel, while SpCas9 achieved 7.65% (Fig 3L). Furthermore, no off-targets were detected for FrCas9 using the PEM-seq method. **Conclusion:** These results collectively point to the enhanced specificity and efficiency of FrCas9 in the context of large genomic edits, which is crucial for DMD therapeutic applications.

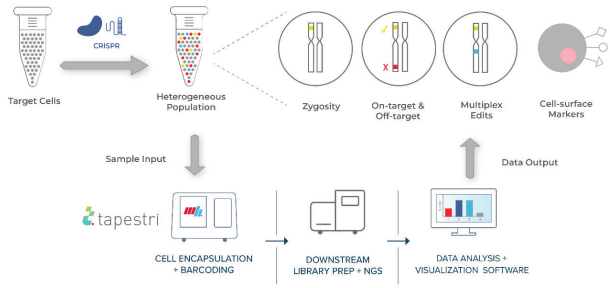


1688. Navigating Heterogeneity in Genome Editing Outcomes Using Single-Cell Analysis

Brittany Enzmann, Saurabh Gulati, Chieh-Yuan (Alex) Li, Benjamin Miltz, Qawer Ayaz, Joanne Nguyen, Shu Wang, Jacqueline Marin, Kathryn Thompson, Benjamin Geller, Benjamin Schroeder

Mission Bio, South San Francisco, CA

Genome editing has emerged as a revolutionary force within the life sciences, wielding transformative potential in applications such as cell and gene therapy development, disease modeling, and functional genomics. Despite the promise of precision of advanced genome editors, editing outcomes remain largely unpredictable. Different cells subjected to the same editing regimen can yield distinct combinations of edits, varying not only across multiple on-target sites but also between on-target and off-target locations. From the perspective of the fundamental biological unit — a single cell — the zygosity disparity (mono-allelic, bi-allelic), heterogeneity in variants (homozygous, heterozygous, compound heterozygous), and their functional impact all contribute to the layer of complexity in the mosaicism of editing outcomes. In addition, different combinations of on-target and/or off-target edits within cells may impact the function and toxicity of edited cells. Moreover, editing efficiencies in mixed cell populations may differ across cells based on cell type or state. Current genome editing analyses primarily rely on bulk methods which provide only an average editing efficiency at the allelic level of a population. The nuanced cell-to-cell variation of edits remains elusive with these traditional approaches. Here, we present compelling evidence that the Tapestri Genome Editing (GE) Solution assesses multi-omic information across 1000s of single cells with high accuracy. To evaluate the single-cell platform's performance in detecting editing events, we used a combination of orthogonally validated CRISPR-Cas9 edited clonal cell lines and pools targeting *PDCD1* and/or *TRAC* genes. Using a targeted panel design strategy for measuring on- and off-target edits, we demonstrate the detection sensitivity and specificity for indels (99.87% and 99.93%) and SNVs (98.71% and 95.27%) with a limit of detection of 0.1% (or, 1 in 1000 cells). This capability enriches the analysis for knockout (KO) and base editing (BE) experiments by providing single-cell information regarding zygosity and the co-occurrence of on- and predicted off-target edits. Additionally, immunophenotypic (cell-surface protein) analysis of edited cells reveals not only differences in editing across cell type/state but also can be used to validate knockouts of cell-surface markers. Additionally, we introduce cell hashing, a method that uses antibody-oligo conjugates (AOCs) against highly expressed cell-surface proteins to enable sample multiplexing. This capability increases the throughput of analysis while lowering reagent cost and processing time. This solution ultimately enables the assessment of cell-to-cell variation in genome editing and provides valuable insights for evaluating the safety and efficacy of cell and gene therapies.



1689. A Fully Integrated and Highly Sensitive Approach to Predicting and Measuring Off-Target Variation in CRISPR Edited Gene Therapy Products

Christopher J. Tompkins[1], Douglas Smith[2]

[1]KromaTiD, Longmont, CO,[2]SequreDx, Waltham, MA

Over the last 6 months, the guidelines and requirements for evaluation of gene therapy product have become clearer and to include the use of orthogonal methods, the effects of genetic variation across target populations, increased levels of assay sensitivity, testing on cell types from target donors, assessment of genomic rearrangements, and evaluation of biological consequence. These guidelines have been further highlighted by recent adverse event findings reported to the FDA. While the cause of these events has not yet been established, one potential cause for these findings is off-target editing. Understanding this association requires measuring all variation of any size and type in gene therapy drug products. We propose a comprehensive and integrated suite of analysis enabling the evaluation of all genomic variation induced by CRISPR editing systems including structural variants formed from recombination of edit site and off-target site double strand breaks. Inherent to our approach is a comprhensive

off-target nomination and biochemical confirmation strategy which allows us to estimate risk to the actual genomes being edited. When paired with unbiased cytogenetic methods and cytogenetic methods using sensitive DNA probes designed to the on-target and confirmed off-target editing sites it is possible to nominate, confirm and test for all edit associated variation, even the low-frequency highly heterogenous variation associated with optimized editing employed in the production of Drug Product. Beyond edit associated variation, this suite of tools can also measure phenomenon such as chromothrypsis, sub-clonal outgrowth, aneuploidy or genomic instability, that may or may not be edit related but poses a risk to patients. By combining best in class methods for nomination, confirmation and routine testing of all classes of variants, this integrated suite of tools meets the needs of guide selection, donor selection, guide characterization, drug product QC and patient testing.

1690. Quantifying Allele-Specific CRISPR Editing Activity with CRISPECTOR2.0

Nechama Kalter[1], Guy Assa[2], Michael Rosenberg[1], Ayal Hendel[1], Zohar Yakhini[2]

[1]The Institute for Advanced Materials and Nanotechnology, The Mina and Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel,[2]Arazi School of Computer Science, Reichman University, Herzliya, Israel

The detection and quantification of adverse off-target events is an important component in the field of CRISPR-Cas genome editing. A widespread approach to assessing this risk involves utilizing unbiased detection methods, such as GUIDE-seq, to identify potential off-target sites, followed by targeted amplification of the previously nominated sites (e.g. rhAmpSeq). Allele variance, namely single nucleotide variants (SNVs) or small insertions or deletions (inDels), can alternate the guide-RNA (gRNA) target site or the protospacer adjacent motif (PAM) sequence and thus drastically alter nuclease efficiency. The possible variant-dependent effects mandate precise nuclease activity quantification in an allele-specific fashion. However, currents tools which quantify CRISPR-Cas9 editing activity (EA) using next-generation sequencing (NGS) data, do not quantify editing differences associated with allele variants or are limited to previously known alleles. Hence, we have developed CRISPECTOR2.0, an extension of CRISPECTOR that supports allele-specific quantification of on- and off- target genome-editing. The computational pipeline performs *de-novo* identification of variants and allele phasing, based solely on an unedited sample, and then allocates the treatment reads to their respective alleles. Finally, the software produces an allele-specific inDel report. In addition, the CRISPECTOR2.0 version benefits from the original features developed for the previous version of the CRISPECTOR software package, including (1) precise and sensitive quantification of CRISPR EA through the utilization of a probabilistic Bayesian classifier; (2) confidence intervals (CIs) representing the statistical certainty (now also per allele); and (3) translocation detection feature. To assess CRISPECTOR2.0 performance, we have generated a novel dataset consisting of rhAmpSeq experiments, examining 40 distinct gRNAs in the HEK-293T-Cas9 human cell line. From a total of ~1,500 on- and off- target tested sites, we identified dozens of sites with allele variance, 40% of them not previously reported. Notably, in one off-target site for the gRNA targeting the *CD33* locus, an SNV in the PAM sequence resulted in one PAM-disrupted allele. The PAM-preserved allele had detectable EA (at a level of 0.1%), whereas the second allele was not edited. Interestingly, for certain alleles we detected variations in EA although the SNVs were located more than 10bps away from the cleavage site. This observation implies that EA differences may arise even in the absence of SNVs strictly within the nuclease recognition sequence, and can be attributed to other genomic factors, such as chromatin status or DNA structure. In summary, our software tool, CRISPECTOR2.0, represents significant progress beyond the current gold standard, as it enables reference-free assessment of allele-specific CRISPR-Cas9 EA. This breakthrough paves the way for a more personalized evaluation of both on-target and off-target effects, thereby enhancing the safety and precision of genome editing experiments for therapeutic applications.

1691. Nomination of Genome-Wide CRISPR-Cas9 Cleavage Activity Using rhAmp Technology

Rolf Turk[1], Kyle Kinney[1], He Zhang[1], Thomas Osborne[1], David Wang[2], Garrett Rettig[1]

[1]Integrated DNA Technologies, Coralville, IA,[2]Integrated DNA Technologies, Redwood City, CA

CRISPR-Cas9 nucleases have transformed the genome editing space because of their ease of use and programmability to create targeted double-stranded breaks. The technology has been widely adopted for basic research, in particular for loss-of-function screening applications. In experiments and applications beyond basic research, characterizing and understanding the genome-wide off-target effects (OTEs) becomes increasingly important and remains a significant hurdle. To interrogate off-target activity, many methods have been developed to empirically nominate off-target editing sites, each one with certain advantages and disadvantages. *In cellulo,* tag-based assays, such as GUIDE-seq, provide an advantage in OTE nomination due to a lower false-positivity rate compared to competing *in vitro* nomination methods. To complement GUIDE-seq, we implemented a cellular tag-based assay rooted in our RNaseH2-dependent amplification technology. The rhAmp nomination strategy allows for a high throughout workflow in 96-well plate format, increases OTE amplification, and shortens total hands-on time by implementing single-tube amplification using rhAmp technology. To compare our rhAmp nomination approach to literature precedent, published guides were used with our workflow and compared to sites nominated by GUIDE-seq. Upon identification of OTEs, rhAmp nomination was able to nominate the highest number of OTEs, which enables the design of rhAmpSeq multiplex targeted amplification pools for downstream confirmation studies. We use this opportunity to report the comparison of methods through identification of sites driven by our rhAmp nomination and confirmation of editing with rhAmp amplicon sequencing methods.

1692. Hybrid Deep Learning Approach to Predict guideRNA Editing Efficiency with Low Volume and High Dimensional Data

Rakesh Kappagantu, Pavan Kodamanchili, Nikhil Panchal, Shamik Mitra, Rohini Kalvakuntla, Shoko Iwai, Poulami Chaudhuri

Helex, Inc., New York, NY

The advent of sophisticated gene-editing technologies such as CRISPR/Cas has enabled a plethora of applications including its use in therapeutic settings. CRISPR/Cas9-based editing starts with DNA breaks at the target site complementing the designed Cas9-guide RNA (gRNA) sequence. However, selecting optimal gRNAs for efficient gene editing out of the pool of multiple gRNAs remains a major challenge due to the complexity of parameters affecting the editing efficiency. As a result, machine learning methods have been used to construct tools that predict gRNA activity and help select the optimal ones. Although these tools have advanced significantly, their performance and accuracy remain questionable.Due to the lack of significant publicly available data concerning gRNA activity for CRISPR/Cas9, common issues such as small sample sizes, poor representativeness, data leakage, and/or overfitting issues lower the effectiveness of data-driven solutions. To create an improved tool predicting gRNA's activity, we developed HelexOn, a new hybrid deep learning model for precisely detecting gRNA activity trained on low volume, high dimensional data. Both stacked attention-based multilayer perceptron MLP units (CNN-Att-MLP) and stacked 1D convolutional neural network (1D-CNN) blocks were included in HelexOn. To extract features from the input sequences, we first employed the 1D CNN layer. The average pool layer then assisted in reducing the dimensionality of the features that the 1D CNN had extracted, thereby conserving the crucial information that fastened the model's computational time. Focusing or emphasizing on features that are essential for outcome prediction was made easier by the attention layers. Dropout layers kept the model from overfitting due to low data availability. HelexOn demonstrated a remarkable performance on both training and testing datasets.To generalize and evaluate the effectiveness of our HelexON model, we tested it on publicly available CRISPR/Cas9 edited datasets from different cell lines, including HeLa, HCT116, RPE-1, A375, HL60, KBM-70, HEK293T, Mouse-EL4, MOLM13 and on Endogenous site on K562, HEK293 and Human Embryonic stem cells (H1). We also tested twelve published prediction models for cleavage activities of gRNAs on the above datasets and compared the performance using the Spearman Correlation between the predicted and actual output values as a metric. HelexON demonstrated comparable performance to existing top predictors even with a smaller training dataset. As more data is generated in the future, hybrid deep learning models such as HelexON will significantly outperform shallow neural networks. Such superior models help in precisely predicting gRNA efficiency and advancing the development of CRISPR/Cas9-based gene therapies.

1693. Comprehensive Safety Analysis Using Spatial Genome Organization Data Assures Long Term Safety of Gene Editing

Shoko Iwai[1], Shamik Mitra[1], Nikhil Panchal[1], Kuljeet Sandhu[2], Rakesh Kappagantu[1], Pavan Kumar Kodamanchili[1], Rohini Kalvakuntla[1], Poulami Chaudhuri[1]

[1]Helex, Inc., New York, NY,[2]Biological Sciences, IISER, Mohali, Mohali, India

CRISPR-Cas technology is promising in providing targeted gene editing for therapeutic outcomes. Careful assessment is necessary to ensure its safety as it advances toward clinical applications involving permanent modifications in the genome. Our understanding of how the editing and potential off-targets interact with other vicinity regions in the genome and their consequent short- and long-term effects are under-explored. Recent studies showed the three-dimensional (3D) spatial organization of chromatin in tissues and cells plays a critical role in gene regulation and function. In our previous study, we demonstrated the proximity between on- and off-targets and between off-targets are associated with CRISPR off-target events and translocations. Here, we assessed guide RNA safety for a kidney target by using tissue- and cell-specific 3D spatial organization data. First, target (kidney) and off-target (liver) tissue and cells were identified based on the in vivo non-viral LNP vector biodistribution assay, and corresponding spatial genome data (Hi-C) were retrieved from public repositories such as ENCODE. About 200 primary off-targets were identified based on sequence similarity, chromatin accessibility, and corresponding gene expression at the targeted tissue (kidney) as potential sites that could be edited unintentionally. Over 1,000 secondary off-targets were identified based on the proximity to the target or primary off-targets in the 3D genome space. To assess the risk associated with the off-target sites and assess the safety of the gene editing apparatus, the following steps were performed. Off-target sites were first annotated by locational information (exon, UTRs, intron, intergenic, splice junction, or deep intronic), then searched for any known regulatory elements (miRNA, lncRNA, promoter, enhancer, TF binding, or CTCF binding). For the regulatory elements, the number of genes that the region affects was also examined. Known clinical significance was searched with the corresponding tissue context in ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/). Potential association with cancer was assessed by searching annotated genes of the off-targets against onco- and tumor suppressor genes in the COSMIC database (https://cancer.sanger.ac.uk/cosmic), cancer-related structural variants and fusion genes in the Mitelman database (https://mitelmandatabase.isb-cgc.org/), known chromatin remodeling genes, and known fragile sites. The CRISPR-mediated structural variant risk was assessed by comparing sequence homology and 3D proximity of the sites between target, off-target, and fragile sites. Rare variants were also searched against the gnomAD database (https://gnomad.broadinstitute.org/). An over-representation analysis was conducted to assess pathway-level effects of the primary- and secondary- off-targets. Finally, safety score was calculated for each category and overall safety was evaluated. Considering 3D spatial genome organization in a tissue and cell-specific manner provided a comprehensive view of potential functional and long-term effects as a consequence of CRISPR edits. This approach is essential in developing precision medicine to deliver safer gene editing therapy.

1694. ONE-seq for Variant-Aware Therapeutic Guide Selection

Douglas R. Smith, Alyssa Ferreira, Ashley Lester, Shankar Shankaracharya, Ravindran Ravindran, Vijetha Vemulapalli, Andrew Hollinger, Thomas E. Mullen
SeQure Dx, Inc., Waltham, MA

Choosing a guide RNA with the lowest off-target impact across human populations for therapeutic CRIPSR gene editing is a challenging task. Computational tools for selecting candidate guides can provide rapid results, even across multiple genomes, but their algorithms are unable to accurately predict off-target editing efficiency. Biochemical methods, on the other hand, are able to quantify off-target editing but are limited in their ability to assess population-scale variation. Here, we present an application of the ONE-seq assay (Oligonucleotide Enrichment and Sequencing) for guide selection to identify guides with the lowest potential off-target risk. ONE-seq leverages both computational and biochemical components for a highly sensitive screen of candidate off-target sites across thousands of genomes. The in vitro cleavage data are augmented with biological annotation of off-target sites and classification of ONE-seq scores to enable prioritization of high scoring sites by impact. We applied ONE-seq in a variant-aware manner to identify *in vitro* off-target sites across global human populations for three therapeutically relevant guide RNAs targeting the PCSK9 gene. Comprehensive ONE-seq libraries were generated including sites with up to 6 differences relative to the on-target site that were identified by screening the HG38 reference sequence and over 4000 genomes from the 1000 Genomes and Human Genome Diversity Project data sets. Candidate off-target sites were identified by ONE-seq analysis and classified into multiple tiers based on their ONE-seq editing score and combined annotation concern score. The latter was based on functional annotation including proximity to the on-target sequence, variant effect prediction for a 2-base deletion, genetic constraint (tolerance to loss-of-function variation), potential gene expression impact (ENCODE elements, LncRNAs, and GTEx tissue-specific expression profiles for genic sites), and finally cancer and disease gene hits with associated inheritance information. Of the three guides, PCSK9-1 had by far the lowest number of sites with ONE-seq scores above 0.001 (43 out of 505,370 sites screened) including the lowest number of disease genes with dominant inheritance (2), cancer genes (1), and sites on the same chromosome (0). Of those high scoring sites, two (with ONE-seq scores of 0.15 and 0.002) represented variant off-target sites with high global allele frequency (26% and 17%, respectively) and elevated editing of the variant allele (874-fold and 68-fold, respectively). These variant sites represent multiple alleles of a single gene - ENSG00000232325 (a LncRNA of unknown function) that is expressed at significant levels in reproductive tissues and whole blood, based on GTEx data. This approach has the potential to improve the safety and reliability of guides for therapeutic CRISPR gene editing.

1695. SAFER-Detection for Efficient Interrogation of Rearrangements in Gene-Edited Human Cells

Hongyao Yu, Shankar Shankaracharya, Ricardo Petroni, Vijetha Vemulapalli, Douglas R. Smith
SeQure Dx, Inc., Waltham, MA

Gene editing therapy utilizing programmable nucleases shows great promise for treating genetic diseases, yet its progress is impeded by the risk of off-target editing. An essential criterion for evaluating human genome editing drug products involves the thorough examination of genomic integrity. This comprises the assessment of chromosomal rearrangements, large insertions or deletions, integration of exogenous DNA, and the potential for oncogenicity or insertional mutagenesis. In this study, we introduce Selective Amplification for Efficient Rearrangement Detection (SAFER-detection), a tagmentation and next-generation sequencing based method designed to enable quantitative detection of chromosomal rearrangement breakpoints with single base resolution, which is capable of classifying rearrangements resulting from on-target and off-target editing by programmable nucleases such as CRISPR/Cas and TALENs. SAFER detection using Cas9 with a CCR5 guide RNA readily identified intra-chromosomal deletions, insertions, and inversions between the on-target site (CCR5) and off-target or homologous sites in a nearby homolog (CCR2). Inter-chromosomal translocations between the CCR5 target site and off-target sites on chr1 and chr13 were also captured, and further validated by PCR. SAFER detection exhibits high sensitivity in detecting intra-chromosomal and inter-chromosomal aberrations mediated by off-target activity or by homologous recombination, and is applicable to samples containing low cell numbers. When combined with a sensitive off-target nomination technology such as ONE-seq, SAFER detection provides a valuable method to assess the risk of chromosomal rearrangements in therapeutic genome editing.

Friday Posters: Gene Targeting and Gene Correction New Technologies

1697. Genome Integrity Assessment by Optical Genome Mapping for Research in Cell and Gene Therapy Applications

Alexander Chitsazan, Joseph Estabrook, Andy Wing Chung Pang, Alex Hastie, Alka Chaubey
Bionano, San Diego, CA

Many cell lines and manufactured cell-based products are used in research, such as transformed cell lines for constitutional and oncological research. Cells in culture, or cells that have undergone gene editing can lose genome integrity, often seen as structural and numerical abnormalities, which can result in improper function and even transformation to malignancy. QC methods are critical to identify potentially detrimental events. While karyotyping is used for detecting large-scale DNA rearrangements, it misses many events due to its low clonal sensitivity, low resolution, and dependency on size. Additionally, PCR and targeted sequencing can characterize small variants but are

not sensitive to off-target or most structural genomic variants. Finally, while whole genome sequencing can identify small variants genome-wide, it has limited sensitivity with structural variants (SVs) at low variant allele fraction (VAF). Optical genome mapping (OGM) is an unbiased, genome-wide, high-resolution approach that can be used as a single assay alternative to multiple cytogenetic techniques to get a comprehensive view of the genome. To spot clonal variants, 400 Gbp of data is collected from the edited and control samples. A de novo assembly is then constructed, and SVs are called. Afterward, a dual variant annotation pipeline can be used to call differences between the edited and control samples. Alternatively, to discover somatic SVs down to VAFs of 5% or approximately 1% VAF, one can generate 1.5 or 5.0 Tbp of data. Here, a series of in silico simulations was performed to examine the limit of detection of OGM at deep coverage (5.0 Tbp), and the SV analysis workflow was run to detect variants at different known VAFs, down to 1%. The resulting data showed sensitivity to detect at ~1% VAF deletions >50 kbp, insertions >20 kbp, duplications >100 kbp, and translocations. Here, the clonal and somatic workflows were applied to verify the integrity of genomes after cell line immortalization, induce pluripotency, transgene-integration, and gene-editing. This data proves the effectiveness of OGM as a comprehensive solution for genome modification and integrity QC.

1698. Cell-Specific Genome Editing Using Receptor-Targeted Virus Like Particles

Surapathrudu Kanakala[1], Melissa Moy[1], Christopher Ziegler[2], Gopal Naik Nenavath[1], Stephen J. Russell[2]

[1]Imanis Life Sciences, Rochester, MN,[2]Vyriad, Rochester, MN

Clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR associated proteins) technology has accelerated the development of gene editing therapies. Enveloped viral and nonviral vectors have shown great promise for delivering CRISPR reagents to ex vivo cultured target cells. However, a major goal for future gene editing protocols is to develop vectors that can directly deliver Cas nucleases with associated guide RNAs to hematopoietic stem cells (HSC) in vivo. Retroviral and lentiviral VLPs (Virus-Like Particles) have recently emerged as attractive vehicles with which to target the delivery gene editing nucleases along with guide RNAs. Here we report the use of our novel VSV-G protein targeting technology to achieve receptor-specific delivery of gene editing ribonucleoprotein complexes using engineered lentiviral VLPs. Untargeted, EGFR targeted and cKit-targeted lentiviral VLPs were generated, each incorporating a Gag-Cas9 fusion protein bound to a single guide RNA targeting the start codon of the DEAD-box helicase 3 (DDX3). Receptor-targeting of the VLPs was achieved by pseudotyping them with VSV-G proteins that had been mutated to destroy their LDL receptor binding activity and further engineered to display epidermal growth factor (EGF) or stem cell factor (SCF) ligands as N-terminal fusions. These retargeted VLPs were shown to specifically deliver their payloads into EGFR-positive or cKit-positive K562 cells (respectively). Deep sequencing (>10,000 reads) of PCR products encompassing the DDX3 target site was used to estimate the efficiency of targeted gene editing of the DDX3 start codon in the transduced K562 cells. On-target editing was readily detected in EGFR-positive, cKit-positive and parental K562 cells transduced with the nontargeted VLP (~18% of reads in each case). In contrast, on-target editing following transduction with the EGFR-targeted VLP was detected exclusively in the EGFR-positive K562 cells (16% of reads). Similarly, on-target editing following transduction with the cKit-targeted VLP was detected exclusively in the cKit-positive K562 cells (79% of reads). Our findings demonstrate the feasibility of achieving cell-specific genome editing through the utilization of receptor-targeted VLPs and highlights their prospective future application for in vivo cell-specific gene editing.

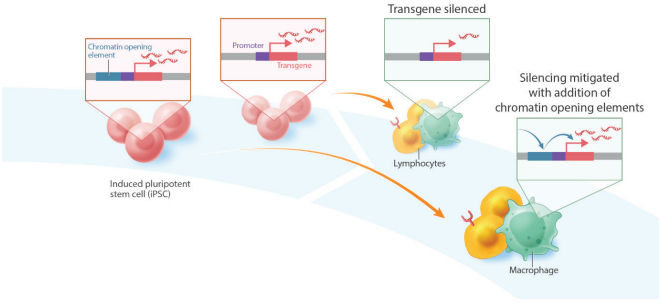
1699. Chromatin Opening Elements Mitigate Silencing of Transgenes During iPS Cell to Macrophage or Lymphocyte Differentiation

Claire Aibel, Abigail Blatchford, Christopher B. Rohde, Matthew Angel

Factor Bioscience Inc., Cambridge, MA

Induced pluripotent stem cell (iPSC)-derived therapies hold potential for increased scalability, broader accessibility, and superior production of genetically identical cells compared to traditional allogeneic approaches. Clonal populations of immune cells expressing therapeutic transgenes can be generated from gene-edited iPSCs. Synthetic promoters are often paired with such transgenes, as they can drive high levels of protein expression and avoid genomic disruption associated with the use of endogenous promoters. However, synthetic promoters can become silenced during differentiation or, conversely, induce expression above a therapeutic range. Therefore, there is a need to engineer regulatory elements that can withstand differentiation and drive clinically relevant levels of a desired protein in multiple therapeutic cell types. We designed novel sequences consisting of a synthetic promoter, EF1α or SFC, and an upstream ubiquitous chromatin opening element (UCOE). We generated iPSC lines containing a GFP reporter under EF1α alone, EF1α with a UCOE (uEF1α), or SFC with a UCOE (uSFC) inserted into the AAVS1 safe harbor locus. The iPSCs were differentiated via static culture into lymphocytes (CD7+) or macrophages (CD14+, CD33+), which displayed expected surface marker expression. We found that uEF1α, compared to EF1α alone, promoted not only higher baseline GFP expression levels in both iPSCs and differentiated cells, but also less differentiation-induced silencing. In iPSCs, the percentages of GFP+ cells were similar (EF1α = 97.9%, uEF1α = 98.1%), but median fluorescence intensity (MFI) among the GFP+ cells was 1.6-fold higher under uEF1α than under EF1α. In the iPSC-derived lymphocytes, the percentage of GFP+ cells was 36.5-fold higher under uEF1α (73%) than under EF1α (2%). Comparison of GFP expression before and after differentiation to lymphocytes indicated that 96% of EF1α cells, but only 25% of uEF1α cells, underwent silencing. Of the GFP+ lymphocytes, the MFI was 7.5-fold higher under uEF1α than under EF1α. In the iPSC-derived macrophages, the percentage of GFP+ cells was 13-fold higher under uEF1α (78%) than under EF1α (6%). These data indicate that, during differentiation to macrophages, 92% of EF1α cells, but only 20% of uEF1α cells, underwent silencing. Of the GFP+ macrophages, the MFI was 2.1-fold higher under uEF1α than under EF1α. Furthermore, we found that the addition of a UCOE did not confer constitutively high gene expression but rather could be modulated through promoter selection to achieve clinically relevant expression levels. uSFC iPSCs were differentiated in parallel with the uEF1α iPSCs into macrophages. The percentage of GFP+ macrophages under uSFC was 17% (compared to 78% under uEF1α), and among the GFP+ population, the MFI was

4-fold higher under uEF1α than under uSFC. These results suggest that pairing a UCOE with a weaker promoter, such as SFC, could be beneficial when lower transgene expression is desired. In summary, we demonstrate novel UCOE-containing regulatory sequences that mitigate silencing during differentiation of iPSCs into lymphocytes or macrophages. These sequence elements may prove useful in engineering cells with functional transgenes encoding therapeutic proteins, and could contribute to the development of effective engineered iPSC-based therapies.



1700. Efficient Ex-Vivo Selection of Gene Edited Hematopoietic Stem Cells by Leveraging on Artificial Transactivators and Lipid Nanoparticle-Mediated Delivery

Samuele Ferrari[1], Martina Fiumara[1], Daniele Canarutto[1], Angelica Varesi[1], Luisa Albano[1], Chiara Gaddoni[1], Erika Zonari[1], Angelo Amabile[1], Angelo Lombardo[1], Bernhard Gentner[1], Pietro Genovese[2], Luigi Naldini[1]

[1]SR-TIGET, Ospedale San Raffaele, Milan, Italy,[2]Dana-Farber Cancer Institute, Boston, MA

Hematopoietic stem/progenitor cell (HSPC) gene editing by homology directed repair (HDR) holds the promise of precise, targeted integration of a therapeutic transgene in genomic safe harbor loci or *in situ* gene correction of disease-causing genes. However, several hurdles have hindered clinical translation of these strategies in HSPCs, including: i) low HDR editing efficiencies in the rare fraction of long-term repopulating HSPCs; ii) toxicity and loss of stemness potential upon delivery of editing components and ex-vivo culture; iii) safety concerns related to frequent occurrence of unintended, potentially genotoxic, repair outcomes at the nuclease on and off target sites. Here, we tackled these limitations by combining a novel enrichment strategy for HDR-edited HSPCs, clinically compliant culture conditions supporting HSPC expansion, and optimized lipid nanoparticle (LNP)-mediated delivery of RNAs. To enrich for a pure population of HDR-edited HSPCs, we designed a Selection by Means of Artificial Transactivators (SMArT) strategy based on HDR donor templates comprising a selector gene, whose expression is conditional to precise on-target integration and confined to a limited ex vivo processing window. Basal expression of the selector was nearly undetectable given the presence of only a minimal promoter in the template and was induced to actionable level upon transient mRNA delivery of an ArT that binds the genome nearby, but outside, the homology region. This strategy enriched the HSPC product to >80% HDR-edited alleles. Transactivation efficiency, however, was halved in the most primitive HSPCs, suggesting lower permissiveness of this subset to ArT delivery and/or transcriptional activation of the edited locus. PCR drop-off assays on hundreds of single colonies derived from sorted and bulk edited HSPCs confirmed that >95% of the clonogenic, enriched HSPCs carried the intended HDR edit. Moreover, SMArT efficiently purged out HSPC clones bearing on-target long-range deletions from the positive fraction, alleviating the safety concerns related to genotoxic byproducts of editing. Xenotransplantation of SMArT-enriched HSPCs resulted in fully HDR-edited grafts, albeit to a lower human chimerism compared to a control group receiving the same number of bulk unselected cells. As expected by design, selector expression was no longer detectable in the xenografts. By leveraging on a clinically compliant protocol for HSPC culture, and improved LNP-mediated co-delivery of Cas9 nuclease and editing enhancers, we then achieved improved preservation of phenotypically primitive HSPCs and higher HDR efficiency prior to enrichment, further increasing the yield of selectable HSPCs. We anticipate that the combination of SMArT enrichment strategies, optimized culture conditions, and harmless delivery modalities may overcome current limitations and concerns of HDR editing, facilitating its clinical translation.

1701. Precision Editing of PLNR14del Mutation Using a Self-Inactivating, All-in-One AAV Vector to Rescue PLN-R14del-Associated Cardiomyopathy

Emma Xu, Max Levine, Uma Lakshmanan, Melissa Van Pell, Laura Lombardi, Amara Greer-Short, Kathryn Ivey, Timothy Hoey, Huanyu Zhou

Gene Therapy, Tenaya Therapeutics, South San Francisco, CA

The *Arg14del (R14del)* mutation in the *Phospholamban* (*PLN*) gene is a common genetic cause of familial dilated or arrhythmogenic cardiomyopathy. *PLN*'s role negatively regulating sarco/endoplasmic reticulum Ca2+-ATPase (SERCA) in cardiomyocyte calcium homeostasis is disrupted by the *R14del*mutation, leading to both super-inhibition of SERCA activity and PLN protein aggregation. *PLN-R14del*-associated cardiomyopathy has a poor prognosis, high adolescent mortality, limited therapeutic options other heart transplantation, and no therapies that address the underlying genetic cause of the disease Genome editing, particularly with the CRISPR-Cas9 system, has opened avenues for modifying genes associated with genetic disorders, ushering in a potential era of curing and preventing human genetic diseases. *In vivo* delivery of CRISPR-Cas9 components holds promise for treating *PLN-R14del*-associated cardiomyopathy by specifically addressing the *R14del* mutation. Indeed, we previously demonstrated the efficacy of this approach in the *PLN-R14del* mouse model, using an All-In-Oneadeno-associated virus (AAV) vector. However, a significant challenge in gene editing applications is the immunogenicity arising from the bacteria-originated gene editing machinery, along with concerns about off-target events. This becomes pronounced when employing an AAV vector for genome editing, as it results in the continuous expression of the therapeutic transgene, unlike other gene editing methods, such as lipid nanoparticles (LNPs) with only transient expression. Our approach grapples with the distinct challenge of prolonged transgene expression through the AAV vector. To overcome this challenge, we designed a Cas9 self-inactivating editing

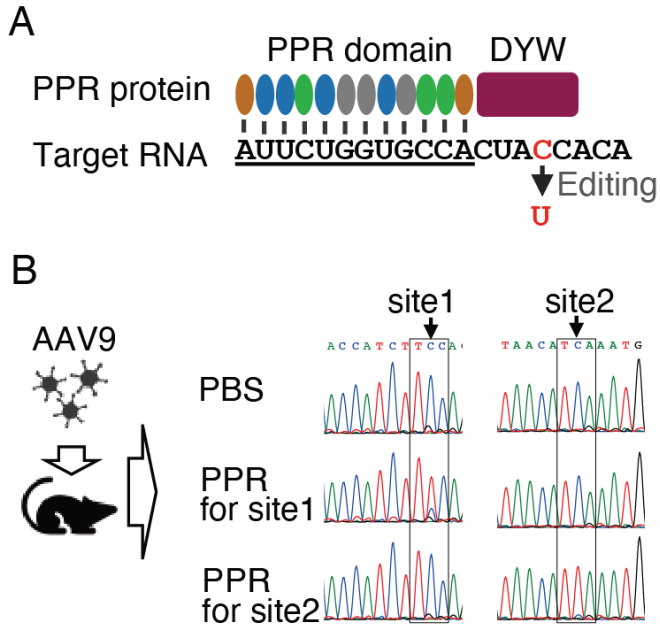
system, that achieves high editing efficiency even with transient Cas9 protein expression. We have developed a gene therapy utilizing a single AAV vector to deliver both a self-inactivating Cas9 and *PLN-R14del*-specific sgRNA. Initial testing in patient-specific human induced pluripotent stem cell (iPSC)-derived cardiomyocytes demonstrated precise and efficient editing of the *PLN-R14del* allele without affecting the wild-type *PLN*allele. Further testing in a mouse model at various doses revealed a rescue of heart function in *PLN-R14del* mice to near wild-type levels, along with a significant prolongation of survival. Notably, these positive effects were observed even at low doses in the range of E13 vg/kg with near-complete inactivation of the Cas9 enzyme by four weeks post-viral administration. These preclinical results suggest that *PLN-R14del* gene editing holds promise as a therapeutic approach for *PLN-R14del*-associated cardiomyopathy.

1702. Efficient *In Vivo* C-to-U RNA Editing Using Pentatricopeptide Repeat (PPR) RNA-Binding Proteins

Yusuke Yagi[1], Masaru Ota[1], Takayuki Tamai[1], Mizuho Ichinose[1], Bernard Gutmann[1], Takahiro Nakamura[2]

[1]EditForce, Inc., Fukuoka, Japan,[2]Kyushu University, Fukuoka, Japan

The development of RNA base-editing technologies has predominantly focused on ADAR-mediated A-to-I substitutions. However, efficient *in vivo* C-to-U editing remains relatively undeveloped. Our research pioneered this by harnessing pentatricopeptide repeat (PPR) proteins, which are involved in C-to-U RNA base editing in plants. PPR proteins, characterized by their tandemly repeated PPR motifs, recognize RNA sequences in a manner dictated by a unique PPR code that links specific amino acids within each motif to the corresponding RNA nucleotides (Fig.A). The C-terminus of PPR proteins lies in a domain known as the DYW domain, which resembles deaminase. This domain is known for its role as a C-to-U RNA-editing enzyme in plant chloroplasts and mitochondria. This understanding of the functional structure of PPR proteins provides a scaffold for designing artificial C-to-U RNA-editing enzymes to enable targeted and precise RNA modifications. To develop artificial base-editing enzymes (PPR-editors), we initially analyzed various PPR motif sequences to understand their RNA-binding characteristics. This led to a methodology for designing PPR proteins capable of specifically and accurately binding to various target RNA sequences. Subsequently, we identified a highly active DYW domain in the plant PPR protein family. To evaluate our technology, we designed multiple PPR editors targeting the mouse *Gapdh* gene and assessed their RNA editing efficiency in Hepa1-6 cells. This assessment revealed that up to 40% of the target sites were successfully modified by our PPR editors.To measure the *in vivo* RNA editing activity of the PPR-editor, we incorporated PPR-editor genes into an AAV9 viral vector and systemically administered them to mice. Two months after administration, we isolated total RNA from various organs to measure editing activity. Remarkably, over 70% of the target RNA underwent editing in the muscle tissues, including the gastrocnemius, tibialis anterior, and heart (Fig. B).These findings underscore the potential of our designed PPR editors, delivered via AAV vectors, to achieve high-efficiency RNA editing, heralding a promising avenue for gene therapy. Although the efficacy of PPR technology in editing RNA across different organs remains to be fully ascertained, optimizing the serotype and administration methods could potentially enable targeted delivery of the PPR gene to specific organs.



1703. Systemic AAV-Delivered Base Editing of ACTA2^{R179H} Improves Pupillary Smooth Muscle Cell Contraction in Mice: A Promising Target Engagement Biomarker to Predict Clinical Efficacy

Claire Elyse Fong[1], Elyssa Alber[1], Shruti Gajjar[1], Vijai Krishnan[1], Sabya Das[1], Kangsan Roh[1,2], Christiano Alves[1], Shuqi Yun[1], Lauren Fox[1], Leillani Ha[1], Hannah Stutzman[1], Benjamin Kleinstiver[1,2], Casey Maguire[1,2], Mark Lindsay[1], David Chung[1,2], Patricia Musolino[1,2]

[1]Massachusetts General Hospital, Boston, MA,[2]Harvard Medical School, Boston, MA

Introduction**:** A missense variant in the gene encoding the smooth muscle specific actin isoform α-SMA (ACTA2) at arginine 179 (most commonly replacement by histidine) causes one of the most severe forms of vascular disease and a multisystemic smooth muscle dysfunction syndrome (MSMDS). Children with the disease suffer recurrent strokes, aortic dissections, and death in the first 2 decades of life. Since first described in 2010, most patients can be diagnosed soon after birth by identification of congenital mydriasis (fixed pupillary dilation) caused by loss of smooth muscle cell (SMC) contractility due to the mutant α-SMA. While the ocular phenotypes remain understudied, we have previously shown that gene correction by AAV-delivered CRISPR/Cas base editors can restore vascular function and prevent early neurodegeneration and death in a mouse model of the disease. Here, we studied SMC function in the eye of the mouse and its response to treatment. Methods**:** We have developed a knock-in murine model of MSMDS in which a Cre-inducible mutant allele (*ACTA2*$^{R179H/fl}$) is expressed in heterozygosity once activated by a Cre recombinase driven by the smooth muscle cell (SMC)-specific *Myh11* promoter,

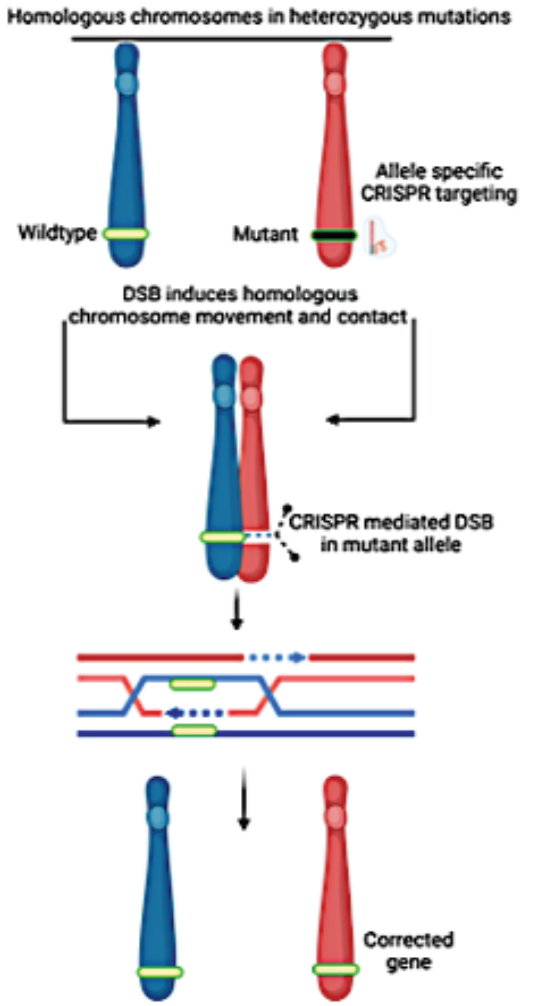
which faithfully recapitulates the human disease. To investigate *in vivo* gene editing mediated by AAV capsid in mice, we customized a Cas9-directed adenine base editor (ABE) and injected mice retro-orbitally at P3 with both AAVPR and AAV9 vectors packaging the VRQR-ABE8e-pegRNA in a split intein design. We imaged the eyes of control, untreated mutant, and AAV9- and AAVPR-treated mutant mice at 8 weeks of age using a Basler imaging system consisting of a high-resolution camera, infrared illumination, and red LED light to observe pupillary reaction to light stimulus. Videos of the eye were captured and resting pupil diameter, degree of pupillary constriction at light stimulus, and degree of recovery dilation after the removal of stimulus were quantified. Results**:** We found that control mice have reliable and quantifiable pupillary reaction to light stimulus while ACTA2^{R179H} mutants showed large, dilated pupils with little to no response. Remarkably, eight weeks following injections, 80% of both AAVPR- and AAV9-treated mice showed decreased resting pupillary diameter resembling that of control mice. Pupillary constriction to light, a direct biomarker of restoration of α-SMA function and therefore SMC contraction, was restored in 60% of AAVPR- and 80% of AAV9-treated mice. These results demonstrate that AAV is capable of transducing SMC in the iris following systemic intravenous delivery and significantly restoring SMC contractility following gene editing. Conclusions**:** Mydriasis and pupillary nonreactivity are hallmarks of MSMDS in humans. Assessment of pupillary response to light, a non-invasive biomarker of SMC target engagement, could guide future clinical studies. Future studies in the mouse model of ACTA2^{R179H} MSMDS will focus on correlating ocular phenotypes to functional and histological analysis of vascular disease and neurodegeneration, which may help identify direct dose-response correlations in their treatment.

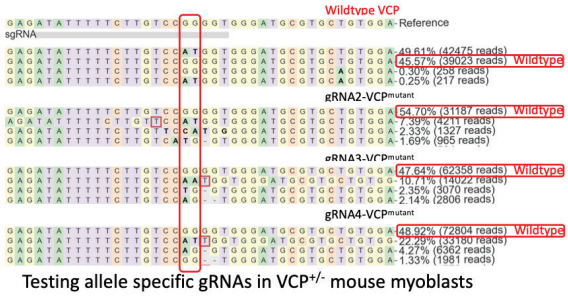
1704. REMEDY, a Novel CRISPR-Based Allele Specific Approach Corrects VCP Mutations without Exogenous DNA Template

Eleonora D'Ambrosio, Yasemin Sezgin, Burcak Ozes, Noushin Saljoughian, Genesis Snyder, Colin Maguire, Joshua Robinson, Jerry R. Mendell, Zarife Sahenk, Meisam Naeimi Kararoudi

Nationwide Children's Hospital, Columbus, OH

Introduction: Valosin-containing protein (VCP) multisystem proteinopathy (MSP) is a neurodegenerative and ultimately fatal disease. The genetic background of the disease (dominant negative pathology) allows for allele-specific targeting of the heterozygous mutation. *Methods:* We developed a strategy, called REMEDY -REpair of heterozygous Mutations independent of Exogenous Donor template with high efficiencY- to precisely target and correct the mutant allele in VCP-MSP by recruiting the wild-type homologous chromosome as an endogenous template (Figure 1). We screened mutant allele-specific gRNAs to target one of the most frequent mutations in VCP, R155H (c.464G>A). We first tested this approach in patient-derived fibroblasts. We then focused on myoblasts derived from a mouse model of VCP-MSP. After optimizing the electroporation conditions, both patient-derived fibroblasts and mouse myoblasts were electroporated with Cas9/RNP. *Results:* We observed the correction of the mutant allele without an exogenous template. In patient-derived fibroblasts the frequency of the wild-type allele targeted with one of the gRNAs tested increased from 50 to 71%. In mouse myoblasts the frequency of the wild-type allele increased from 50% to 58% (Figure 2). The analyses were done by next generation sequencing and CRISPResso2. *Conclusions:* We observed that 21% of the patient-derived fibroblasts and 8% of the mouse myoblasts had reverted to homozygosity for the wild-type allele. REMEDY has the potential to efficiently repair other diseases caused by heterozygous mutations and without the necessity of an exogenous donor DNA template.





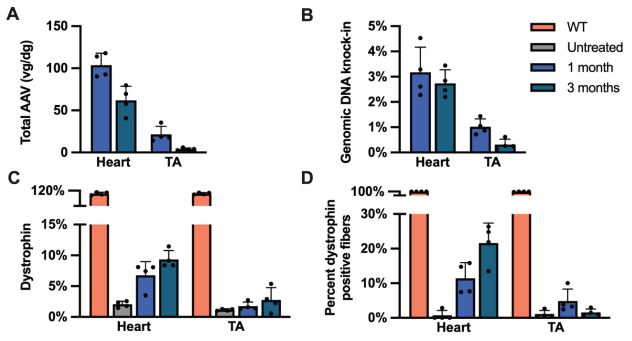
Testing allele specific gRNAs in VCP$^{+/-}$ mouse myoblasts

1705. Gene Editing by Homology-Independent Targeted Integration in Duchenne Muscular Dystrophy is Hampered by Loss of Edited Genomes and Fragmentary AAV Integration

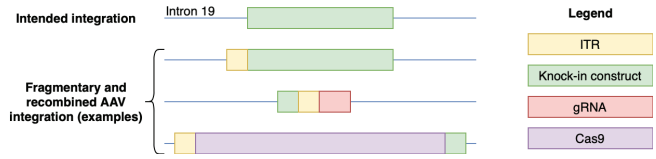
Stefan Nicolau[1], Anthony A. Stephenson[1], Tatyana A. Vetter[1,2], Jessica E. Sarff[1], Gabrielle P. Dufresne[1], Emma C. Frair[1], Kevin M. Flanigan[1,2,3]

[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[2]Department of Pediatrics, Ohio State University, Columbus, OH,[3]Department of Neurology, Ohio State University, Columbus, OH

Background: Duchenne muscular dystrophy is the most common muscle disease in childhood, affecting 1 in 5000 boys. Mutations in the N-terminal region of the *DMD* gene account for 25% of all patients. We previously developed a CRISPR/Cas9-based gene editing system employing homology-independent targeted integration to knock into the endogenous intron 19 of *DMD* a construct consisting of the coding sequence of exons 1-19 under the control of a new promoter. Translation from this new promoter effectively bypasses any upstream mutations and enables full-length dystrophin expression. **Methods:** The gene editing system was delivered to mice carrying an exon 2 duplication using a pair of AAV9 or MyoAAV3A vectors. Biodistribution, editing efficiency, editing outcomes, and dystrophin expression were measured at different time points. **Results:** With both AAV9 and MyoAAV3A, the highest biodistribution was observed in the heart, with significantly lower levels in the diaphragm and tibialis anterior (MyoAAV3A shown in Figure 1A). MyoAAV3A achieved 3-fold higher biodistribution in the heart compared to AAV9, but its performance was similar to AAV9 in skeletal muscles. The highest editing efficiency was 3% in the heart, enabling dystrophin expression levels of 9% of normal (Figure 1B-D). In skeletal muscles, there was a loss of both AAV and edited genomes between 1 month and 3 months post-injection. As a result, there was only a minimal increase in dystrophin expression.



Long-read sequencing identified integration of recombined and fragmentary AAV genomes at the target site, most frequently involving fragments of the inverted terminal repeats (Figure 2). These included both potentially therapeutic and non-therapeutic integrations, and collectively occurred at rates higher than the intended integration outcome.



Conclusion: This gene editing system results in an increase in cardiac dystrophin expression that is expected to be clinically meaningful. Efficiency is lower in skeletal muscles, potentially owing to a combination of vector tropism, immune mechanisms, and turnover of dystrophic muscle. Fragmentary AAV integration may occur at the site of Cas9-induced double-stranded breaks.

1706. Translating AAV-Delivered CRISPR-Cas13 Therapy for FSHD May Require Overcoming Size and Immune Challenges

Manal Ali[1], Afrooz Rashnonejad[1,2], Kate Neal[1], Gerald Coulis[3], Armando Villalta[3], Scott Q Harper[1,2]

[1]Center for Gene Therapy, Abigail Wexner Research Institute, Nationwide Children's Hospital, Columbus, OH,[2]Department of Pediatrics, The Ohio State University College of Medicine, Columbus, OH,[3]UC Irvine, Irvine, CA

Facioscapulohumeral muscular dystrophy (FSHD) is caused by aberrant expression of the toxic *DUX4* gene in muscle, leading to myofiber death and dysfunction. No approved disease-modifying treatments currently exist. We hypothesized that a FSHD treatment should center on *DUX4* inhibition, and we are developing a CRISPR-Cas13-based approach to accomplish this goal. Cas13 has emerged as a potent alternative to Cas9-based genome editing strategies, as Cas13 cuts RNA instead of DNA, thereby reducing potential genotoxicity. In this study we used Cas13b and Cas13d proteins from *Prevotella sp. P5-125* and *Ruminococcus flavefaciens XPD3002* bacterial species, respectively. Cas13b is too large to allow co-packaging of a guide RNA (gRNA) expression cassette in the same AAV genome. The first-generation system required injection of 2 AAVs, one expressing the gRNA from a U6 promoter and a second carrying the Cas13b protein expression cassette. In contrast, Cas13d is sufficiently small to co-package with a gRNA expression cassette in the same AAV. Although Cas13b and Cas13d function similarly, they arise from different bacterial species and utilize different gRNA structures. This necessitated designing two sets of gRNAs targeting *DUX4* mRNA, which we then tested *in vitro* and *in vivo*. We began with Cas13b. Following identification of lead gRNAs using various outcome measures in FSHD cell models, we tested the system *in vivo* using two FSHD mouse models. Following intramuscular injection in a rapidly progressing AAV-based FSHD mouse model, we measured significant *DUX4* mRNA reductions in AAV.Cas13b/gRNA1 co-treated muscles. This knockdown was accompanied by histological improvements, as well as reductions in DUX4 protein, DUX4-activated biomarkers and apoptotic nuclei at early timepoints (2 wks). In our genetic mouse model of FSHD (TIC-DUX4), Cas13b/gRNA1 also reduced DUX4-induced myopathy but protection waned over time. In wild-type mice, loss of Cas13b expression was accompanied by cytotoxic T-cell responses in skeletal muscle, suggesting an immune response to the Cas13b protein. We are now exploring several strategies to overcome this immune challenge, including testing Cas13 proteins from other bacterial species, such as Cas13d. For the smaller Cas13d enzyme, we

designed and tested 50 gRNAs, all of which caused significant *DUX4* knockdown compared to non-targeting controls using several *in vitro* assays. We then selected 3 leads, based on their ability to consistently reduce DUX4 transactivation activity and FSHD biomarker expression, and knockdown DUX4 protein by 80-90% *in vitro*. *In vivo* testing of the potency and durability of single AAV-Cas13d+gRNAs in our FSHD mouse models are still ongoing. We conclude that CRISPR Cas13 is a robust *DUX4* RNA-targeting tool *in vitro* and a promising strategy for *DUX4* mRNA silencing *in vivo*, if immune response challenges can be overcome.

Friday Posters: Oligonucleotide Therapeutics

1708. Development of a microRNA-Based Gene Therapy Platform for Laminopathies

Neel D. Lachhwani[1], Nizar Y. Saad[1,2]

[1]Center for Gene Therapy, Nationwide Children's Hospital, Columbus, OH,[2]Department of Pediatrics, The Ohio State University, Columbus, OH

Laminopathies are a group of genetic diseases caused by mutations in the *LMNA* gene. These are ultra-rare diseases with a prevalence range of 1 in 20 million (i.e., the Hutchison-Gilford Progeria Syndrome) to 1 in 45 thousand (i.e., dilated cardiomyopathy). The laminopathy group of diseases encompasses at least 27 ultra-rare diseases with spontaneous or inherited mutations in the *LMNA* gene. Most of them possessing an autosomal dominant pattern of expression, leading to a spectrum of clinical manifestations affecting various organs, including skeletal muscle, cardiac muscle, connective and adipose tissue. We estimate the number of the autosomal dominant laminopathy patients to be around 20,000 in the US and 500,000 worldwide. To this date, these patients have no approved effective therapies. Therefore, the development of a gene therapy for these diseases is still an unmet need. Due to their ultra-rare prevalence, tackling them, one disease at a time is not a viable model of interest to the biotech industry and private investors, whose support is essential to translate prospective gene therapies to the clinic. Accordingly, we think that the development of a microRNA-based gene therapy platform would make a more compelling and efficient strategy with unified and adaptable framework. This strategy will address commonalities in the design of the gene therapy by using the same miRNA, same target gene (*LMNA*) and delivery vehicle, reducing the cost of toxicology studies. It will also address the treatment of common clinical features i.e., cardiac, muscular and connective tissue abnormalities, streamlining and accelerating the translational process. Hutchinson-Gilford Progeria Syndrome (HGPS) or Progeria is the first disease to be targeted by this strategy. HGPS is an autosomal dominant genetic disease affecting 400 patients worldwide. It is caused by a single C to T substitution in exon 11 of the *LMNA* gene, leading to the formation of a cryptic splice site and the expression of a modified Lamin A nuclear membrane protein isoform called "Progerin". Progerin is toxic and elicits pathology in multiple tissues, leading to premature physiologic aging and eventual death at the current average age of 14 years. Our therapeutic strategy consists of knocking down *Progerin* expression in affected tissues using *Progerin*-targeting microRNAs (miRNAs) that use the RNA interference (RNAi) pathway to silence gene expression. The *LMNA* gene encompasses exons 1-12. Through physiological alternative splicing of the *LMNA* transcript, two isoforms are produced, *Lamin A* and *C*. *Lamin A* encompasses exons 1-12, and *Lamin C*, exons 1-10. The *Progerin* transcript is produced through the activation of a cryptic splice donor site upon the presence of a C>T point mutation at position 1824 (c.1824C>T; p.G608G), leading to a transcript encompassing exons 1-12, but having a deletion of 150 nucleotides in exon 11. Accordingly, our RNAi-based gene therapy aims to target exon 12, knocking down both *Progerin* and *Lamin A* transcripts. Previous studies showed that the absence of lamin A is dispensable. Using a miRNA prediction algorithm, we identified 46 artificially designed miRNAs predicted to target exon 12. To validate target engagement and assess silencing efficiency, we screened these miRNAs in HEK293 cells using a dual luciferase assay in which *Progerin* transcript is expressed. Most miRNAs were able to knockdown *Progerin* and had an inhibition efficiency ranging from 18-60%. *miPRG1*, had the highest inhibition efficiency, reducing by 60% *Progerin* expression. We also tested *miPRG1* in patient-derived fibroblasts and showed similar silencing efficiency. In future steps, we will test *miPRG1* in additional patient-derived fibroblasts and *in vivo* using the $Lmna^{G608G/G608G}$ HGPS humanized mouse model.

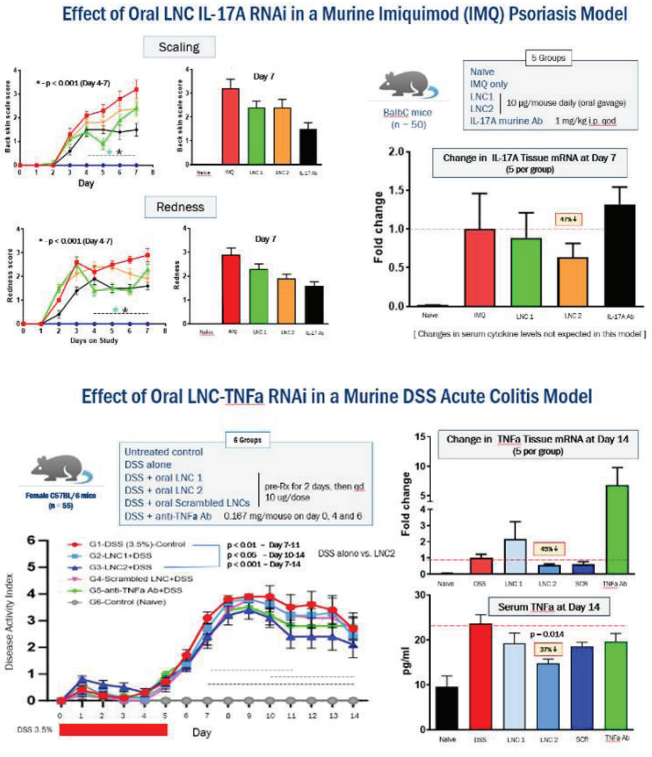
1709. Successful *In Vivo* Oral Delivery of Biologically Active and Therapeutic Anti-Inflammatory mRNA-Targeted Oligonucleotides with a Lipid Nanocrystal Delivery Platform

Hui Liu[1], Vinod Ramgolam[2], Jeffrey Bender[2], Miriam Mikhael[1], Amra Tabakovic[1], Tzong-Jen Sheu[1], Partha Samadder[1], Thomas Hoover[1], James J. Ferguson[1]

[1]Matinas BioPharma, Bedminster, NJ,[2]TargetSite Therapeutics, New Haven, CT

Background: There has been little progress in the oral delivery of nucleic acid therapeutics beyond the liver. Matinas BioPharmas' lipid nanocrystal (LNC) platform successfully delivers oral amphotericin-B in patients with cryptococcal meningitis, and other ex vivo work has shown avid LNC uptake by innate immune cells. Prior studies have shown the in vitro efficacy of two mRNA-targeted oligos - one knocking down IL-17A, the other knocking down TNFa. LNC formulations of both have shown greater cytokine knock-down than "naked" oligos in vitro. The present work evaluated the in vivo efficacy of oral LNC formulations of these oligos in two inflammatory disease models. **Methods:** Psoriasis in BALB/c mice was induced with 31.25 mg of 5% Imiquimod (IMQ) applied daily for 6 days. There were 5 treatment groups (n=10/group): untreated controls, IMQ alone, IMQ plus one of two different LNC-oligo formulations administered daily by oral gavage, and IMQ plus anti-IL-17A antibodies. Skin Erythema and scaling was scored daily. The study was terminated at day 7; cytokine mRNA levels in the psoriatic skin lesions were determined by qRT-PCR. Colitis in C57BL/6 mice was induced with 3.5% DSS in drinking water for 5 days. There were 6 treatment groups: untreated controls, DSS alone, DSS plus one of two different LNC-oligo formulations, DSS plus LNC formulated scrambled oligos, and DSS plus a TNFa neutralizing antibody. Daily disease activity scores were measured; animals were

sacrificed at day 14 and serum TNFa and tissue (colon) TNFa mRNA (qt-PCR) were measured. **Results:** In the imiquimod psoriasis model (**Figure 1**) daily oral administration of an LNC formulation of the IL-17A targeted oligo resulted in both knock-down of skin IL-17 mRNA, and significant improvement in clinical parameters of redness and scaling. Similarly, in the DSS colitis model (**Figure 2**), daily oral administration of an oral LNC formulation of the TNFa targeted oligo resulted in reductions of colon TNFa mRNA and significant reductions in serum TNFa levels, as well as significant improvements in disease activity scores. Thus, we have shown successful oral delivery of two RNAi oligos targeting different inflammatory cytokines in two different disease models; with documented biological/molecular activity as well as therapeutic efficacy. **Conclusions:** The LNC delivery platform can successfully orally deliver biologically active (and potentially therapeutic) oligonucleotides targeting key cytokines in inflammatory disease models. While these initial results are promising, there is still some individual heterogeneity of response; future work will be focused on optimizing the LNC formulations to improve delivery efficiency, increase their potency, and extend the application of oral cytokine-targeting oligo therapeutics in other inflammatory diseases. models.



1710. Novel Antisense Therapy to Durably Treat USH2A Patients

Stephanie A. Mauriac[1], Yu-Han Huang[2], Jiahe Jin[2], Jen Phillips[3], Jeremy Wegner[3], Monte Westerfield[3], Karl Koehler[4], Timothy Yu[2], Gwenaelle Geleoc[2]

[1]Otolaryngology, Boston Children's Hospital, Boston, MA,[2]Boston Children's Hospital, Boston, MA,[3]University of Oregon, Eugene, OR,[4]Boston Children's Hospital/Harvard Medical School, Boston, MA

Usher syndrome (USH) is the most common inherited genetic disease of combined deafness and blindness. Mutations in *USH2A* account for half of USH cases. The size of *USH2A* (~15.6Kb) prevents the development of classic gene augmentation therapy mediated by adeno-associated virus (AAV)-based vectors, because it exceeds the capacity of single or even dual AAVs. Thus, we took advantage of an antisense oligonucleotide (ASO) strategy to correct hearing and vision loss in patients living with USH2A. We target an out-of-frame mutation, an *USH2A* founder mutation identified in patients at Boston Children's Hospital, and a major cause of USH in the French-Canadian population. To restore the proper reading frame, we developed a strategy leading to dual exon skip of human *USH2A*. Our hypothesis is that in-frame deletion of two exons in human will result in expression of slightly shorter yet still functional USH2A pseudo-protein. *In silico* analysis was performed using Alphafold2 to predict the 3D structure of both wild-type and pseudo-USH2A protein. We designed ASO sequences based on computational analyses of cis-regulatory sequences predicted to impact splicing. Candidate ASOs were synthesized with modified 2'-O-methyl nucleotides and phosphorothioate backbones. ASOs were either (1) transfected into retinoblastoma cells (WERI-RB1) or (2) applied gymnotically to inner ear and retinal cells derived from human mutant and isogenic iPSCs. We performed RT-PCR to validate ASO-induced *USH2A* dual-exon skipping. To test function, two *ush2a* zebrafish mutant models were generated using CRISPR-Cas9 and sgRNA to mimic (1) the patient's mutation or the (2) dual exon skipping strategy. Phenotype analysis was performed to assess vision (OKR), hearing (acoustical startle reflex), and balance (swimming behaviors) along with histology to assess inner ear and retinal structures. Our exon skipping strategy leads to an in-frame internal deletion of 105 amino acids out of 5202 amino acids. This deletion excises a single fibronectin domain. *In silico* analysis demonstrates the preservation of protein folding properties suggesting that protein function would be preserved. Our data demonstrate successful single and dual exon skip with different combinations and dosages of ASOs. The homozygous zebrafish models with truncating mutations in *ush2a* are viable, fertile and display normal startle response and vestibular behavior, but show a decrease in OKR and loss of Usherin protein localization, consistent with published zebrafish *ush2a* models. Histologic and functional assessments on the dual exon skipping zebrafish model are ongoing. ASOs have great promise as a platform to treat genetic disorders, including Usher syndrome specifically. They are simple to customize to different targets, inexpensive to manufacture, and can be efficiently delivered without special formulation. By demonstrating the high efficiency and safety of this approach, we hope to bring this novel antisense therapy from the bench to the bedside for USH2A patients.

1711. Design and Production of Circular RNAs (circRNAs) with Validated Functional Activity in Cell Models

Nicholas Dreisbach, Isabella Darke, Patrick Magunga, Meemanage De Zoysa, Miranda Yang, Dana Cairns, Stacie Seidel, Austin Thiel, Melissa Carpenter, Cherylene Plewa

ElevateBio, Waltham, MA

The Covid-19 pandemic has highlighted the therapeutic potential of Ribonucleic Acid (RNA) after messenger RNA (mRNA) was

successfully used in vaccines developed to combat the virus. Despite this success, mRNA technology has challenges including reactogenicity, poor stability, and high cost of manufacturing. Circular RNA (circRNA), which is a covalently closed RNA, is being developed to overcome the shortcomings of mRNA to achieve longer therapeutic expression. However, generating circRNAs with low impurities at a large scale has proven to be a challenge in the RNA field. At ElevateBio, we developed a robust and scalable method to produce high quality circRNAs using a Permuted Intron-Exon (PIE)-based construct design. A set of characterization assays were also developed and implemented to ensure the quality of final circRNA products. Initial testing validates the circRNA platform and demonstrates that ElevateBio circRNAs have low toxicity in cells, express robust levels of transgene products and show functional activity in cell models. The current circRNA design and production method are undergoing further development to be used in a range of therapeutic applications both ex-vivo and in vivo.

1712. One-Step Selection of High-Affinity Aptamers Using a Non-Fouling Porous Hydrogel

Yixun Wang, Yong Wang

Biomedical Engineering, The Pennsylvania State University, State College, PA

Aptamers are "chemical antibodies". They have garnered great attention across different fields owing to their economical production and robust structural stability compared to antibodies. However, the discovery of aptamers presents a substantial challenge due to numerous problems related to PCR amplification bias, ineffective partition, and nonspecific binding to the selection matrices. The purpose of this presentation is to discuss a novel aptamer enrichment and selection method - hydrogel for aptamer selection (HAS). In this method, aptamer candidates are loaded into a macroporous polyethylene glycol (PEG) hydrogel that is conjugated with target proteins. After loading, the candidates are allowed to diffuse out of the hydrogel. Low-affinity aptamer candidates are rapidly released from the hydrogel; however, high-affinity aptamers are slowly released. This difference in diffusion is attributed to their difference in the strength of binding to the immobilized proteins. Therefore, different aptamer candidates can be automatically separated during this diffusion-binding process for aptamer enrichment and selection. Methods The macroporous PEG hydrogel was synthesized by cryogelation. The target protein was conjugated to the hydrogel via the standard carbodiimide crosslinker chemistry. After the hydrogel was partially dehydrated, a single-stranded DNA (ssDNA) library was loaded to the hydrogel. The aptamer-loaded hydrogel was incubated in the selection buffer for diffusion. Sixty hours post diffusion, the selection buffer was replaced with a concentrated salt solution to elute the enriched ssDNA library. The enriched library was PCR amplified and purified for next-generation sequencing. The 50 sequences with the most abundant hits were grouped into approximately ten families. One member was chosen from each family for the binding analysis based on multiple assays. Result With thrombin as a model system, we found that the one-step enriched library exhibited a higher affinity than the original ssDNA library (**Fig. a).** An aptamer candidate T.7 exhibited a 1.5 nM Kd value (**Fig. b**). T.7 also showed a high specificity in binding thrombin compared to the other six protein targets (**Fig. c**). The full-length T.7 was further truncated to shorter sequences based on its stem-loop secondary structures (**Fig. d**). Four truncated aptamers exhibited a high binding capability compared to the two most widely used thrombin aptamers -15mer and 29mer that were both selected over ten cycles based on traditional aptamer selection methods (**Fig.e**). Conclusion Therefore, this work has demonstrated a novel HAS method for one-step aptamer enrichment and selection based on a diffusion-binding process in a non-fouling hydrogel matrix. This method is simple, fast, and low-cost. In addition, it does not need the use of sophisticated instruments. This one-step HAS method holds great potential to advance number fields ranging from nucleic acid delivery to biomolecular analysis.

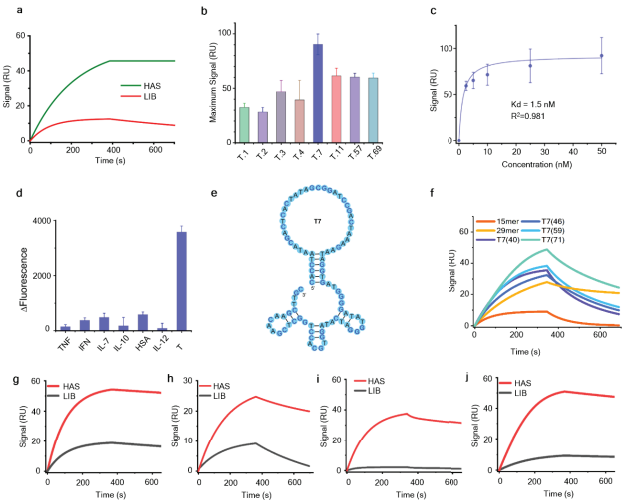


Fig a) Comparison of the initial and enriched library binding affinity. **b)** SPR analysis of nine thrombin aptamer candidates **c)** SPR analysis of T.7's binding affinity. **d)** Evaluation of T.7's binding specificity. **e)** Secondary structure of T.7. **f)** Evaluation of the truncated aptamers using SPR. **g) ~ h)** SPR analysis of enriched library and the initial library in binding to GM-CSF, IL-12, IL-10, and BDNF.

1713. Optimization and *In Vivo* Performance of circVec, a Vector-Based Circular RNA Expression Platform

Eoghan T. O'leary[1,2], Sara Vikberg[1,2], Juan Zhang[1,2], Daisy Hjelmqvist[1,2], Erik D. Wiklund[1], Victor Levitsky[1], Thomas B. Hansen[1]

[1]RnD, Circio AB, Huddinge, Sweden,[2]Clintec, Karolinska Institutet, Huddinge, Sweden

Circular RNA (circRNA) is a class of endogenously expressed non-coding transcripts found in most eukaryotes. In contrast to mRNA, circRNA is resistant to the intra-cellular exo-nucleolytic RNA turnover, which results in increased stability and persistence within cells. With enhanced understanding of circRNA biogenesis and functionality and recent engineering of circRNAs for highly efficient translation of proteins, circRNA is rapidly emerging as a next generation RNA platform that is expected to outperform mRNA-based therapies. Circio is developing a novel spliceosome-dependent expression system, circVec, designed for potent and durable vector-based intracellular production of circRNA, where the circVec cassette design and sequence elements have been optimized for high-yield circRNA biogenesis. Furthermore, to maximize the translation rate of the resulting circRNA, internal ribosomal entry sites (IRESs) have been screened for enhanced protein expression across a panel of cell lines in vitro. The circVec platform is vector agnostic, and we have shown effective expression

from a variety of non-viral and viral DNA vector systems. Here, we show that protein expression from the optimized circVec expression cassette accumulates over time when delivered transiently and reaches 10x higher expression rate compared to mRNA-based vectors one week after transfection. The observed circRNA advantage was consistent in a luciferase reporter mouse model, where the circVec-based system shows significantly enhanced accumulation over time compared to the mRNA benchmark following intra-muscular vector delivery. We therefore propose that the circVec expression platform is a promising new technology for various therapeutic areas requiring high and durable gene expression, such as monogenetic disorders, infectious disease and oncology.

1714. Simultaneous, Multiplex Detection of Sense and Antisense Strands of Therapeutic siRNA Using an Ultrasensitive Electrochemiluminescence Assay

Rohini Datta, Timothy J. Break, Seth B. Harkins, Jeff Debad, Jacob N. Wohlstadter

Meso Scale Discovery, Rockville, MD

Introduction: Small interfering RNA (siRNA) molecules play a key role in gene regulation and therapeutic applications. Currently, there are at least five FDA-approved siRNA drugs, with many more in various stages of the clinical trial pipeline, making their precise detection of paramount importance in preclinical research and translational medicine. Measuring both strands of therapeutic siRNA is essential for optimizing efficacy, ensuring specificity, and addressing safety concerns, including off-target effects and immunostimulation. Most pharmacokinetic assays for the detection of duplex siRNA rely on low-sensitivity formats like LC-MS or singleplex measurements of only the antisense strand (AS) and a separate assay for the sense strand (SS). We sought to develop an assay that can measure both strands of a duplex siRNA, in a single well, with high sensitivity. Methods: Here, we present a rapid and efficient approach for the multiplex detection of both strands of duplex siRNA using an ultrasensitive electrochemiluminescence (ECL) assay. Simultaneous detection of both strands of duplex siRNA in a single well is challenging using existing technologies, since the strands share a high degree of sequence complementarity, leading to cross-hybridization between capture probes and high false positive signals. We address this using specially-designed capture probes, tailored to target the SS and AS strands of siRNA, while eliminating the risk of cross-hybridization. These capture probes are shorter than the SS and AS strands by two or more nucleotides. Shortened probes specific for SS or AS hybridize to their target strands with high stringency in a single well, instead of cross-hybridizing to each other. Any residual probe-probe interactions are also degraded by the added RNases at the free single-stranded RNA sections not covered by the shortened probes. Hybridized probe-analyte complexes are then detected via an RNase protection assay using ECL-based readout. Assay conditions were optimized to achieve reproducible and highly sensitive detection for both siRNA strands. Results: Using this approach, we were able to achieve multiplex detection of duplex siRNA at remarkably low sub-pM concentrations in a single well. The lower limits of detection (LLODs) were 0.15 pM for AS and 0.28 pM for SS strands respectively. Experimentally-determined lower limits of quantitation (LLOQs) were 0.8 pM for both SS and AS strands. We demonstrate the versatility and robustness of this detection strategy in complex biological matrices such as mouse plasma, brain, and liver. Recoveries of high-, mid- and low-spikes in mouse brain and liver tissue lysates ranged between 83% - 118%. In plasma, the assay showed excellent linearity of dilution throughout the dynamic range. Conclusions: This method, combining siRNA strand specificity with highly sensitive ECL detection, represents a significant advancement in siRNA analysis techniques for pharmacokinetic studies.

Friday Posters: Nonviral Therapeutic Gene Delivery and Synthetic/Molecular Conjugates

1716. A Novel, Synthetic DNA Alternative for mRNA Manufacturing

Gustavo Lou, David Wilson, Ivana Pastierikova, Luca Distefano, Marco Guarrera, Alexander Pekarsky, Jorge Omar Yanez Cuna, Joel de Beer

Anjarium Biosciences AG, Schlieren, Switzerland

The emergence of messenger RNA (mRNA) as a transformative platform for gene therapy and vaccine applications, especially in the wake of the COVID-19 pandemic, has ignited a quest for innovations in mRNA production. A pivotal challenge in this space revolves around the choice of a DNA template for in vitro transcription (IVT) to generate mRNA. While bacterial-derived plasmid DNA has been the go-to template, its utilization has presented a significant bottleneck in mRNA manufacturing due to issues of cost, turnaround time, and purity concerns associated with the incorporation of bacterial-derived sequences into the final mRNA product. We have developed a novel, optimized synthetic linear double-stranded DNA with customizable hairpin-ended structures designed to serve as an alternative to plasmid DNA for mRNA production, completely devoid of any bacterial elements. The platform offers unparalleled advantage in terms of scalability, delivering high-fidelity, high-yield, and high-purity DNA within a remarkably brief timeframe. Here we show that our synthetic DNA is a versatile template for in vitro transcription that accommodates a diverse range of mRNA designs, and encompasses variations in length, capping technologies, and degrees of chemical modification. The use of our synthetic DNA for IVT affords significantly higher mRNA yields as compared to conventional plasmid DNA, while exhibiting comparable integrity, purity and levels of dsRNA contaminants. Furthermore, the functional activity of mRNA produced from our novel DNA is equal or superior to benchmark mRNA synthesized from plasmid DNA. The implications of these results underscore our novel DNA as an innovative and robust solution to overcome the bottleneck encountered in mRNA manufacturing.

1717. A Novel, Synthetic DNA for Cell and Gene Therapy Application

Alexander Pekarsky, Luca Distefano, Marco Guarrera, Ivana Pastierikova, David Wilson, Martin Cusack, Anna Krutyholowa, Roxanne Lourman, Fabian Trick, Gustavo Lou, Andreia M. Silva, Ileana Guerrini, Nicolas Meier, Jorge Omar Yanez Cuna, Joel de Beer

Anjarium Biosciences AG, Schlieren, Switzerland

In the last decade, the field of cell and gene therapy has undergone a remarkable expansion with unprecedented growth in research, clinical trials, and therapeutic applications. Central to this transformative development is the increased recognition of DNA as the essential input material. As demand for advanced therapies continues to rise, there is increasing need for efficient and scalable platforms for manufacturing DNA constructs that are used directly as therapeutic agents or as input material for therapeutic products. We have developed a novel optimized synthetic linear double-stranded DNA with customizable hairpin-ended structures (e.g. synthetic and natural inverted terminal repeats) for cell and gene therapy applications. Our proprietary one-pot, enzymatic and cell-free production process has a small footprint, seamless scalability and allows production of synthetic DNA constructs of variable size and sequence complexity without residual bacterial backbone sequences. Additionally, as the generation of the hairpin ends is not sequence specific, the DNA platform allows application-oriented customization of the hairpin structures on both DNA ends. Here, we show that our cell-free, enzymatic DNA production process offers high product purity and flexibility coupled with faster production times and minimal footprint requirements compared to plasmid DNA production process. Additionally, we show the broad versatility of our synthetic DNA for cell and gene therapy applications by demonstrating its use as input for mRNA, recombinant adeno-associated virus, and lentivirus vector production, as well as for use in transgene expression *in vitro* and *in vivo*. Our synthetic DNA is well-positioned to replace plasmid DNA as key starting material for cell and gene therapy modalities.

1718. Non-Viral Gene Editing Solutions for Efficient Cell Therapy Engineering

Lumeng Ye, Fan Zhou, Zhaoyang Mao, Hong Li

R&D Department, GenScript Biotech, Nanjing, China

With the fast progress and deeper understanding of CAR-T cell therapy, finding a better solution other than lentivirus transduction is more requested, to overcome the functional limitation on random insertion, uncontrolled copy number of CAR insertion and to overcome the capacity limitation of GMP lentivirus manufacture. Here we report two non-viral DNA payloads, for highly efficient non-viral cell therapy engineering and manufacture. GenExact™ ssDNA, linear single-stranded DNA produced by proprietary isothermal enzymatic reaction process, with high purity and sequence integrity proved by NGS and HRMS. This high purity single-stranded format DNA payloads also proved with lower cytotoxicity than dsDNA, could facilitate high knock-in efficiency of CAR sequence at specific location when coupled with CRISPR gene editing system, as about 50% at TRAC with CTS (Cas9 Targeting Sequence) modification, and >90% at GAPDH with SLEEK (SeLection by Essential-gene Exon Knock-in) design. For other types of immune cells, GFP knock-in can be achieved as 50% on iPSC and 30% on NK cell as well, and the editing efficiency by HDR (homology-directed repairing) can be further increased by selected chemical compounds. With non-viral CRISPR editing and electroporation process, different kinds of immune cell therapy can be manufactured with more defined design and unified final product. For insertion of large cargo that longer than 5kb, GenCircle™ dsDNA, circular supercoiled dsDNA with minimized backbone (430bp), is designed for large insertion coupled with transposon system. With the reduced backbone size and immunogenicity, GenCircle can be used for the delivery of large cargo without trigger severe cytotoxicity, and can achieve around 60% insertion rate on T cell by dual vector process. This protocol paves the way for non-viral CAR-T manufacture with multiple CAR design and precise modulation with logic control design.

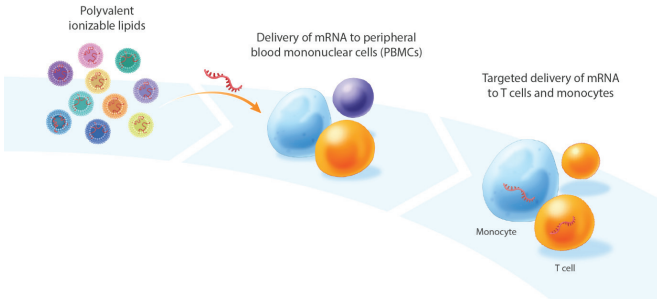
1719. Novel Polyvalent Ionizable Lipids Enable Targeted Delivery of mRNA to T Cells and Monocytes

Ariadna Lubinus, Joseph Pisano, Christopher B. Rohde, Matthew Angel

Factor Bioscience Inc., Cambridge, MA

Ionizable lipids are established nucleic acid delivery vehicles capable of delivering mRNA to induce protein expression in target cells. These lipids deliver their mRNA cargo into the cytosol, where it can be directly translated into proteins. As a result, lipid-based mRNA delivery does not carry the risks of mutagenesis that limit the clinical use of both viral vectors and DNA-containing delivery systems. Yet, current lipid-based delivery systems suffer from low transfection efficiency, especially for cell types such as primary cells and immune cells, which are notoriously resistant to transfection due in part to their immune response to foreign nucleic acids and selective uptake of molecules by specialized endocytic machinery. To address this, we designed a library of 4,200 ionizable lipids of which a panel of 84 potential candidate vehicles were synthesized and screened in human peripheral mononuclear cells (PBMCs) and other difficult-to-transfect immune cells including THP-1 monocytes. Human PBMCs were transfected with green fluorescent protein (GFP)-encoding mRNA formulated into complexes with the candidate lipids. 43 of these lipids possess a spermine-derived backbone, a structural characteristic of the ToRNAdo™ Nucleic-Acid Delivery System which has effectively delivered mRNA to a variety of cell types both ex vivo and in vivo. We also tested these lipids on PBMCs that had been exposed to T-cell activators IL-2 and anti-CD3/CD28 antibodies for 72 hrs to mimic innate immune activity in a pathogenic microenvironment. 1.33 ng/uL of GFP mRNA was delivered per 100,000 PBMCs at a lipid:mRNA wt/wt ratio of 3:1. High content cellular imaging and fluorescent microscopy showed peak GFP fluorescence intensity 24-36 hrs after transfection. Flow cytometry was performed 24 hrs after transfection to quantify GFP expression and viability in transfected cells. Of the lipids tested, those containing spermine headgroups and hexyldecanoate ester tails yielded high levels of GFP expression in CD3+ cells with a four- to eleven-fold increase in GFP positive cells relative to Lipofectamine™ 3000. The same lipids transfected THP-1 monocytes at greater than 90% efficiency. Our data collectively indicates that spermine-derived polyvalent ionizable lipids target transfection of mRNA to primary

human immune cells, particularly T cells and monocytes. These novel lipids may prove useful in the development of mRNA therapeutics based on the transfection of immune cells.



1720. Effect of LNP Components on mRNA Mediated Protein Expression of Lipid Nanoparticles in Different Organs

Anitha Thomas, Malathi Anantha, Ruchi Sharma, Cece Nazaripour, Rehan Higgins, Kobe Tickner, Zhengyu Chen, Nikita Jain, Jay Paquette

Cytiva, Vancouver, BC, Canada

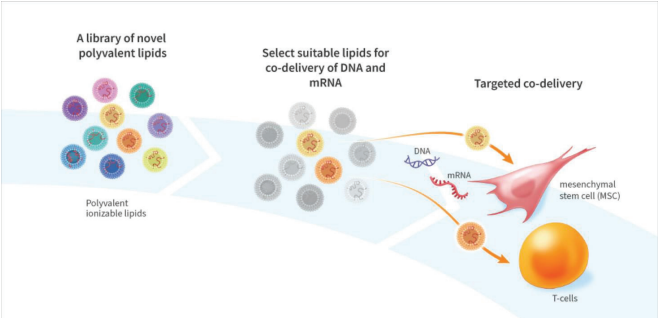
Introduction: In recent years, RNA therapeutics have received tremendous attention. To fully realize the potential of RNA therapeutics, lipid nanoparticles (LNPs) have been developed as an efficient delivery system. While LNPs have been widely used for delivery of RNA therapeutics into liver, non-hepatic delivery remains highly challenging. To better understand the mRNA expression of LNPs in different organs, we have evaluated the translation of luciferase encoded mRNA using various LNP systems. **Objectives:** To evaluate the effect of various parameters of compositions constituting LNPs for protein expression in different organs. **Methods:** LNP formulations were manufactured using NanoAssemblr® Ignite with different compositions with Luciferase encoded mRNA. Size and PDI were determined using Dynamic Light Scattering. The encapsulation efficiency was determined using the Ribogreen™ assay. The pKa of mRNA LNPs were measured by TNS assay. Luciferase expression as total flux in various organs were evaluated in vivo and ex vivo using IVIS imaging. Briefly, CD-1 mice were dosed with luciferase encode mRNA LNP intravenously at a 0.1 mg/kg dose. Four hours later mice were injected with D-luciferin and imaged using IVIS. After euthanasia, different organs were dissected for ex-vivo luminescence imaging using IVIS. **Results:** Size of the LNP was reported to be between 60-150 nm with PDI<0.2. The experimental pKa of different ionizable lipids were between 4.9 to 7.4. All LNPs tested demonstrated high luciferase protein expression in the liver. Liver expression levels were comparable to other clinically demonstrated lipids. Conclusions: For ionizable lipids, there is a correlation between overall protein expression and pKa of ionizable lipids. We have also seen that various components in the LNP compositions have implications with respect to protein expression in liver, spleen, and other organs. These parameters affect the biodistribution and translation of the lipid nanoparticles.

1721. A Library of Novel Polyvalent Ionizable Lipids for Targeted Co-Delivery of mRNA and DNA

Joseph Pisano, Ariadna Lubinus, Christopher B. Rohde, Matthew Angel

Factor Bioscience Inc., Cambridge, MA

Lipid delivery systems containing ionizable lipids have entered broad clinical use with the introduction of COVID-19 mRNA vaccines and are now generally considered the gold standard for in vivo mRNA delivery. However, intravenously administered lipid delivery systems are still limited by undesirable toxicity profiles, poor accumulation at in vivo target sites, minimal uptake by cells of interest, and low levels of endosomal escape. To address these deficiencies, we have investigated strategic incremental modifications to the chemical structure of DLinDHS, a key component of the ToRNAdo™ Lipid Delivery System. ToRNAdo™ has shown effective delivery of mRNA to a variety of cell types both ex vivo and in vivo, including mRNA encoding chimeric antigen receptors (CARs) targeting CD19 and the receptor tyrosine kinase like orphan receptor 1 (ROR1), a receptor found on many solid tumor cancers. iPSC-derived macrophages transfected with such mRNA efficiently bound recombinant CD19 or ROR1 and demonstrated improved cytotoxicity towards tumor cells. We hypothesized that combining various structural elements with the polyvalent DLinDHS backbone could lead to improved transfection efficiency and enhanced tissue targeting. In designing the novel lipids, structural characteristics such as biodegradability, lipid tail length, level of branching, branched tail symmetry, and increased hydrogen bonding ability were considered. Ester functional groups were incorporated to increase potential for in vivo biodegradability via hydrolysis by esterases, generating byproducts consisting of functional groups which may be metabolized more readily. Lipid tail design was influenced by length, level of branching, and branched tail symmetry in order to optimize the physicochemical characteristics of the resulting lipid-nucleic acid complexes. Increased hydrogen bonding capacity was achieved through the introduction of hydroxyl substituents, promoting stronger intermolecular association with anionic nucleic acid phosphate groups. Using these principles, we designed 4,200 novel ionizable lipids and synthesized a library consisting of 84 polyvalent ionizable lipids sorted into 11 families. We tested this library for delivery of mRNA to cells and identified candidate vehicles for further development, which will ideally yield an effective and versatile lipid delivery system with potential to enable effective therapies that can positively impact patient outcomes for countless diseases.



1722. Auto-Circulating RNA for Protein Expression

Qianbo Ma[1], Chuanqing Tang[1], Sheng Zhao[1], Xiulian Sun[2]

[1]uBriGene Biosciences International Co., Nanjing, China,[2]uBriGene Biosciences International Co., Suzhou, China

Compared with linear RNA, circular RNA is a closed-loop structure that doesn't require a cap or polyA tail. Without the free ends, circular RNA is resistant to RNA exonucleases, and therefore more stable in cells. At present, the manufacture of circular RNA mostly uses an in vitro circulation strategy by utilizing self-splicing introns from viral or other known genomes, which is often called PIE (permuted introns and exons) based method. Here we reported a linear but auto-circulating RNA (acRNA) produced by *in vitro* transcription with 5' hydroxyl and 3' 2, 3-cyclic phosphate ends formed by the self-cleavage of ribozymes or DNAzyme on both ends. After transfected / delivered into the cell, acRNA can be circulated by the cellular endogenous ligase automatically through a human tRNA splicing mechanism. The auto-circularized RNA is driven by the IRES element to express the coded protein. The results showed that acRNA with different human tRNA stems exhibited different expression activities, indicating different auto-circulating efficiencies of these tRNA stems in cells. To minimize the safety issue raised by using non-human tRNA sequences (like the Tornado system), the acRNA was designed based on the human tRNA stems. An acRNA-LNP-based gene editing system including a spCas9-acRNA was also demonstrated to function well in tested cells. Without the need for capping and polyA tail (for mRNA), or the RNase R digestion and multiple purification steps (for those *in vitro* circulating circRNA, PIE circRNA), the manufacture of acRNA is low-cost when compared to either mRNA or *in vitro* circulating circRNA. Subsequent studies will further optimize the circulating stems to improve the auto-circulating efficiency and transient expression level of the acRNA.

1723. Determination of mRNA Capping Rate and polyA Length by High-Resolution Capillary Electrophoresis

Ye Sun[1], Sheng Zhao[1], Xiulian Sun[2]

[1]uBriGene Biosciences International Co., Nanjing, China,[2]uBriGene Biosciences International Co., Suzhou, China

mRNA therapy has steadily grown to be one of the most popular fields in pharmacological research. It is anticipated to be an efficient treatment for a wide range of illnesses. The *in vitro* transcript mRNA must be capped and have the polyA tail for protein translation in eukaryotic cells. One of the gold standard techniques to measure the rate of capping is to perform RNase H digestion on hybridized RNA with a biotin-labeled DNA probe near 5'-end mRNA followed by purification and mass spectra. A similar analysis can be done to measure the length of polyA. However, these LC-MS-based methods are costly and sophisticated. Here, we introduced a DNAzyme-based high-resolution capillary electrophoresis to quickly and precisely estimate the mRNA capping rate and the length of polyA tail. The site-specific DNAzymes were first screened according to the candidate mRNA sequence near both ends, usually within 5'-UTR and 3'-UTR regions. The selected DNAzyme with enough cleavage activity cut the mRNA near the ends of mRNA, producing small fragments with/without a cap on the 5'-end, or fragments with various lengths of polyA tails on the 3'-end. DNase I treatment was then performed to digest the DNAzyme and the resulting RNA fragments were purified before analyzing by high-resolution capillary electrophoresis (CE) which could distinguish RNA fragments with minimal 1 nt difference. The capped mRNA will produce a 1~2 nt longer CE peak than that of uncapped mRNA. The ratio between the longer peak and the shorter peak of the 5'-end can be used to estimate the mRNA capping rate. On the other hand, the range of the length of the polyA tail can be calculated by the position of peaks of 3'-end cut fragments after subtracting the length of the sequence before the polyA tail and after the cutting site. The DNAzyme-based high-resolution capillary electrophoresis is a cheap, easy-to-use, and accurate method for mRNA quality control for both capping rate and polyA tail length.

1724. Revolutionizing Neurofibromatosis Treatment: mRNA Delivery in Schwann Cells with Lipid-Based Formulation

Nabila Laroui[1,2], Sarah Meresse[1,3], Laura Colar[2], Christophe Delehedde[2], Cristine Gonçalves[1,2], Fabienne Godin[2], Beatrice Vallée[2], Patrick Midoux[1], Helene Benedetti[2], Chantal Pichon[1,2,3]

[1]ART ARNm US55 Inserm, Orleans, France,[2]CBM UPR 4301 CNRS, Orleans, France,[3]Faculty of Sciences and Techniques, University of Orleans, Orleans, France

Neurofibromatosis (NF), an inherited disorder, arises from mutations within the ~8.0 kb coding gene sequence of the NF1 tumor suppressor located mainly in Schwann cells. NF1 hallmark is the development of both benign and malignant nerve sheath tumors including cutaneous neurofibroma (cNF). Currently, cNF treatment is limited to drugs, surgical resections or involves ablation approaches which are invasive and poorly efficient. The only gene replacement therapy work using NF1-GTPase-activating protein-related domain (GRD; an important functional domain of NF1) plasmids and adeno-associated viruses (AAVs) has shown significant progress during pre-clinical studies [1]. However, AAVs have limited capacity in terms of the size of genetic material; around ~ 4.5 kb. In this study, we propose an innovative approach, based on the introduction of the synthetic messenger RNA (mRNA) of NF1 to restore the production of a normal Nf1 protein. The delivery of *NF1* mRNA is much simpler and does not require this nuclear import step as the translation machinery takes place in the cytosol. Most importantly, the *in-situ* expression of Nf1 will allow all post-translational modifications and interactions required for its function. First, we determined and optimized the delivery and expression of mRNA in mutated NF1 cells comprising human Schwann cells (HSCs), fibroblasts and keratinocytes using a range of polymeric and lipid vectors, including our original lipids. With both modified and unmodified mRNA, they achieved the highest transfection rate (~90%), without inducing cytotoxicity. In a remarkable way, intradermal injection of lipid-based luciferase mRNA formulation in human skin explant, a highly relevant organotypic model, demonstrated efficient luciferase expression for up to 5 days, using. In addition, we were able to successfully transfect mutated Schwann cells (*NFI* -/-) with the optimized lipid-based formulation containing GRD mRNA construct enabling the restoration of GRD biological activity. To the best of our knowledge, this study shows for the first time the feasibility of Schwann cells transfection with mRNA GRD-NF1 where the biological activity

will be evaluated on cells and tissue biopsies from neurofibromatosis patients. Thereby, this work will pave the way to a therapeutic paradigm shift based on protein replacement for cutaneous neurofibroma treatment. [1] Bai et al., « Feasibility of Using NF1-GRD and AAV for Gene Replacement Therapy in NF1-Associated Tumors ».

1725. Cargo-Agnostic Lipid Nanoparticles to Correct Cystic Fibrosis Mutations in Airway Stem Cells

Ruth Foley[1], Paul Ayoub[1], Colin Juett[1], Alicia Sanoyca[1], Vrishti Sinha[1], Caroline Cherry[1], Emily Duggan[1], Beate Illek[2], Donald B. Kohn[3], Brigitte Gomperts[1], Steven J. Jonas[4]

[1]UCLA, Los Angeles, CA,[2]UCSD, San Diego, CA,[3]Unversity of California, Los Angeles, Tarzana, CA,[4]University of California, Los Angeles, Los Angeles, CA

Aim: Cystic fibrosis (CF) is a monogenic disorder arising from mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that leads to chronic lung infections & respiratory failure. Genetic diseases like CF are appealing targets for gene therapies, but mutation-agnostic gene correction strategies rely on genomic integration of large double-stranded DNA (dsDNA) cassettes that are difficult to package and deliver to target cells. We report the design and testing of a gene therapy strategy that utilizes lipid nanoparticles (LNPs) configured to transport CRISPR/Cas9 payloads designed for site-specific insertion of CFTR in *in vitro* models of the human airway. Methods: LNP formulations were synthesized *via* microfluidic mixing and characterized with dynamic light scattering. We systematically screened LNPs with varying ratios of mRNA encoding Cas9, single guide RNAs (sgRNAs), and donor DNA templates to optimize gene editing in a human bronchial epithelial cell line (16HBE14o-). 16HBE14o- cells stably expressing blue fluorescent protein (BFP) were applied to evaluate editing at the *BFP* locus *via* flow cytometry. In this model, non-homologous end joining (NHEJ)-mediated repair at the locus results in loss of fluorescence whereas homology-directed repair (HDR) can be detected following successful integration of donors designed to convert BFP to green fluorescent protein (GFP). Next, we applied HDR donors designed to insert a *mCitrine* reporter within the endogenous 5' untranslated region of *CFTR* in 16HBE14o- cells to assess whether dsDNA cassettes should be co-encapsulated with Cas9-mRNA and sgRNA in a single LNP or packaged separately. The dsDNA:Cas9-mRNA and nitrogen-to-phosphate (N/P) ratios were systematically varied to improve the integration of a *CFTR*-encoding cassette in 16HBEge-G542x CF mutant cells. Integration of the reporter *mCitrine* and *CFTR*-correction cassettes was measured with digital droplet PCR. Successfully edited 16HBEge-G542x colonies were transferred to TransWell inserts and evaluated in an Ussing chamber to validate restoration of CFTR function. Results: In the presence of an HDR donor, *ca.* 90% NHEJ-mediated repair and up to 35% HDR were observed in 16HBE14o-BFP cells treated with LNPs packaged with BFP editing cargoes. LNPs with a sgRNA:Cas9-mRNA w/w ratio of 1.2:1 achieved the highest overall editing efficiencies. Encapsulation of dsDNA donor cassettes with Cas9-mRNA and sgRNA in a single LNP yielded *ca.* 14% integration of the mCitrine reporter in 16HBE14o- cells compared to *ca.* 10% when the donor was packaged separately. In 16HBEge-G542x cells, LNPs with a dsDNA:Cas9 mRNA w/w ratio of 3:1 demonstrated up to 3.5% integration, compared to <2% integration for 2:1 and 4:1 w/w ratios. Increasing the N/P of the LNPs to 9 further improved CFTR dsDNA integration. Initial validation of *CFTR* dsDNA donors electroporated in 16HBEge-G542x cells restored CFTR-dependent Cl- current to *ca.* 80% of wild-type, confirming functional correction. Conclusion: We demonstrate a LNP platform capable of transporting large gene editing biomolecules to airway epithelial cells that can achieve mutation-agnostic, site-specific correction of *CFTR* in a CF-deficient cell line. These nanotechnologies offer solutions to overcome limitations in the cargo-carrying capacity of existing gene delivery vectors. Our work sets a new precedent for packaging and delivering large dsDNA templates with LNPs that is poised to accelerate progress toward definitive cures for CF and other genetic diseases.

1726. Lung Delivery for Cystic Fibrosis

Miriam L. Greenwood, Hannah Mitchison, Stephen L. Hart

UCL Great Ormond Street Institute of Child Health, London, United Kingdom

Background: Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The dysfunctional CFTR protein leads to thickened mucus, bacterial infections, and reduced lung function. Modulators are effective treatments for many CFTR genotypes, but ~20% of patients remain untreatable. A major obstacle to developing a CF gene therapy is efficacy and safety of lung delivery. Aims: The aim was to compare the transfection efficiencies in different *in vitro* lung models, with respect to uptake and gene expression in response to nucleic acid transfection. As CF involves inflammation of the lung, a further aim was to determine whether delivery exacerbates inflammation. Methods: GFP and luciferase pDNA were packaged into receptor-targeted nanocomplexes (RTNs), comprising liposome, ditetradecyl trimethyl ammonium propane (DTDTMA) and dioleoyl L-α phosphatidyl ethanolamine (DOPE) at a 1:1 molar ratio, and an epithelial targeting peptide, K_{16}-GA-CSERSMNFCG. The cell types transfected were the human bronchial epithelial cells: 16HBE14o-, and primary CF and non-CF basal cells transduced with BMI-1 (CFBE F508del and NHBE). CFBE cells were grown both in submerged culture and in air-liquid interface (ALI) cultures alongside NHBE cells. Interleukin 6 and 8 (IL-6 and IL-8) protein secretion were measured by ELISA as a marker of inflammation. GFP pDNA was labelled with Cy5 using Label IT® to evaluate nuclear uptake and gene expression by confocal microscopy and flow cytometry. Luciferase assays were also utilised to determine transfection efficiency. Results: Transfection efficiency with GFP pDNA was 21.7% in 16HBE cells and 37.1% in CFBE cells, and the uptake efficiency of Cy5-labelled pDNA was 62.4% and 99.1% respectively. ALI cultures demonstrated some successful transfections after two or three consecutive transfections with secretable luciferase when measured from the apical surface. Successful transfection of individual well in ALI cultures correlated with elevated IL-6 and IL-8 levels. There were no significant differences in cytokine levels between CFBE and NHBE cells. In CFBE submerged culture, there was a significant increase in IL-6 (μ = 99 pg/ml compared to 13 pg/ml negative control), but not IL-8 in response to RTN transfection. However, co-transfection with 1000 nM dexamethasone, a glucocorticoid anti-inflammatory, significantly reduced IL-6 to a mean

of 73 pg/ml. Dexamethasone also reduced IL-8 levels. Comparatively, in the 16HBE14o- cell line, IL-6 levels were significantly increased ($p<0.0001$) in transfected cells 24 hours post-transfection but had decreased by 72 hours. However, incorporating dexamethasone into RTNs in 16HBE transfections significantly reduced IL-6 levels from a mean of 1956 pg/ml to 963 pg/ml. Discussion: After comparing different *in vitro* lung models, we found that submerged NHBE and CFBE cells transfect more easily with RTNs than 16HBE14o- cells in both uptake and gene expression efficiencies. In ALI cultures, not all wells achieved successful transfection, likely attributed to substantial mucus production but there was also evidence of an inflammatory response to RTN pDNA transfection, due to increased IL-6 and IL-8, although the effect was transient and can be reduced with dexamethasone, a widely used anti-inflammatory agent. Conclusion: RTN pDNA transfection is highest in primary cells compared to a cell line, and causes an inflammatory response in epithelial cells associated with transfection efficiency but this can be modulated by transient treatment with dexamethasone, a widely used anti-inflammatory agent.

1727. Development of *In Vitro* Transcribed RNA Therapeutics for Cystic Fibrosis

Ruhina Maeshima[1], Aristides D. Tagalakis[1], Drew Weissman[2], Robin J. McAnulty[3], Chris O'Callaghan[1], Deborah Baines[4], Stephen L. Hart[1]

[1]UCL Great Ormond Street Institute of Child Health, London, United Kingdom,[2]University of Pennsylvania, Philadelphia, PA,[3]UCL Respiratory Centre for Inflammation and Tissue Repair, London, United Kingdom,[4]St George's University of London, London, United Kingdom

Background: Cystic fibrosis (CF) is an autosomal recessive rare disease, caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Absent or nonfunctional CFTR leads to thick, sticky mucus in the lungs, which results in chronic bacterial infection and inflammation. *In vitro* transcribed mRNA has emerged in the last few years as a new approach for CFTR protein replacement therapy, but problems of delivery must be overcome. **Methods:** We have developed novel receptor-targeted nanocomplex (RTN) formulations consisting of liposomes and receptor-targeting peptide for delivery of CFTR IVT mRNA. The RTNs were first optimized in transfections of primary CF bronchial epithelial (CFBE) cells using reporter IVT mRNAs. We then assessed delivery efficiency of CFTR mRNA in CFBE cells at submerged culture and ALI culture and assessed uptake by quantitative reverse transcription polymerase chain reaction and demonstrated protein expression by Western blot. **Results:** We first optimized the RTN formulations, comparing combinations of three cationic liposomes and five peptides for their biophysical properties and transfection efficiency. We identified a novel formulation (size 130 nm, polydispersity index (PDI) 0.28, charge +38 mV) for mRNA delivery that achieved almost 100% cellular uptake efficiency and 90% transfection efficiency. There were no differences in transfection efficiency between primary normal human bronchial epithelial (NHBE) cells and CFBE cells. The same RTN formulation was able to deliver the mRNAs in air-liquid interface (ALI)-cultured cells and mouse lungs. The luciferase expression in mouse lungs was approximately 200 times as high as plasmid DNA encoding luciferase. CFTR mRNA was successfully delivered to NHBE and CFBE cells. In addition, we co-packaged the CFTR corrector VX-809 with CFTR mRNA into RTNs and found that this increased expression of CFTR protein in both NHBE and CFBE cells in submerged culture approximately 2 to 2.5 times. CFTR protein translated from the IVT mRNA was detected by immunoblotting from 4 hours to at least 48 hours after the transfections. CFTR protein expression was also shown in transfections of ALI culture of CFBE cells by functional analysis of ion transport in an Ussing chamber. The change of short circuit current (ΔIsc) in CFBE transfected with CFTR mRNA once, was 10-fold larger than in untransfected cells before and after forskolin was added, and it was approximately 83% of that seen in NHBE. CFBE transfected three times with CFTR mRNA achieved larger ΔIsc than NHBE after CFTR inhibitor 172 was added into the cells. **Conclusions:** CFTR IVT mRNA delivery is a promising novel therapeutic for CF. The flexibility of the RTN formulation allows co-delivery of CFTR mRNA with VX-809, which significantly improved CFTR expression. It can induce the same level of CFTR expression as NHBE in CF cells.

1728. Interleukin-27 (Il-27)-Based Gene Therapy to Reduce Inflammation in Acute Respiratory Distress Syndrome

Grace E. Mulia, Janelle W. Salameh, Marxa L. Figueiredo

Basic Medical Sciences, Purdue University, West Lafayette, IN

Acute Respiratory Distress Syndrome (ARDS) is an acute inflammatory lung injury associated with pulmonary edema and characterized by the presence of alveolar damage and lung stiffness. Globally, about 40% of ICU patients suffer from ARDS, half of which are categorized as severe ARDS with 90% mortality rate. Current therapy is limited to supportive care in the form of mechanical ventilator, which highlights the dire need of targeted therapies. In ARDS, lung injury triggers activation of immune cells, which release pro-inflammatory cytokines aimed at eliminating the cause of injury. Prolonged upregulation of IL-1β, IL-8, IL-6, and TNF-α contribute to macrophage and neutrophil recruitment and accumulation, promoting further release of toxic mediators that perpetuate chronic inflammation and alveolar damage. Therefore, therapies capable of modulating cytokine production while stimulating tissue repair may improve ARDS patients' prognosis. Interleukin-27 (IL-27), a multifunctional cytokine involved in innate and adaptive immunity and in promoting tissue repair, has been hypothesized as a candidate to rebalance an ARDS inflammatory microenvironment. To test this hypothesis, we first established an LPS-induced in vitro model and characterized gene expression responses in lung epithelial cells (BEAS-2B) and macrophages (THP-1). Cytokines typically have short half-lives, thus we utilized a gene therapy approach to deliver IL-27 by transfecting an IL-27-expressing plasmid into human adipose mesenchymal/stromal cells (hASC). Our results showed that IL-27-hASC conditioned media reduces pro-inflammatory cytokine gene expression in LPS-induced BEAS-2B and THP-1, both in monoculture and co-culture settings. We are currently testing our hypothesis in an in vivo model of LPS-induced ARDS, further determining the potential of IL-27 therapy in reducing inflammation in an ARDS model.

Friday Posters: Other Nonviral Delivery

1729. Pulmonary Endothelial Cell- Selective Ionizable Lipid and Biodegradable Polymer-Based Stability Enhanced Nanoshell (SENS™) Technology for RNA Therapeutics

Jihye Han, Seungju Yoo, Kyeongdae Kim, Eujung Kim, Kyuhee Yum, Jongmin Park, Joungpyo Nam, Sojin Lee, Helen Cho

Samyang Biopharmaceuticals, Seongnam, Korea, Republic of

Recent rapid advances in technology of mRNA-based therapeutic has the potential to treat a wide range of uncurable pathologies including infectious diseases, cancers, immune diseases, and genetic disorders. Ionizable lipid nanoparticles (LNPs) have been widely utilized and developed for the delivery vehicles of RNA-based therapeutics but have limited applications due to accumulation in the liver after systemic administration. Systemic delivery of mRNA to target organs in vivo remains the major challenge in the development of mRNA-based therapeutics. Here we report a unique technology termed Stability Enhanced Nanoshell (SENS™) as an RNA delivery platform which contains polymer and proprietary ionizable lipid as essential components. By employing different compositions of lipids and polymers, SENS™ deliver RNAs selectively to various organs by intravenous administration. Lung- selective SENS™ (refers to PulSENS™) enabled selective delivery of RNA to the lung of mice after systemic injection. Importantly, the lung distribution and expression profile of SENS™ translated well in larger species such as rabbits and ferrets that better resembles the architecture of human lungs. Within the lung, mRNA expression was found evenly distributed throughout the endothelial cellular population with minor expression in surrounding immune and epithelial cells in both healthy and fibrosis-induced mouse models. These results suggested a potential therapeutic regimen of PulSENS™ treat pulmonary diseases caused by the dysfunction of multiple cell types. Indeed, bleomycin-induced pulmonary fibrosis were ameliorated after PulSENS™ with therapeutic mRNA administration. Treatment with the PulSENS™ confirms significant decrease in accumulation of fibronectins (FNs) extracellular matrix (ECM) components in the fibrosis mice models. Taken together, SENS™ platform has great potential as translatable therapeutics for the development of RNA therapeutics to treat lung diseases by lung selective delivery.

1730. The Endosomal Escape Vehicle Platform Enhances Lipid Nanoparticle-Mediated Delivery of Genomic Medicines

Nan Cher Flo Yeo[1], Mah Kheirabadi[2], John Buzzo[1], Lucas Bongarzone[1], Pinakin Sukthankar[1], Zekun Li[1], Anushree Pathak[1], Xiang Li[2], Ziqing Leo Qian[1]

[1]Entrada Therapeutics, Boston, MA,[2]Entrada Therapeutics Inc, Boston, MA

Lipid nanoparticle (LNP) technology has emerged as a promising delivery method for nucleic acids therapeutics, including mRNA vaccines, small interfering RNA and gene editing modalities. Despite tremendous success, LNP delivery platforms still face major challenges, such as limited tissue tropism and poor delivery efficiency due to low endosomal escape. Endosomal escape is especially important for mRNA therapeutics, because different from vaccines, as much as a 1,000-fold higher level of functional protein expression is required to reach a therapeutic threshold. To address these concerns, we have applied our Endosomal Escape Vehicle (EEV™) technology to LNP engineering, with the goal of improving the delivery of genomic medicines. The EEV platform consists of a family of proprietary cell penetrating peptides (CPPs), which has been shown previously to improve intracellular delivery of oligonucleotides in various tissues and cell types. We found that EEV-modified LNP (EEV-LNP) significantly enhanced the efficiency of mRNA delivery and gene editing compared to unconjugated LNP *in vitro*. Through mechanistic studies, we further established the mechanism of uptake for EEV-LNP and indicate its ability to target a broad range of cell types and tissues. Overall, these results indicate the potential of our EEV-LNP platform for improved functional delivery of genomic medicines, with applications in the areas of mRNA delivery and gene editing.

1731. A Platform for RNA-Based and Genomic Cell Engineering: Simplifying Multiplexed Delivery to Human Primary Immune and Stem Cells

Zhihui Song, Sophia Hirsch, Rachel Conover, Athira Pillai, Andrew Laroque, Jacquelyn Hanson, Alec Barclay, Mathias Pawlak, Armon Sharei

Portal, Watertown, MA

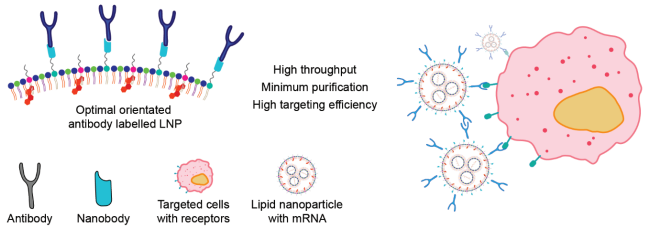
Cell therapy has the potential to revolutionize many areas of unmet clinical needs including cancer, degenerative and metabolic diseases, genetic disorders, infection, inflammation and autoimmunity. Both RNA-based and genomic editing technologies (e.g. CRISPR/Cas9) are actively investigated to design cellular therapies with customized functionalities. One of the key advantages of these new technologies is their highly adaptable nature that enables sophisticated cellular engineering, rational design and rapid pursuit of new therapeutic targets. At Portal, we have developed a gentle silicon membrane-based delivery technology that facilitates mechanoporation of cells and can be easily integrated with existing clinical-scale workflows. Our results in primary immune cells using monocytes, T, B and NK cells demonstrate over 80% efficiency and viability of cellular engineering. CRISPR/Cas9 RNPs, mRNAs and siRNAs have been successfully delivered as single cargos or in a multiplexed fashion, demonstrating the potential for either single- or multi-step cell engineering workflows which can address several targets. Furthermore, data in iPSCs and HSCs have also shown significant potential in stem cell engineering and differentiation capabilities. This technology is currently being implemented for research and clinical scale use - including in an integrated Point-of-Care manufacturing system. With continued progress in simplifying cell engineering using both RNA-based and genomic editing technologies, we aim to unlock vast biological potential in the field of cell therapy while simultaneously reducing or even eliminating safety or manufacturing concerns such as inadvertent genome alteration associated with other delivery modalities such as lentiviral vectors.

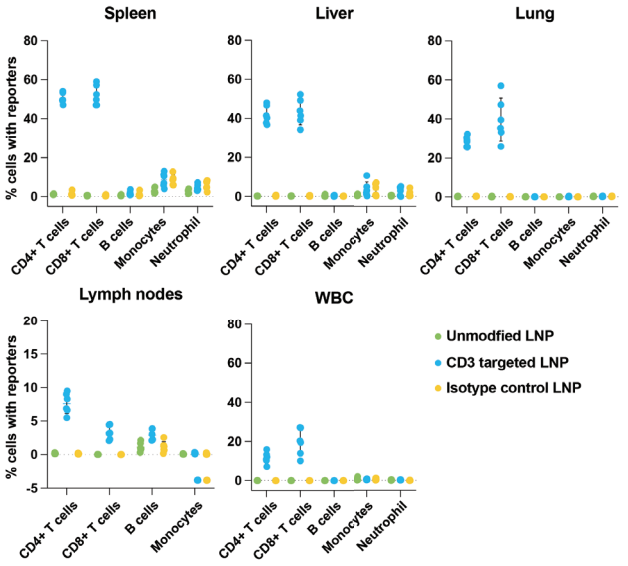
1732. A Versatile, Receptor-Targeted mRNA/LNP System for *In Vivo* Immune Cell Delivery

Zhe Chen[1], Daniel Yuen[1], Victoria M. McLeod[1], Ken W. Yong[1], Cameron H. Smyth[1], Bruna Rossi Herling[1], Thomas J. Payne[1], Stewart A. Fabb[1], Matthew J. Belousoff[2], Patrick M. Sexton[2], Christopher J. Porter[1], Colin W. Pouton[1], Angus P. R. Johnston[1]

[1]Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences, Parkville, Australia,[2]Drug Discovery Biology, Monash Institute of Pharmaceutical Sciences, Parkville, Australia

Efficient and more precise delivery of mRNA is critical to advance mRNA therapies beyond their current use as vaccines. One of the problems lies in its limited targeting ability, namely the off-targeting delivery to bystander cells. This limitation can be overcome by functionalising LNPs with antibodies that target mRNA to the specific cells where it is required. While greatly improving delivery efficiency, antibody modification typically requires complex conjugation and purification methods that lead to poor yields and decreased antibody specificity. To overcome this, we present here a simple method to generate receptor-targeted LNPs, where the LNPs are functionalised with antibodies oriented in their optimal orientation in a process that does not require antibody modification or complex purification (Figure 1). This antibody conjugation strategy leads to protein expression levels >1000 times higher than non-targeted LNPs and >10 times higher than conventional, less optimal antibody functionalization techniques. We have successfully demonstrated the precise delivery of mRNA to T cells via receptor-mediated endocytosis using ex vivo PBMC from healthy donors. We found that by targeting CD3, CD5, and CD7, our targeted LNP can efficiently transfect human primary T cells with minimal delivery to bystander PBMCs. We also successfully advanced this method in vivo, with precise targeting delivery towards T cells. Expression of a reported gene was observed in T cells across multiple organs (spleen, liver, lung, lymph nodes, and circulating blood) with minimal delivery to other immune cells such as B cells, monocytes, and neutrophils. In summary, we have demonstrated a versatile system to engineer LNP with precise delivery to specific cells in vivo. Our targeted LNP system underlines the potential to develop new therapeutic modalities that precisely deliver mRNA cargo to immune cells.





1733. Efficient Scalable Manufacturing of Virus-Like Particles for the Delivery of CRISPR-Cas9 Ribonucleoproteins Using a cGMP-Compliant Electroporation Platform

Tien Nguyen, Peter Gee, Sean Menarguez, Andrew G. Mancini, Marianna Romito, Ashley B. Strickland, Lauren E. Unsworth, Cenk Sumen, Jason Papademetriou, Isabel Daher, James Brady

MaxCyte Inc., Rockville, MD

Genome editing tools such as CRISPR-Cas9 nucleases, base editors, and prime editors hold tremendous promise for treating human diseases by being able to specifically modify a targeted region of the DNA genome. However, there are limited *in vivo* delivery options that are safe, efficient, and transient. Moreover, viral vectors such as AAV are hampered by limited cargo size capacity. Virus-like particles (VLPs) offer a solution to these problems. VLPs are derived from retroviral structural proteins, which can be engineered to specifically package a cargo of interest. They are safer than traditional viral vectors because they lack a viral genome but can utilize the traditional virus delivery machinery to target and enter cells. Recently, VLPs have been reported to efficiently package base editors and prime editors for delivery into mice at therapeutically relevant levels. To realize the potential of VLPs for *in vivo* delivery, alternative methods to scale up their manufacturing will be critical for future clinical applications. Here, we utilized the MaxCyte® ExPERT GTx™, a cGMP-compliant electroporation instrument, to manufacture VLPs— packaged with CRISPR-Cas9 RNPs for genome editing in target cells — in adherent and suspension HEK293 cells. We found that electroporation consistently produced significantly higher yields of functional VLPs compared to a commercially available transfection reagent, with up to a 20-fold improvement when using an optimized electroporation protocol. Furthermore, the production of VLPs using electroporation exhibited favorable production kinetics compared to other transfection methods, enabling a shorter VLP manufacturing process. Finally,

we demonstrated the scalability of VLP production across a 15-fold volume range with minimal re-optimization. In summary, our results show that electroporation is a viable means for consistent, efficient, and scalable manufacturing of VLPs for gene-editing applications and has high promise to address needs for future clinical and commercial VLP manufacturing.

1734. Development of Highly Efficient and Safe mRNA Delivery System for the Treatment of Chronic Liver Diseases: Liver-Selective Ionizable Lipid and Biodegradable Polymer-Based Stability Enhanced Nano Shell (HepaSENS™) Platform Technology

Gooyoung Kim, Jihye Choi, Hyosuk Kim, Hyejin Park, Yisak Kim, Jongmin Park, Joungpyo Nam, Sojin Lee, Helen Cho

Samyang Biopharmaceuticals, Seongnam-si, Korea, Republic of

Lipid nanoparticle (LNP) is a clinically approved delivery system for RNA-based therapeutics, initially developed for siRNA liver delivery and more recently for mRNA vaccine approaches. LNPs have also been shown to effectively deliver mRNA to the liver and various mRNA treatments are in clinical development. Depending on the function of the payload, these systems have facilitated the advancement of treatments for rare genetic disease and acute/chronic liver diseases. Considerable efforts to enhance the potential of these systems by 1) enhancing the target tissue and subcellular cell type selectivity, 2) designing formulations that deliver to different cell types, 3) increasing protein expression efficiency and durability, and 4) improving safety are in progress. Here we report the development of Stability Enhanced Nano Shell (SENS™) as an mRNA delivery platform to overcome these challenges. SENS can effectively deliver mRNA to selective tissues such as liver, lung, and spleen by employing different compositions of novel ionizable lipids and biodegradable polymer. Liver-selective SENS (refers to HepaSENS™) exhibits selective delivery of mRNA to hepatocytes, which are the most prominent and key cell target in variety of liver diseases. HepaSENS™ efficiently delivers mRNA to the liver not only in mice with healthy livers but also in mice with diseased liver by fibrosis. Importantly, different from systems that uses PEG-conjugated lipids, HepaSENS™ does not induce anti-drug antibodies which permits consistent protein expression after each repeat administration. Moreover, to increase the therapeutic index typically seen by traditional LNPs, HepaSENS™ is designed to significantly lower liver toxicity. Human transcription factor gene HNF4A, has been known as a master regulator of the hepatocyte phenotype/metabolism and an effective therapeutic target for the treatment of hepatic disease including liver fibrosis. We compared the efficacy of repeated systemic administration of HNF4 mRNA delivered by HepaSENS™ (HNF4A/HepaSENS™) with that delivered by conventional LNP (HNF4A/LNP) in a thioacetamide (TAA)-induced hepatic damaged mice (TAA-mice) model to overcome liver damage and fibrosis. We confirmed that HNF4 expression is higher in HNF4A/HepaSENS™-treated TAA-mice liver compared to HNF4A/LNP-treated mice liver after repeated systemic administration. Importantly, blood biochemistry (alanine aminotransferase), histology (Sirius red staining), and fibrogenic gene expression profile clearly revealed effective recovery of liver damage and fibrosis in liver of HNF4A/HepaSENS™ -treated mice but not in HNF4A/LNP-treated mice. The data confirmed HepaSENS™ as an advanced hepatocyte-selective RNA delivery system that can be repeat dosed with a high safety profile. To provide new and better treatment options for patients, development of mRNA-based therapeutics using to HepaSENS™ for various liver diseases requiring short and long-term administration are in progress.

1735. Cellular Phenotyping of Non-Human Primate and Mouse Kidney and Liver Following a Novel Targeted Transcutaneous Ultrasound-Mediated Gene Delivery Demonstrates Gene Expression in Clinically Relevant Cell Types

Katie Benthall, Nana Ingram, Margarita Krivega, Julia Poniatowski, Ivan Krivega, Bert Frederich, Surabhi Rao, Victor Foster, Barry Campbell, Adel Alonso, Glenda Arauz, Celia Ortiz, Oliver Chen, Dhruv Bole, David Kim, Elizabeth Del Greco, Charlotte He, Anagha Rao, David Satyadi, Zoya Gluzman-Poltorak, Steven B. Feinstein, Ken Greenberg

SonoThera, Inc., South San Francisco, CA

Introduction: Robust delivery of genetic payloads to the kidney and liver remains a significant challenge for the development of safe and effective genetic medicines. Current limitations include the ability to 1) achieve durable episomal gene expression in a safe, titratable, and redosable manner, 2) deliver large DNA payloads (>5kb), 3) bypass endosomal and cytoplasmic immune recognition and effectively transit the nuclear envelope, and 4) achieve targeted transgene expression in clinically relevant cell types. Transcutaneous ultrasound-mediated gene delivery (UMGD) is a noninvasive *in vivo* gene delivery approach that may overcome the key delivery challenges facing genetic medicines. SonoThera is developing a novel ultrasound-guided nonviral gene therapy platform based on UMGD that allows selective targeting of specific organs and tissues within the body in a safe, redosable, durable, and titratable manner. A body of literature suggests UMGD may be an effective approach for transgene delivery into various organs, however relatively few studies have been performed to analyze the biodistribution of payload delivery and expression. Methods: To evaluate the potential of UMGD for safe and efficient transgene delivery to the non-human primate and mouse kidney and liver, we developed a technology platform utilizing novel acoustic profiles, next-generation genetic payloads, and FDA-approved ultrasound components. The delivery process involves intravenous co-administration of nucleic acid payloads (e.g. DNA or RNA) and ultrasound contrast agents (a.k.a microbubbles), coupled with targeted application of externally applied ultrasound energy to guide the DNA into specific tissues via sonoporation. To perform cellular phenotyping following sonoporation in mouse and non-human primate preclinical models, we developed three orthogonal assays, namely HALO analysis of RNAscope stained tissue slides, single nucleus RNA-seq, and immunohistochemistry. Results: Upon systematic investigation of multiple mice and NHP liver tissue samples, we confirmed that our novel UMGD platform enabled delivery of genetic payloads to hepatocytes and liver sinusoidal endothelial cells (LSECs) following liver targeting. In the mouse and NHP kidneys, we identified podocytes, tubular epithelial, and

endothelial cells as major targets for UMGD-mediated genetic payload delivery. Furthermore, we confirmed robust delivery and expression of genetic payloads in the polycystic kidney of a mouse ADPKD disease model (Nek8^{jck}). Conclusion: These biodistribution data establish a novel UMGD platform as an effective approach for non-viral delivery of genetic medicines to the most clinically relevant cell types in the liver and kidney.

1736. ARMMs as Non-Viral Vehicles that Enable *In Vivo* Functional Delivery of Cas9 to Disrupt NLRP3 in Kupffer Cells and Ameliorate Acute Liver Injury

Mike Thomas, Ryan von Kleeck, Qiyu Wang, Emmanuel Appiah, Carson Semple, Lucy Sun, Pearl Moharil, Komal Vyas, Chou-Wei Chang, Shu-Lin Liu, Qin Yu, Joseph F. Nabhan

Vesigen Therapeutics, Cambridge, MA

Acute liver injury (ALI) can be initiated through a variety of stimuli and is often the initial trigger to multiple chronic liver diseases. The NLRP3 inflammasome plays a central role in ALI to exacerbate liver disease pathogenesis thus making NLRP3 a potential target for therapeutic intervention. In this study, the non-viral delivery vehicle ARMMs (ARRDC1-mediated microvesicles) were loaded with Cas9 and gRNA targeting NLRP3 and were administered intravenously in a CCl4-induced mouse model of ALI. This resulted in up to 60% genomic editing of NLRP3 in liver tissue-resident Kupffer cells. Liver damage was assessed 24 hours post-administration of CCl4. Notably, we observed significantly diminished %TUNEL+ staining in the tissue sections of ARMM-treated mice compared to control treated mice. Flow cytometry analysis of harvested non-parenchymal cells from dissected liver tissue revealed significantly greater cell viability of cells isolated from ARMM-treated compared to control-treated mice. Circulating liver enzymes ALT and AST were also monitored in this study. In conclusion, we demonstrate that ARMMs efficiently deliver the gene editing complex (Cas9/gRNA) to disrupt NLRP3 expression in a large proportion of Kupffer cells and yield diminished liver inflammation in a mouse model of ALI. Our data suggest ARMMs as a superior vehicle for precise gene editing in renewable immune cells, such as macrophages, and as a therapeutic strategy for multiple inflammation-related disorders of the liver, including fibrosis.

1737. Novel Biodegradable Lipid Nanoparticles (LNP) for Co-Encapsulation of Complex Nucleic Acid Payloads for *In Vivo* Genome Editing

Alicia Davis, Lijun Huang, Ling Li, Khalid Hajj, Mu Li, Sang Jun Lee, Fernando L. Espinoza, Eliana Estrada, Stephanie Kearney, Kevin Johnson, Tuyen Nguyen, Thien Le, Chris Deng, Oscar Alvarez, Brian Sloat, Michael Bennett, Joshua Rychak

Poseida Therapeutics, San Diego, CA

Our Cas-CLOVER™ Gene Editing System comprises a next-generation nuclease able to achieve precise genomic edits with exceptionally high fidelity. Ensuring that the targeted genomic edits occur in the desired tissue, in particular to avoid genomic alterations that would be heritable, is of key importance for this class of gene therapy. This requirement for high organ specificity presents a challenge to conventional delivery platforms. Here, we present the results of our efforts aimed at developing a lipid nanoparticle capable of robustly co-encapsulating and delivering the Cas-CLOVER payload (consisting of a single mRNA and two gRNAs) to liver, while avoiding uptake in gonads. A novel class of biodegradable ionizable lipids was discovered and characterized as part of this effort, and methodical formulation optimization studies were performed to understand how LNP formulation and process parameters influence *in vivo* biodistribution and performance. We first aimed to discover novel ionizable lipids capable of supporting robust encapsulation and delivery of the multi-component Cas-CLOVER payload. A library of novel diamine lipidoids was designed, synthesized, formulated, and screened for delivery of a luciferase mRNA. The highest-performing lipidoid was explored using design-of-experiment (DoE) modelling to generate a panel of LNPs that were evaluated in adult mice by BLI and by measuring cytokine release. We observed a promising formulation parameter space, though subsequent studies revealed this lipidoid demonstrated unacceptably long residence time in the liver. Structure-activity relationship studies were conducted to accelerate liver clearance while preserving potency and liver tropism. One resulting ionizable lipid with ideal clearance characteristics was selected and further explored in an *in vivo* formulation discovery campaign. We conducted a DoE formulation optimization campaign using Cas-CLOVER mRNA and gRNAs specific for mouse *Pcsk9* (as a tooling locus). The model evaluated four formulation variables (% ionizable lipid, % phospholipid, % DMG-PEG, and lipid-to-payload ratio), and each run was evaluated in mice. A formulation able to reproducibly encapsulate the Cas-CLOVER payload components and mediate *Pcsk9* editing with high potency was identified from the DoE model. Benchmarking studies demonstrated superior potency and tolerability for this LNP formulation using the Cas-CLOVER nuclease. The formulation was subsequently transferred from the benchtop microfluidic/dialysis process to one utilizing scalable unit operations, and successfully scaled up by 10X. This work resulted in the generation of a novel ionizable lipid and LNP formulation capable of supporting application of the Cas-CLOVER nuclease for liver-directed genome editing. These results demonstrate the utility of rational design, chemical syntheses and formulation optimization when seeking to deliver complex nucleic acid payloads.

1738. Inhibition of IFNAR-JAK Signaling Enhances Tolerability and Transgene Expression of Systemic Non-Viral DNA Delivery

Cameron Baptista, Sujata Senapati, Mike Minnier, Matt Walsh, Sean M. Armour, Pedro J. Cejas

Spark Therapeutics, Philadelphia, PA

Lipid nanoparticles (LNPs) have demonstrated great potential for non-viral delivery of nucleic acid therapeutics, with mRNA delivery used for vaccines and siRNA to treat genetic disease. There is considerable interest in applying LNPs to therapeutic delivery of DNA (DNA-LNPs) to achieve long-term gene expression for a broad range of genetic diseases. A significant obstacle to systemic DNA-LNP delivery is the potential for acute inflammatory responses, which present

significant safety concerns and potentially limit therapeutic transgene expression. However, the mechanisms and immune signaling pathways underlying DNA-LNP-triggered inflammatory responses are not well characterized. We employed gene-targeted mouse models and identify cGAS-STING and IFNAR as major molecular mediators of acute inflammation triggered by systemic DNA-LNPs. cGAS-STING activation induces expression of numerous JAK-STAT cytokines, and we show that treatment of mice with JAK inhibitors, ruxolitinib or baricitinib, significantly improves tolerability to systemically delivered DNA-LNPs. Further, specific inhibition of IFNAR signaling significantly enhances DNA-LNP tolerability and transgene expression. Therefore, utilization of JAK inhibitors or IFNAR blockade present potentially useful approaches to improving safety and efficacy of non-viral DNA delivery for gene therapy.

1739. Human Organoids for Optimization of LNP Mediated Targeting of Non Parenchymal Patient Liver Cell Types

Tobias Raabe[1], Jilian Melamed[1], Mohamad-Gabriel Alameh[1], Andy Liu[2], Drew Weissman[1]

[1]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA,[2]School of Engineering and Applied Science, University of Pennsylvania, Philadelphia, PA

Background and Rationale: Lipid nanoparticles (LNPs) have emerged as pivotal tools in gene therapy and drug delivery, epitomized by the success of mRNA LNP-based COVID-19 vaccines and the FDA-approved LNP-mediated gene therapy for transthyretin amyloidosis. However, most intravenously administered LNP's target hepatocytes, limiting their potential applications. Targeting non-parenchymal liver cells, which are crucial mediators in liver diseases, poses a significant challenge and urges the development of effective LNP strategies. Since mouse models of LNP targeting are often unable to predict optimal LNPs for use in humans, and primate models are time consuming and expensive, we reasoned that targeting human liver derived organoids with LNPs may be helpful to efficiently and systematically develop clinically relevant LNPs for nonparenchymal liver cells. **Experimental Setup:** We incorporated two excipient lipids with candidate myeloid tropism - as determined by mouse studies - into LNPs, in the hope to effectively modulate cellular distribution away from patient hepatocytes and towards patient liver myeloid cells. We employed non alcoholic steatohepatitis (NASH) patient liver - derived organoids in co-culture with primary human macrophages and tested the two types of candidate myeloid tropic LNP preparations under several culture conditions. To asses myeloid targeting capacity of the candidate LNP types we co-transfected them with known hepatotropic LNPs. To directly and simultaneously visualize both hepatotropic and candidate myelotropic LNPs in the co-cultures we formulated the hepatotropic LNPs with GFP mRNA and the candidate myelotropic LNPs with tdTomato mRNA. **Results:** Detailed quantitative analysis of immunofluorescence images revealed that our candidate myelotropic LNPs are indeed myelotropic compared to the LNPs with known hepatocyte specific tropism. Importantly, we also show that organoid-macrophage co-culture media composition is a major determinant of the degree of myelotropism and especially the remaining capacity to target hepatocytes. We show that our system has the capacity to directly test the effect(s) of media components such as ApoE, serum and others on our candidate LNPs myeloid specificity. Importantly, even under optimal media conditions our candidate myeloid tropic LNP formulations targeted unexpectedly only about 50% of all primary human macrophages. We are currently improving our myeloid specific LNPs which includes investigating if our candidate myeloid specific LNPs target only specific subtypes of human macrophages. **Conclusions:** Co-cultures of primary human myeloid cells with patient liver derived organoids have the capacity to precisely assess LNPs patient liver cell type specific tropism and to optimize LNP formulations to direct them toward non parenchymal patient liver cells and away from patient hepatocytes. Combining advanced LNPs and patient liver organoids holds the potential to revolutionize targeted gene therapy and drug delivery, paving the way for precision medicine in the treatment of liver diseases.

1740. Kidney-Targeted Non-Viral Gene Delivery Using a Novel Noninvasive Transcutaneous Ultrasound Delivery Platform Enables Redosable, Titratable, and Durable Gene Expression in Healthy Mice and NHPs, and Polycystic Kidneys Mouse Model

Bert J. Frederich, Surabhi Rao, Victor Foster, Barry Campbell, Adel Alonso, GLenda Arauz, Celia Ortiz, Oliver Chen, Charlotte He, Elizabeth Del Greco, Anagha Rao, David Satyadi, Dhruv Bole, David Kim, Katie Benthall, Margarita Krivega, Julia Poniatowski, Ivan Krivega, Zoya Gluzman-Poltorak, Steve Feinstein, Kenneth Greenberg

Sonothera Inc, South San Francisco, CA

Introduction: Gene delivery to the kidney remains a significant impediment to the development of genetic medicines for renal diseases. Transcutaneous ultrasound-mediated gene delivery (UMGD) presents a noninvasive *in vivo* gene delivery approach that may overcome the key delivery challenges facing genetic medicines. Despite a body of literature supporting the effectiveness of UMGD in introducing transgenic DNA into target organs, a comprehensive understanding of the efficacy, durability, and safety in healthy and diseased kidneys has yet to be established. SonoThera is developing a novel ultrasound-guided nonviral gene therapy platform based on UMGD that allows selective targeting of specific organs and tissues within the body in a safe, redosable, durable, and titratable manner. Methods: To evaluate the potential of UMGD for safe and efficient transgene delivery to the kidney, we developed a technology platform utilizing novel acoustic profiles, next-generation genetic payloads, and FDA-approved ultrasound components. The delivery process involves intravenous co-administration of nucleic acid payloads (e.g. DNA or RNA) and ultrasound contrast agents (a.k.a microbubbles), coupled with targeted application of externally applied ultrasound energy to guide the DNA into specific tissues via sonoporation. In vivo bioluminescence imaging of firefly luciferase reporter gene expression was evaluated using an IVIS imaging system at multiple timepoints in kidneys of wild type mice and a mouse model of polycystic kidney disease ($Nek8^{jck}$) following UMGD. Mouse and monkey kidney tissues were analyzed for reporter gene presence and expression. Multiple clinical and molecular safety assessments were evaluated in mice and monkeys using established methods. Results: The delivery of next-generation

non-viral genetic DNA payloads via UMGD resulted in rapid, robust, durable, redosable, and dose-responsive transgene expression in wild-type mouse kidney. Durable gene expression with no loss of signal was observed beyond 9 months following a single treatment in mice. Additionally, in a mouse model of polycystic kidney disease (Nek8^{jck}) exhibiting advanced cystic pathology, UMGD resulted in significant microvascular perfusion, efficient gene delivery, and robust expression in cystic structures. Reporter gene presence and expression were detected in the analyzed mouse and NHP kidney tissues. Safety assessments including clinical observations, blood urea nitrogen, creatinine, and a proinflammatory cytokine panel indicate an excellent tolerability and safety profile following single and repeat treatments in mice and monkeys. These findings hold potential significance for the application of this technology in genetic medicines for multiple types of kidney diseases. Conclusion: This study positions a novel UMGD platform as a promising strategy for *in vivo* transgene delivery using next-generation non-viral genetic payloads, thereby laying the foundation for the development of future genetic medicines for renal diseases.

1741. Next-Generation DNA-Based Delivery of Therapeutic Proteins Using MYO Technology: Preclinical Results on Incretins Receptor Agonists

Linda Sasset, Marek M. Drozdz, Andrew D. Cameron, Delcora A. Campbell, Debnath Maji, Xin Yao, Carleigh Sussman, Andy T. Thompson, Robert Miller, Rachel Liberatore

RenBio, New York, NY

Therapeutic proteins, a large class of drugs including antibodies, hormones, and enzymes, have seen a substantial increase in both clinical development and use across many disease areas. However, significant drawbacks continue to limit access to many of these drugs, and can include: i) high manufacturing costs; ii) administration via time-consuming intravenous infusions; iii) frequent dosing, sometimes even daily; iv) requirements for low temperature storage, hindering the ability to get therapeutics to large numbers of people, a problem amplified in low and lower-middle income countries. MYO Technology™, a DNA-based platform for the delivery of therapeutic proteins, was developed to overcome these barriers. The MYO Technology platform consists of therapeutic-encoding plasmid DNA (pDNA) and a proprietary medical device for the intramuscular injection of pDNA, followed by the delivery of very short electrical pulses to the muscle tissue surrounding the injection site. These pulses promote the *in vivo* electroporation of muscle cells and uptake of pDNA, leading to production and secretion of the therapeutic protein, and ultimately uptake into peripheral circulation.MYO Technology™ offers several advantages over standard delivery of therapeutic proteins, in part because pDNA manufacturing is a simpler and less specialized process as compared to protein manufacturing, and pDNA is very stable and lacks most cold chain requirements. Furthermore, administration using MYO Technology takes just a few minutes, and the serum level of a therapeutic protein can potentially be maintained for many months, perhaps even years, without the need for redosing. A subclass of therapeutic proteins that have recently come to prominence are incretin (GLP-1, GIP) receptor agonists (IRAs). IRAs are emerging as powerful glucose control and weight loss drugs, used for the treatment of type 2 diabetes (T2D) and obesity. The FDA-approved drugs semaglutide (Ozempic/Wegovy) and tirzepatide (Mounjaro/Zepbound) are very potent molecules but have a short half-life, requiring weekly administration by subcutaneous injections. Moreover, since their effects rapidly disappear once the treatment ends, T2D and obese patients may have a life-long dependency on IRAs, and this requirement for weekly injections can negatively affect the quality of life and the adherence to therapy, as well as create a significant financial burden.Here, we present our preclinical studies on delivery of IRAs with MYO Technology. Animal proof-of-concept studies demonstrate that expression of MYO Technology-delivered IRAs is pDNA-dose dependent and remains stable for several months or longer in vivo. Importantly, MYO Technology-produced IRAs are functional and efficacious in stimulating weight loss in animal models of diet-induced obesity.Through significant improvements in administration frequency, MYO technology has the potential to dramatically improve quality of life and therapy adherence among T2D and obese patients. Furthermore, MYO Technology has the potential to present the same advantages when applied to other therapeutic proteins with short half-lives.

1742. Risks and Benefits of Multiplexed Gene-Editing Strategy for Generation of Hypo Immune Universal Pluripotent Stem Cell Platforms for Off-the-Shelf Cell-Based Therapeutic Applications

Miguel A. Tellez[1], Joe Geraghty[1], Luis Borlado[1], Saumyaa Saumyaa[2], Noorul S. Sheikh Farid[2], Gargi Roy[1]

[1]Biopharmaceutical Development-Cell Culture and Fermentation Sciences, AstraZeneca, Gaithersburg, MD,[2]Safety Sciences, AstraZeneca, Gothenburg, Sweden

We seek the development of allogeneic regenerative cell therapy products to make cell therapies accessible to wider patient populations. But this approach requires the product to escape immune system activation to obtain a sustained therapeutic benefit. We and others have shown that major histocompatibility complex (MHC) class I and II removal prevent T-cell recognition, however this approach requires the expression of cloaking genes on the cell surface to avoid elimination by natural killer (NK) cells and macrophages. Herein in a single electroporation-based gene editing step, we generated hypo immune universal human embryonic stem cells (hESCs) by knocking out MHC-I and MHC-II genes and inserting NK cell and macrophage evasion molecules in beta-2-microglobulin and CIITA loci respectively, utilizing a non-viral transgene delivery in combination with synthetic guide RNA and Cas9-mediated double strand DNA breaks. This single electroporation strategy significantly reduced the number of cell passages and timeline required for the establishment of clonal hypo immune hESCs, resulting in the reduction of overall timeline for generation of gene-edited lines by 3 months, as compared to a multistep editing approach. Among the clones screened, 38% contained site-specific knock-ins, expressed high levels (>90%) of pluripotency markers with normal karyotype and were able to efficiently differentiate into the three germ layers. Interestingly, 1 of 3 clones evaluated by

long-range whole genome analysis (optical genome mapping) showed no post-editing structural and copy number variations across the genome. Overall, when multiplex gene editing is used in tandem with clonal expansion, there is no observed excessive risk of chromosomal translocations, but rather is balanced by the reduced risk of genomic instability as observed in extended passages of stem cells.

1743. Polycistronic Modified mRNA Encoding Epigenome Rejuvenating Oct4, Sox2 and Klf4 Factors for Genome-Safe Rejuvenation of Age-Defective Kidneys from Life-Supported Organ Donors

Daniel Chevallier[1], Roger Bertolotti[2]

[1]Department of Urology and Kidney Transplantation, University Hospital of Nice, Nice, France,[2]Faculty of Medicine, University of Nice-Sophia Antipolis, Nice, France

Breakthrough retinal rejuvenation has been recently achieved by others upon AAV-mediated ectopic expression of 3 Yamanaka factors (Oct4, Sox2 and Klf4: OSK) in mouse retinal ganglion cells. Unlike the full set of Yamanaka factors (OSKM, M: cMyc), polycistronic OSK transduction has been shown to maintain cellular identity while restoring youthful DNA methylation patterns and relevant transcriptomes, consistent with the described promotion of axon regeneration and reversion of vision loss in aged and glaucoma mice. Such a stable in vivo epigenetic gene therapy achievement relies on an hit-and-run protocol in which transient expression of the long-lasting AAV-OSK transgene is under the control of doxycycline, thereby prompting us to devise a genome-safe strategy based on intra-vitreal delivery of short-lived nucleoside-modified mRNA (modRNA). Importantly, similar rejuvenation results have been recently published for mouse kidney with the above AAV-OSK vector, a result that could broaden the availability of kidney transplants upon applying our above protocol to rejuvenate kidneys from aged donors. Indeed, the shortage of kidney grafts is such a dramatic burden that we have transplanted kidneys from an 80 years-old donor. Of course, therapeutic rejuvenation will most likely target patient's organs themselves in a non-distant future. However, such a clinical translation requests costly time-consuming trials with large animal models. Therefore, rejuvenating age-related defective donor kidneys during the transplantation process stands as an exciting option because we believe that delivery of OSK modRNA before transplantation can be efficient enough to induce significant post-transplantation rejuvenation while avoiding potential OSK mRNA hazards to the patient himself. Indeed, under our proposed modRNA transfection protocol, safety hazards are mainly restricted to the allogeneic graft, thereby securing a positive benefit/risk balance. Our approach is thus discussed in terms of genome-safe rejuvenation of aged kidney transplants mediated by delivery of synthetic OSK mRNA modified by N1-methylpseudouridine as a substitute for uridine and optimized with appropriate capping, 3'/5'UTRs, improved coding sequence and poly(A) tail. Kidney delivery of such purified modified polycistronic OSK mRNA will rely either on lipid nanoparticles or on a citrate-saline formulation. Of note, as described by others, hydrodynamic delivery of DNA/mRNA to the kidney has been shown to be effective on mice/rats as long as it is performed on the clamped renal vein (with synergistic renal artery clamping) or on the renal pelvis. Hydrodynamic delivery appears to be perfectly fitted to the rejuvenating trial since it can be done in situ, before transplantation, on the kidneys from aged organ donors maintained under life support. Design optimization of the synthetic modRNA will be done while the compassionate use of early transfected donor kidneys will be consistent with the acute dramatic organ shortage. Importantly, we believe that kidneys from life-supported donors can accommodate several courses of mRNA transfection before transplantation, thereby easing the optimization of the rejuvenation process under compassionate conditions. Thus, the idea is to reduce kidney organ shortage upon epigenome rejuvenation of kidneys from aged organ donors maintained under life support, first upon compassionate conditions, then under optimized rejuvenation conditions of donor kidneys. Our proposed compassionate clinical trial is now ready for evaluation.

1744. Nanoparticles in the Biomimetic Extracellular Matrix Coated on the Cell Surface Remotely Regulate Gene Expression

Kyungsene Lee, Brandon Davis, Xuelin Wang, Yong Wang

The Pennsylvania State University, University Park, PA

The regulation of gene expression is critical to various applications. In this study, we introduce a novel approach for nanoparticle (NP) immobilization on the cell surface for cell regulation utilizing an ultrathin biomimetic extracellular matrix as a nanocapsule for carrying nanoparticles (CN2). This biomimetic extracellular matrix not only encapsulates cells but also carry gene-regulating nanoparticles. Encapsulated cells demonstrate sustained high viability and normal metabolic activity. By employing gold nanoparticles as a model, we found that exposure to light induced a transient temperature increase, activating the heat shock protein 70 (HSP70) promoter and modulating the expression of reporter genes. Given the versatility of the biomimetic nanocapsule for decoration with various nanoparticles, CN2 emerges as a promising platform for the regulation of gene expression and cell functions. Methods: Oligonucleotides used in this study were purchased from Integrated DNA Technologies (Coralville, IA). The DNA initiator was introduced to the cell membrane through lipid insertion. The combination of biotinylated DNA monomer 1 and alginate-conjugated DNA monomer 2 resulted in the formation of a DNA template through hybridization chain reaction. The decoration and stability of nanoparticles facilitated by CN2 on the cell surface were assessed using confocal microscopy and flow cytometry. In vivo imaging system demonstrated the successful transfection of a heat-inducible HSP70 promoter-based DNA plasmid into mesenchymal stem cells (MSCs) under laser irradiation. ELISA and in vitro wound healing assays revealed improved VEGF secretion from CN2 engineered MSCs with the HSP70-VEGF plasmid. Laser speckle contrast imaging on the chicken chorioallantoic membrane model study demonstrated enhanced angiogenesis in the CN2 group compared to other groups. Results: Confocal laser scanning microscopy and flow cytometry results demonstrated the formation of CN2 on the cell surface. CN2 stably retained NPs on the cell surface with minimal dissociation and internalization. Cell viability assays and cytokine array results demonstrated that CN2 did not compromise cellular behaviors of the host cell. MSCs transfected with heat-inducible HSP70-luciferase DNA plasmid showed significantly increased luminescence after laser

irradiation, demonstrating the stimulated luciferase expression from the plasmid. Kinetics analysis demonstrated that the expression of protein was time post laser irradiation dependent, with showing plateau after 12 hours. ELISA results showed the more than 3-fold increase in HSP70-VEGF plasmid transfected group after laser irradiation compared with other groups. Cells were transplanted on the chicken chorioallantoic membrane and blood flow was analyzed. CN2:VEGF group showed significantly increased blood flow after laser irradiation with showing over 270% increase. Conclusions: CN2 synthesis on the cell membrane represents a promising approach for nanoparticle immobilization on the cell surface for gene regulation. Nanoparticles can be displayed at the vicinity of encapsulated cells. This system offers a facile means to regulate cellular functions, including gene expression. The results show versatility and generality of CN2 in encapsulating any cell type while carrying single or multiple nanoparticles. Consequently, CN2 is a promising technique leveraging the features of both polymeric nanocoating and inorganic NPs, presenting a novel pathway for advancing cell-based applications, such as stem cell delivery for regenerative medicine, immune cell delivery for immunotherapy, and cell-mediated nanoparticle delivery for cancer treatment.

1745. Highly Efficient Non-Viral Gene Editing of Unactivated Human T cells and Hematopoietic Stem and Progenitor Cells Using Microfluidic Transfection

Eric Ouellet[1], Manreet Chehal[1], Ulrike Lambertz[1], Elinor Binson[1], Tina Liao[1], Gil Paik[1], Phillip Chau[1], Kevin Chen[1], Bob Dalton[1], Allen C. Eaves[1,2], Sharon A. Louis[1], Andy Kokaji[1]

[1]STEMCELL Technologies, Inc., Vancouver, BC, Canada,[2]Terry Fox Laboratory, Vancouver, BC, Canada

Ex vivo gene editing of primary cells presents great potential for investigating molecular drivers of disease and development of new therapeutics. The increasing variety of bioactive molecules used in these studies continue to push the limits of traditional intracellular delivery methods. Non-viral delivery represents an attractive approach to limiting the risks of insertional mutagenesis associated with viral delivery, but some methods, including electroporation, can be accompanied by increased cell loss and altered cellular physiology. Therefore, careful considerations must be made in selecting the best approach that provides high efficiencies and minimal unwanted cellular perturbations. Mechanoporation has been shown to safeguard critical cell quality attributes including viability, phenotype and function. The CellPore™ Transfection system is a mechanoporation-based system that relies on rapid controlled cell deformation within microfluidic channels to generate temporary pores for direct cytosolic introduction of materials. In this work, we describe the implementation of the CellPore™ technology workflow for genetic manipulation of human unactivated T cells and CD34+ hematopoietic stem and progenitor cells (HSPC). By evaluating the interplay between the delivery of fluorescent dextrans and impacts to cell viability under varying system pressures, the transfection efficiency can be optimized for various biological cargos, including mRNA and CRISPR Cas9 ribonucleoproteins (RNP). Our study first applied this method to optimize the delivery of Cas9 RNP complexes targeting either *B2M* or *TRAC* genes in unactivated T cells from healthy donors. After 2 or 6 days of culture, T cells were analyzed for MHC-I or TCRαβ marker expression, respectively. *B2M* editing resulted in 73.6 ± 4.0% knockout of MHC-I (mean ± SD; n = 12) and 93.9 ± 1.0% knockout of TCRαβ for *TRAC*-edited donors (n = 3). Overall viability for all edited donors remained high (80.3 ± 4.1%; n = 15). Importantly, we further demonstrated that transfection via CellPore™ safeguarded the unactivated T cell phenotype, evidenced by low CD69 activation marker expression (0.8 ± 0.3%; n = 6) and baseline secretion levels of several pro-inflammatory cytokines, including IL-2 (0.11 ± 0.09 pg/mL) and IFNγ (0.14 ± 0.03 pg/ml; n = 3). In contrast, electroporation resulted in elevated levels of CD69 marker expression (41.2 ± 8.4%; n = 6) and proinflammatory cytokines (77.6 ± 23.5 pg/mL IL-2; 8.9 ± 3.5 pg/mL INFγ; n = 3). Finally, the ability for unactivated T cells manipulated by CellPore™ to undergo similar activation and expansion compared to unmanipulated samples is shown. Likewise, we investigated the ability to genetically manipulate cord blood-isolated CD34+ HSPCs via CellPore™ transfection. After two days of culture in StemSpan™ medium, HSPCs were harvested, transfected with Cas9 RNP targeting *B2M*, and returned to culture for an additional 4 days. Editing of *B2M* resulted in 80.6 ± 3.1% knockout of MHC-I (n = 15) in the long-term repopulating hematopoietic stem cells (CD34+CD90+CD45RA-) within the bulk CD34+ HSPC population. Furthermore, a 14-day colony-forming unit (CFU) functional assessment of the edited HSPCs showed minimal impact to their proliferation. These results underscore the important considerations that need to be made when choosing a suitable intracellular delivery method for genetically manipulating primary cells. In this capacity, these results provide a demonstration of a non-viral delivery approach that enables high cellular engineering efficiencies with minimal impacts to downstream cellular function.

1746. Suspension Cell-Based Production and Scalable Purification of Engineered ARMMs as a Platform for Non-Viral Therapeutics

Steven Greenway, Alysia Bryant, Ishaan Tiwari, Zachary Shelnitz, Leah Gens, Adam Francoeur, Arian Pushka, Kristin Luther, Gary Hao, Joseph Nabhan

Vesigen Therapeutics, Cambridge, MA

Extracellular vesicles (EVs) are currently being evaluated as promising platforms for non-viral delivery of therapeutic payloads. The clinical development of EVs is hampered by the challenges in manufacturing of therapeutic-grade material at scale. Here, we report an efficient and scalable platform tailored for production of a specific class of EVs, ARRDC1-mediated microvesicles (ARMMs). During process development, each unit of operation was systematically evaluated for the selection of the optimal reagent, instrumentation, and parameters. The upstream process is comprised of a specific class of transfection reagent for enhanced ARMMs yield, low-shear rocking platform bioreactor, and primary clarification by continuous centrifugation. The downstream process entails tangential flow filtration followed by mixed-mode size exclusion chromatography or monolithic anion exchange chromatography. The platform enables the production of ARMMs loaded with therapeutic molecules including CRISPR/Cas, base editors, mRNA, or short hairpin RNA (shRNA). The final products show high purity, consistent size distribution and payload concentration, as well as functional payload delivery in both in vitro

and in vivo experiments. Our data establishes robust and scalable production and purification processes for ARMMs with multiple payloads. These advances support the potential to utilize these processes for manufacturing of ARMMs for clinical trials.

1747. Regulatory Strategy to Maximize Success Potential of Gene Therapy & Rare Diseases

Mridula Shukla

Poseida Therapeutics, San Diego, CA

The rapidly changing field of Gene Therapies promises several innovative treatments for rare diseases. Given that approximately 80 percent of the 7,000 known rare diseases are caused by a single-gene defect, there has been increased research in developing cell and gene therapies to treat rare diseases. However, several challenges hinder these efforts, including the high cost of bringing these drugs to market, unique manufacturing and supply chain challenges, pricing and reimbursement, and our current limited understanding of disease pathology and progression. In this presentation, we will discuss how we can downplay the complexities associated with gene therapies by employing the optimal regulatory strategy. Questions that will be covered in this presentation include. Q1: Recent trends in gene therapies and key challenges given the complexity & high cost of gene therapy development Q2: Discuss strategies to develop gene therapy products in a cost-effective way Q3: How gene therapy can be impactful for Rare diseases Q4: Successful and Proven Regulatory Strategies to expedite the development of GT drugs Q5: Recent announcements from the FDA on Project OWS-RD & BCGT Plug-n-Play approach Q6: Benchmarking of regulatory pathways for gene therapy drugs Q7: Inflection point and evolving trends in Gene Therapy

1748. Gene Editing in Liver Towards the Treatment of Life-Threatening Cardiovascular Diseases: POC for Efficient *In-Vivo* Genome Editing/Base Editing Using Lipid Nanoparticle Library

Anitha Thomas, Mehar Gayatri Namala, Nikita Jain, Zhengyu Chen, Leanna Lee, Malathi Anantha, Sams Sadat, Tony Wu, Ruchi Sharma, Eric Wu, Jay Paquette

Cytiva, Vancouver, BC, Canada

Introduction: The transformative potential of CRISPR-Cas9 gene-editing technologies, including CRISPR-Cas9 base editors (ABE, CBE), in modifying human genes has paved new path for genomic medicines. This, along with the renaissance of the LNP field hold enormous potential in enabling gene medicines. Efficient gene editing workflows and enabling tools for in vivo and ex vivo gene editing are required to advance genomic medicine innovation. Scarcity of well characterized fit-for-purpose lipids that are suitable for CRISPR-Cas9 gene-editing technologies continue to be a problem in advancing many genomic medicine applications. In this study, we present the development of novel ionizable lipids as functional excipients for designing vehicles for delivering CRISPR-Cas9. We evaluated the genomic medicine potential towards the treatment of life-threatening cardiovascular diseases. **Methods:** The robust LNP delivery system utilized our proprietary lipids and compositions for editing *TTR* and *PCSK9* genes in rodent models. Guide RNA (sgRNA) and messenger RNA (Cas9 or ABE 8.8m) were co-formulated in LNPs using NanoAssemblr® Ignite. Size and PDI were determined using Dynamic Light Scattering. The encapsulation efficiency was determined using the Ribogreen™ assay. Cas9-TTR LNPs and ABE 8.8m-PCSK9 LNPs were administered intravenously into CD-1 and C57BL/6 mice, respectively, at 2-3 mg/kg dose. Serum was collected 7 days post injection and analyzed for *TTR* and *PCSK9* levels through ELISA. Further, we have done NGS sequencing to evaluate editing levels. **Results:** Size of the LNPs were reported to be between 50-140 nm with PDI<0.2. PNI LNPs showed significant editing of the mouse transthyretin (*TTR*) gene and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene in the mouse liver, resulting in approximately 75% and 80% reductions in serum protein levels, respectively, following a single administration of 2mg/Kg LNPs. The robust LNP delivery system used in this current study demonstrated exceptional biodegradability and high tolerability, at efficacious doses tested. It has been shown earlier that ABE-cas9 show off-target editing even with-in the targeted locus. Novel LNPs showed precise base editing of PCSK9 locus without having off targeting effects. Overall, we demonstrated successful gene editing and base editing of *TTR* and *PCSK9*, respectively, in rodents. **Conclusions:** Our proprietary lipid and or LNP compositional library can be used to efficiently deliver CRISPR/Cas9 components. We achieved clinically relevant levels of in-vivo genome editing of TTR and PCSK9 targets as examples. These findings highlight the potential of our lipid library as a highly effective and promising platform tools for genome editing.

1749. Sonogenetic Modulation of Cellular Activities for Treatment of Neurological Disorders

Subrata Batabyal[1], Sanghoon Kim[1], Michael Carlson[1], Darryl Narcisse[1], Adnan Dibas[2], Samarendra K. Mohanty[1]

[1]Nanoscope Technologies LLC, Bedford, TX,[2]Nanoscope Technologies, Bedford, TX

PURPOSE: While electrical modulation of neuronal activity in the central and peripheral nervous systems using neuroprosthetic has shown therapeutic benefits in restoring sensory and motor function in various neurological disorders, it is challenged by complexity due to invasive implantation surgeries and non-specific stimulation. It has been demonstrated that ultrasound can modulate cellular activities with or without the use of exogenous microbubbles or genetic mechano-sensitization. However, the requirement of high-intensity ultrasound poses a challenge in miniaturization for neuroprosthetic applications. By sensitizing specific neurons toward ultrasound using engineered mechanosensitive channels (EMC), low-power sonogenetic simulation provides a potential alternative therapeutic modality to achieve cell-specific stimulation for improving the treatment of various neurological diseases. METHODS: Using pressure-clamp electrophysiology, we have screened several mutated mechanosensitive channels derived from different microbial species that are heterologously expressed in HEK293 cells. Sensitization of cells with EMC was achieved either by viral or non-viral methods. In-vivo expression of EMC in the central nervous system was achieved by AAV delivery. Sonogenetic stimulation of EMC-expressing neurons was carried out by ultrasound

with varied intensity to determine the threshold for activation. The activation was evaluated using electrical measurements *in-vitro* and *in-vivo*. The impact of ultrasound-evoked electrical activities in EMC-sensitized neurons was evaluated for modulating different sensory functions. RESULTS: By molecular engineering, the activation threshold of EMC was significantly reduced compared to wild-type microbial mechanosensitive channels. Our studies show robust EMC expression in the cellular membrane (assessed by analysis of mCherry reporter) leads to activation in response to low-intensity ultrasound (0.2 MPa). In-vivo stimulation of EMC-sensitized neurons by low-intensity ultrasound led to statistically significant ($p<0.05$) activation measured by electrophysiology compared to non-treated controls. Sonogenetic stimulation of the EMC-sensitized brain led to efficient neuromodulation, as assessed by behavioral assays. CONCLUSION: EMC sensitization of specific neurons via genetic targeting combined with external ultrasound stimulation elicited functional responses in multiple animal models. The advancement of this sonogenetic approach will lead to a new treatment approach for debilitating neurological disorders.

1750. Chandipura Viral Glycoprotein (CNV-G) Promotes Gectosome Generation and Enables Delivery of Intracellular Therapeutics

Xuedong Liu[1], Xiaojuan Zhang[1], Quanbin Xu[1], Zeyu Liu[1], Brandon Black[1], Saheli Ganguly[1], Jayson Ball[2], Michael E. Harland[2], Samuel Blackman[3], Stephanie Bryant[3], Kristi Anseth[3], Linda Watkins[2]

[1]Biochemistry, University of Colorado-Boulder, Boulder, CO,[2]Psychology & Neuroscience, University of Colorado-Boulder, Boulder, CO,[3]Chemical and Biological Engineering, University of Colorado-Boulder, Boulder, CO

Achieving cell type-specific delivery of biologics and overcoming the challenge of delivering large macromolecules through endosomal barriers remain significant hurdles in the field of biomedicine. Previously, we developed a versatile approach for delivering proteins and ribonucleoproteins (RNPs) to both in vitro cell cultures and in vivo mouse liver tissue using specially engineered vesicles known as "Gectosomes." These Gectosomes are designed to co-encapsulate the vesicular stomatitis virus G protein (VSV-G) with bioactive macromolecules through split GFP complementation. Our method enables active cargo loading into these Gectosomes, resulting in improved cargo delivery efficiency and simplified purification processes. While VSV-G Gectosomes have broad cellular tropism, for specific therapeutic applications, it is often desirable to have Gectosomes with more restricted cellular tropism and cell type specificity. In this study, we evaluated a subset of viral glycoproteins from a diverse range of viruses for their ability to stimulate the production of Gectosomes and the intracellular delivery of encapsulated cargos. We made a significant discovery that the expression of the viral glycoprotein CNV-G, derived from the human neurotropic pathogen Chandipura virus in 293T cells, significantly shifts the production of extracellular vesicles (EVs) towards CNV-G Gectosomes. Compared to VSV-G Gectosomes, CNV-G Gectosomes exhibit enhanced cell-type selectivity, demonstrating effectiveness in primary cells and tumor cell lines, with a notable preference for neuronal cells. Further investigations revealed that CNV-G Gectosomes enter cells independently of the Low-Density Lipoprotein Receptor (LDLR), which is essential for VSV-G entry. Additionally, CNV-G Gectosomes respond differently to pharmacological modulators. Using a split GFP and chemical-induced dimerization system, we demonstrated that CNV-G Gectosomes can be adapted to deliver various intracellular cargos designed to modify the genomic sequence of target cells or respond to external stimuli, both in vitro and in the brains of mice in vivo. Pharmacokinetics and biodistribution analysis further support the potential of CNV-G Gectosomes as a highly effective programmable macromolecular delivery platform for intracellular therapeutics. Consequently, Gectosome technology holds promise for the treatment of rare human diseases by enabling targeted gene therapy.

1751. One-Step Production of Cas9-Based Precision Gene Editors for *In Vivo* Delivery

Colin D. Robertson[1], Alexandros Poulopoulos[2]

[1]Pharmacology and UM-MIND, University of Maryland School of Medicine, Pikesville, MD,[2]Pharmacology and UM-MIND, University of Maryland School of Medicine, Baltimore, MD

As precision genome editing technologies advance, it is important to broadly consider applications that may benefit patients suffering from genetic diseases. Given the long path towards approval for therapeutic gene editing, a more immediate clinical need might be met through the use of these precision editors to efficiently model disease. Our previous work has demonstrated the use of prime editing in vivo to model an individual rare epilepsy variant in a wild type background. Because gene editing approaches now boast extremely flexible design requirements, allowing efficient and precise modifications spanning an impressive range of sizes, most genetic variants could similarly be targeted. Our work has previously been limited to delivery of plasmids in utero, restricting the target tissues and breadth of impact. Here, we describe an updated system for producing custom Cas9-based ribonucleoproteins (RNPs) in one step in *E. coli*. We assess these RNPs for efficiency and precision in vitro and in vivo assays targeting multiple genomic loci. These RNPs alleviate previous restrictions to our disease modeling platform, decrease the potential for off-target and unintended edits, and allow for higher efficiency editing while providing the possibility of on-demand whole animal editing. This approach could allow for rapid, personalized disease modeling, providing patients with a population of preclinical surrogates tailored to their own genetics, a pseudo n-of-1 trial that could be used to screen approved medications and inform evidence-based medicine for patient treatments. This approach is not limited to any specific tissues, targets, or model organisms, and each variable can be tailored to the disease, condition, and patient.

1752. Co-Quantifying Surface Targeting Antibody and Nucleic Acid Payload of Lipid Nanoparticles by Single Nanoparticle Analysis

Sixuan Li[1], Yizong Hu[2], Jinghan Lin[1], Hai-Quan Mao[1], Tza-Huei Wang[1]

[1]Johns Hopkins University, Baltimore, MD,[2]Massachusetts Institute of Technology, Cambridge, MA

Introduction: Lipid nanoparticles (LNPs) are one of the leading approaches to deliver nucleic acid therapeutics in a wide range of applications, including genetic vaccines, gene editing, regenerative

therapies, etc. To achieve a desired tissue or organ-specific treatment, especially extrahepatic delivery, active targeting by decorating the LNP surface with targeting ligands has been extensively investigated [1]. However, it remains challenging to effectively leverage the rational design of this mechanism due to the lack of tools to characterize ligand configurations on LNPs, which is critical to understand and control the delivery specificity and efficiency of the targeted LNPs. Existing methods provide an estimated average ligand quantity on LNP at best. Here, we describe a technique using a microfluidic-based, single-particle analysis, termed cylindrical illumination confocal spectroscopy (CICS), to achieve statiscally robust and absolute quantifications of targeting antibodies on the surface of LNPs and nucleic acid payloads encapsulated within LNPs. This technique can unveil the heterogeneity of surface ligand density and mRNA payload at a single particle level and provide key information to assess nanomedicine targeting. **Methods:** We adopted our recently developed CICS platform and the LNP characterization protocol [2] to examine the LNPs with fluorescently labeled Cy5-pDNA, and TopFluor-PC lipids and conjugated with Alexa 546 labeled IgG antibody (Ab) by maleimide-thiol chemistry. A DLin-MC3-DMA-based benchmark LNP formulation was formulated by rapid mixing at pH 4, dialyzed against PBS at pH 7.4, and purified after antibody conjugation. **Results:** Antibody conjugation was verified by an approximate 20 nm increase in LNP size measured by DLS, and a 12 mV decrease in zeta potential (**Fig. 1a**), while maintaining a narrow polydispersity index (~0.2) and high DNA encapsulation efficiency (~95 %). The 3-color labeled Ab-conjugated DNA LNPs were screened at the single nanoparticle level by CICS; and the single particle fluorescence coincidence was analyzed to classify 6 LNP subpopulations including Ab-DNA-LNPs, non-Ab DNA-LNPs, Ab-empty-LNPs, non-Ab empty LNPs, unencapsulated DNA and unconjugated Ab. Three Ab to maleimide-PEG molar ratios were used for the conjugation reaction, and we found more DNA-encapsulated LNPs were conjugated with Ab compared to empty LNPs by around 30% consistently across the 10-fold Ab range (**Fig. 1b**). Fluorescence deconvolution analysis was performed to quantify the Ab surface density and DNA payload on single LNP particles. The ligand surface density distribution follows a Poisson distribution (**Fig. 1c**) and the average ligand number increases at higher antibody input (**Fig. 1d**), while the DNA payload distribution follows a lognormal distribution and the payload remains the same before and after conjugation (**Fig. 1e**), suggesting the different LNP assembly and ligand conjugation mechanisms. **Conclusions:** The CICS platform and the single particle assay characterize targeting antibody-functionalized lipid nanoparticles and quantify the surface density and distribution of ligand and nucleic acid payload simultaneously. This technique provides a powerful single nanoparticle analysis tool to facilitate further optimizations of LNPs aiming for more homogeneous payload loading and surface antibody presentation. **References:** [1] Nat. Rev. Mater. 8(4): 282 (2023). [2] Nat. Comm. 13 (1): 5561 (2022).

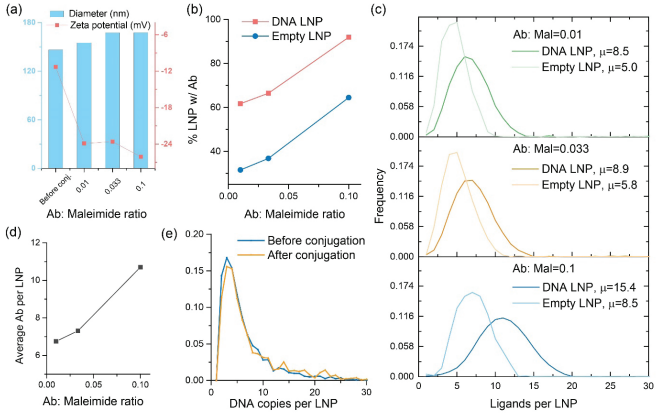


Fig. 1. (a) DLS and zeta potential of LNP before and after conjugation. (b) Percentage of DNA encapsulated LNPs and empty LNPs conjugated with antibody. (c) Antibody quantification and ligand quantity per LNP distribution. (d) Number of Ab per LNP, including both DNA encapsulated and empty LNPs. (e) DNA payload distribution in DNA encapsulated LNPs before and after conjugation.

1753. In Utero Systemic LNP mRNA Delivery Targets the Fetal Brain and Permits Diffuse Gene Editing

Marco D. Carpenter, Rosa Choi, Nicole Kus, Andrew Mudreac, Alanis Perez, Hooda Said, Mohamad Gabriel Alameh, William Peranteau

Children's Hospital of Philadelphia, Philadelphia, PA

No curative therapies exist for the 1% of children affected by genetic diseases of the central nervous system (CNS). Often the pathogenesis of these diseases begins before birth and is irreversible. CRISPR-Cas9 gene editing provides a unique opportunity for a single dose cure if the target cell type and organ are reached. Unfortunately, therapeutic CNS gene editing is limited by the diversity of cell types in the brain, the limited regenerative capacity of neurons and, the impenetrable blood-brain barrier (BBB) which prevents access of systemically delivered gene editing therapies. Non-viral ionizable lipid nanoparticles (LNPs) have emerged as a safe and effective means of mRNA for the correction of genetic liver diseases but they have not been shown to deliver mRNA to the brain following intravenous delivery. In addition to mitigating disease pathology before its onset, in utero gene editing via LNP delivery may take advantage of fetal developmental properties to facilitate delivery of therapeutic editing technology to the brain following systemic delivery. To address this hypothesis, we screened three LNP formulations loaded with CRE recombinase mRNA in a GFP reporter mouse, via intravenous delivery at three developmental stages: fetal, neonatal, and adult. All mice were harvested 4-6 weeks following injection and GFP expression in the brain was quantified using flow cytometry and immunohistochemistry using markers for specific cell types (neurons, endothelial cells, microglia, astrocytes, and oligodendrocytes) and other organs. We identified one LNP (LNP1) with efficient mRNA delivery to endothelial cells (~50%), macrophages (75%) and neurons (5%) in mice who were injected in utero when compared to mice injected as neonates or adults which demonstrated no editing in endothelial cells or neurons. These data suggested the fetal environment has distinct biological features that enhance mRNA delivery via LNPs to the brain. We then applied these findings and used LNP1 to assess CNS gene editing in a mouse model of Friedreich Ataxia (FRDA). FRDA is a neurodegenerative disease in which propagation of

GAA repeats in the frataxin gene results in decreased frataxin protein expression. Gene editing to excise the triplet repeat is one potential therapeutic approach that has been suggested. LNP mediated systemic delivery of SpCas9 mRNA and gRNA targeting the triplet repeat to gestational day 14 FRDA fetal mice result in low level editing in the brain. These initial studies provide a foundation for an in-utero gene therapy approach for FRDA. In conclusion, we leverage our discovery related to basic fetal biology and its influences on LNP-mRNA delivery to develop clinically relevant CNS therapies.

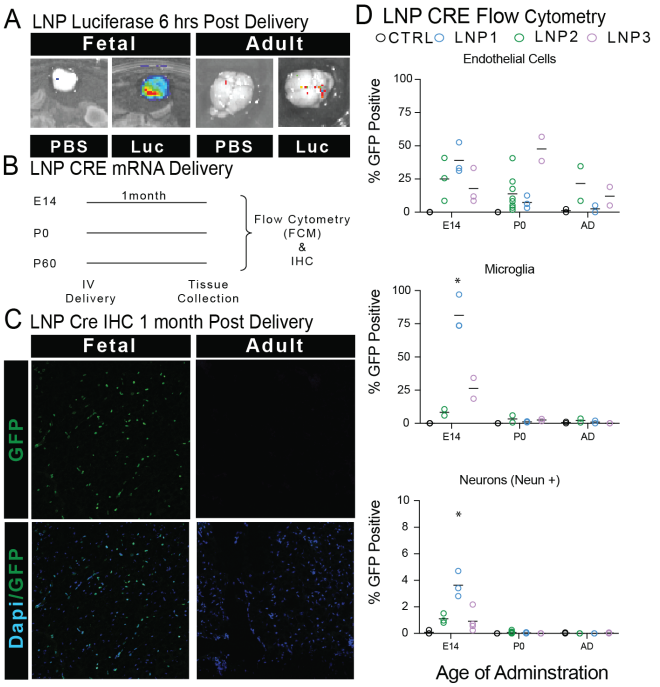


Figure 2. LNP containing mRNA is efficiently delivered to the brain following in utero but not adult IV administration. A) In utero delivery of LNP1-Luciferase mRNA targets the fetal brain. LNP1-Luc mRNA translation is undetected in the adult brain. B) Mice were injected IV with LNP1-3 at E14, P0 or P60 (adult) and tissue was collected for analysis 1 month following injection C) LNP1 efficiently delivers CRE mRNA to the fetal brain. LNP1-CRE mRNA translation is undetected in the adult brain. Sun1GFP is expressed in the nucleus following successful recombination with CRE. Nuclei stained with DAPI. Brain and liver were collected for IHC 1 month post LNP delivery D) In utero delivery of LNP-CRE mRNA targets fetal brain endothelial cells, Microglia and neurons in the brain parenchyma. Editing quantified by Flow Cytometry as percent GFP positive (edited). Two-way ANOVA compares CRE recombinase mRNA delivery in utero vs adult across 3 LNP formulations. P< 0.05. n = 2-9

1754. Engineered Virus Like Particles VLPs Transverse Immune Cells *In Vitro*

Priya Dhole[1], Natalie Correa[1], Isai Leguizamo[1], Michael Kuipa[1], Abubakarr Koroma[1], Matthew R. Gardner[1,2]

[1]Microbiology and Immunology, Emory University, Atlanta, GA,[2]Medicine, Emory University, Atlanta, GA

During the replication of viruses, apart from virus progeny, virus-like particles (VLP) are also generated randomly by selfassembly of viral proteins during infection or by in vitro protein expression. These particles are devoid of proper genetic material, which makes them replication and infection-deficient. Owing to their simultaneous property of mimicking the parent virus and inability to cause disease in the host, VLPs are a viable choice for wide application in the field of gene therapy. Many documented scientific annals have accumulated the usage of VLPs in delivering an array of drugs, peptides, and nucleic acid to the target cell. Advances in the retrovirus research field have helped researchers to use natural HIV-derived VLPs for carrying cargo inside the cells. A unique feature of retroviruses is that they contain the Gag polyprotein, which is capable of self-assembly into VLPs without requiring any other viral protein. Here we describe the production and application of targeted VLPs for various receptors on B and T lymphocyte-derived cell lines. We pseudo-typed an array of different VLPs by co-transfecting human embryonic kidney 293 (HEK-293) with different plasmids combinations, encoding monoclonal antibodies against CCR5, CD4, CD3, CD19, and CD20 receptors on T-lymphocytes and B lymphocytes. All the generated VLPs had luciferase tagged NL4-3delta(env), a full-length proviral DNA construct without a functional env gene, and vesicular-stomatitis virus G (VSV-G) as a fusogenic component. VSV-G is modified to knock out binding to natural receptors, thus only fusing in cells where the VLP enters through specific receptor targeting. VLPs generated in this manner had greater efficiency at entering cells than VLPs produced using a modified Sindbis virus glycoprotein that was modified to bind to monoclonal antibodies targeting similar receptors. Initial results showed targeted VLPs were capable of entering immortalized T and B cell lines like Sup-T1, Jurkat, and Raji cells. We are currently evaluating targeted VLPs for their efficiency in entire primary T and B cells. Overall, targeted VLPs can serve as a useful gene therapy tool, especially for delivering cargo to specific cells without entering off-target cell types.

1755. Pharmacokinetics and Distribution of Lipid Nanoparticle Formulations of mRNA in Gonadal Tissues in Rat and Monkey

Sherry A. Weppler, Merete L. Eisenhardt, Thomas C. Chamberlain, Ying K. Tam, Sean C. Semple

Acuitas Therapeutics, Inc., Vancouver, BC, Canada

With the increasing use of lipid nanoparticles (LNP) for gene-editing and other RNA-based therapeutics, a detailed understanding of LNP distribution is essential to support the safety assessment of these therapeutics and to identify cell types for further investigation of on- and off-target editing. The distribution and persistence of LNP components in gonadal tissue is of particular interest, given the potential for chemical and/or biological effects of LNP components on reproductive status. To address these questions, we have evaluated the pharmacokinetics and distribution of different mRNA-LNP formulations in cynomolgus monkeys and Wistar Han rats, with emphasis on distribution into testes and ovaries. mRNA localization and persistence within tissues and cells was evaluated by *in situ* hybridization, and ionizable lipid and PEG-lipid levels in plasma and tissue were quantified by LC-MS/MS or MALDI-MS. Distinct plasma pharmacokinetic (PK) profiles and large differences in plasma exposure of the ionizable lipid were observed in monkeys for the three different LNP compositions evaluated; however, the PK profiles of the PEG-lipid component of each LNP formulation were nearly identical, consistent with the intended exchange of the PEG-lipid out of the LNP in vivo. Ionizable lipids, PEG-lipid and mRNA were detected mainly in liver, spleen and adrenal glands for all three LNP, and much lower levels of ionizable lipids were detected in ovaries and testes, which further decreased to near the limit of detection by 14 days after dosing. Low levels of mRNA payload were also detected in ovaries and testes, with most of the signal present for only 24 hours post-dose and declining

rapidly thereafter, consistent with mRNA degradation. In ovaries, mRNA was observed mainly in the stroma and in thecal and granulosa cells surrounding the follicles, while in testes, mRNA was detected mainly in connective tissue cells between seminiferous tubules. Importantly, no mRNA was detected within oocytes or spermatogonia at any timepoints, for any of the formulations. These results illustrate that, despite a range of LNP plasma exposures examined, mRNA-LNP components were observed only at low levels in gonadal tissues, and there was no evidence of uptake into germline cells of rats or monkeys.

Friday Posters: Cancer - Oncolytic Viruses

1756. Dual Tumor-Responsive Liposome System Enables Effective Systemic Treatment of Breast Cancer Using Oncolytic Adenovirus

A-Rum Yoon[1], Yuebin Zhao[1], Chae-Ok Yun[2]

[1]Hanyang University, Seongdong-Gu, Korea, Republic of,[2]Hanyang University, Seoul, Korea, Republic of

To improve the efficiency of tumor-targeting systemic delivery of adenovirus (Ad), a dual tumor-targeting liposomal system composed of lipid decorated with epidermal growth factor receptor (EGFR) targeting peptide (GE11) (GE11-DOPE) and a pH-responsive sulfamethazine-based polymer (PUSSM)-conjugated phospholipid (DOPE-HZ-PUSSM) was complexed with Ad (Ad/LP-GE-PS). The replication-incompetent Ad (dAd) complexed with a dual tumor-responsive liposome system (dAd/LP-GE-PS) showed higher transduction efficiency than naked Ad in EGFR-positive cancer cells. Similarly, oncolytic Ad co-expressing short hairpin RNA (shRNA) against Wnt5 (shWnt5) and decorin complexed with dual tumor-responsive liposome system (oAd/LP-GE-PS) induced potent pH-responsive and EGFR-specific breast cancer cell killing. Importantly, systemically administered oAd/LP-GE-PS was shown to accumulate more effectively in EGFR-overexpressing breast xenograft tumor tissue compared to naked oAd, oAd encapsulated in control liposomes lacking tumor-targeting moieties (oAd/LP) or oAd encapsulated in EGFR-targeted liposomes (oAd/LP-GE). Liposome-based systemic delivery of oAd resulted in significantly attenuated hepatic sequestration of the virus compared to naked oAd, ultimately attenuating the induction of hepatotoxicity and lethality associated with systemically administered Ad. Taken together, this systemic delivery of oAd using dual tumor-targeted liposomes may be an effective and safe strategy for the treatment of EGFR-overexpressing breast cancer.

1757. Multifaceted Immunological Response by Combination of Dendritic Cell Therapy with Oncolytic Adenovirus-Based Nanohybrid System

In-Wook Kim[1,2,3], A-Rum Yoon[1,2,3], JinWook Hong[4], Dayananda Kasala[1], Chae-Ok Yun[1,2,3,4]

[1]Department of Bioengineering, Hanyang University, Seoul, Korea, Republic of,[2]Hanyang Institute of Bioscience and Biotechnology (HY-IBB), Hanyang University, Seoul, Korea, Republic of,[3]Institute of Nano Science and Technology (INST), Hanyang University, Seoul, Korea, Republic of,[4]GeneMedicine CO., Ltd, Seoul, Korea, Republic of

To reprogram the immunosuppressive tumor milieu and augment DC-based anticancer immune response, here we investigated polymeric nanocomplex incorporating an oncolytic adenovirus (oAd) expressing antitumor cytokines and arginine-grafted bioreducible polymer with PEGylated paclitaxel (APP) in combination with adoptively transferred DC. The nanohybrid system (oAd/APP) in combination with DC (oAd/APP+DC) induced higher expression level of therapeutic gene over oAd/APP or DC monotherapy in tumor tissues, resulting in higher level T cell and activated DC infiltration. oAd/APP+DC was also shown to effectively promote the migration of DC to secondary lymphoid organs, such as tumor draining lymph nodes and spleen, in comparison to either monotherapy. Effective migration of DCs to the secondary lymphoid organs following oAd/APP+DC treatment led to more robust tumor-specific T cell immune response over the respective monotherapy. oAd/APP+DC-treated mice possessed lowest percentage of immunosuppressive regulatory T cell in tumor infiltrating lymphocyte and splenocyte populations compared to other treatment groups. Taken together, combination of gene therapy, cell therapy and drug delivery nano system effectively overcome immunosuppressive tumor microenvironment, leading to potent and synergistic antitumor immune response.

1758. Tumor Microenvironment-Responsive Nanohybrid for the Controlled Release of Therapeutics

Dayananda Kasala[1], A-Rum Yoon[1,2,3], Jinwoo Hong[4], Chae-Ok Yun[1,2,3,4]

[1]Department of Bioengineering, Hanyang University, Seoul, Korea, Republic of,[2]Institute of Nano Science and Technology (INST), Hanyang University, Seoul, Korea, Republic of,[3]Hanyang Institute of Bioscience and Biotechnology (HY-IBB), Hanyang University, Seoul, Korea, Republic of,[4]Gene-Medicine, Seoul, Korea, Republic of

To improve the synergistic efficacy induced by oncolytic adenovirus (oAd) and paclitaxel (PTX), a reactive oxygen species (ROS)-responsive supramolecular hydrogel (mPEG-*b*-PCL-DPMP/α-cyclodextrin (DPMP-hydrogel)) has been newly designed. Here, we demonstrated that ROS-responsive DPMP hydrogel system can effectively prolong the biological activity of both oAd and PTX in ROS-rich tumor microenvironment to induce potent antitumor effect. DPMP-hydrogel loaded with oAd and PTX was shown to degrade over the span of one week period *in vivo*. Both oAd and PTX released from DPMP hydrogel were shown to retain their biological activity, thus exerting superior antitumor efficacy up to 19 days after single administration

in subcutaneous H460 xenograft tumor model compared with oAd and PTX combination therapy in absence of hydrogel delivery system or respective monotherapy, simultaneously enhancing intratumoral delivery efficiency of oAd and PTX in a ROS-rich tumor microenvironment.

1759. Efficacy of Third-Generation Oncolytic Herpes Virus G47Δ Combined with Immune Checkpoint Inhibitors in Renal Cell Carcinoma Models

Kenichi Sasaki[1], Yuta Takeshima[1], Miwako Iwai[1], Hiroshi Fukuhara[2], Haruki Kume[3], Tomoki Todo[1]

[1]The Institute of Medical Science, The University of Tokyo, Tokyo, Japan,[2]Dept. of Urology, Kyorin Univ. School of Med., Tokyo, Japan,[3]Dept. of Urology, Faculty of Med., The University of Tokyo, Tokyo, Japan

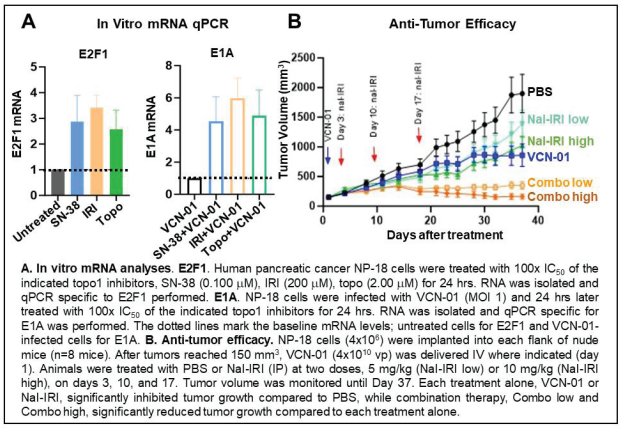
G47Δ (teserpaturev) is a third-generation oncolytic herpes simplex virus type 1 (HSV-1) recently approved in Japan as a new drug for malignant glioma. As a result of its oncolytic activity, G47Δ elicits systemic antitumor immunity, therefore works collaboratively with other cancer immunotherapies. Renal cell carcinoma (RCC) is known to be relatively immunogenic, and immune checkpoint inhibitors (ICIs) are frequently utilized for metastatic RCC. In this study, we aim to investigate the efficacy of G47Δ combined with ICIs and to apply the evidence towards clinical settings. In immunocompetent mice harboring subcutaneous RCC tumors, intratumoral injections of G47Δ and systemic administrations of ICIs were both significantly more efficacious than controls, and the combination therapy was more efficacious than either monotherapy. Analysis of intratumor immune response showed a marked decrease in the proportion of regulatory T cells (Tregs) among CD4-positive cells in the combination treatment group, resulting in a significant increase in the CD8+/Tregs ratio. These results suggest that the combination therapy exhibits high efficacy for RCC via effective remodeling of the immunosuppressive tumor microenvironment.

1760. Enhanced Anti-Tumor Efficacy of Combination Therapy with the Oncolytic Adenovirus, VCN-01, and Liposomal Irinotecan in a Human Pancreatic Mouse Xenograft

Maria Maliandi[1], Paz Moreno[1], Ramon Alemany[1,2], Manel Cascallo[1], Sheila Connelly[3], Ana Mato-Berciano[1]

[1]Theriva Biologics, S.L., Barcelona, Spain,[2]Immunotherapy and Virotherapy, ProCURE Program, Catalan Institute of Oncology (ICO), Barcelona, Spain,[3]Theriva Biologics, Inc., Rockville, MD

VCN-01 is a clinical-stage oncolytic adenovirus engineered to replicate selectively in cancer cells with an aberrant RB1-E2F pathway. The VCN-01 E1A promoter is modified by inclusion of eight E2F1 binding sites and a 24-bp deletion that promotes viral transcription in cells with elevated free E2F1 and renders the virus replication-deficient in RB1-functional, normal cells. Here, human pancreatic cancer cell lines were exposed to topoisomerase I (topo1) inhibiting chemotherapeutics, irinotecan (IRI), its active metabolite, SN-38, and topotecan (topo; see Figure A). Notably, in NP-18 cells, E2F1 mRNA was increased ~3-fold compared to untreated cells, and E1A mRNA was boosted ~5-fold in cells treated with VCN-01 + topo1 inhibitors compared to VCN-01 alone. These data suggest topo1 inhibitor-mediated elevations in E2F1 increase VCN-01 transcription. Based on these promising in vitro results, potential synergy of VCN-01 plus the liposomal formulation of IRI (nal-IRI) was tested in animals bearing subcutaneous human pancreatic tumors . After tumors were established, indicated animals were treated with a single IV dose of VCN-01 ($4x10^{10}$ vp) or PBS (day 1). Designated animals in each group were then treated with either PBS or nal-IRI (IP) on days 3, 10 and 17. In animals with NP-18 tumors, treatment with VCN-01 or Nal-IRI alone at both the 10 mg/kg and 5 mg/kg doses resulted in significant tumor growth inhibition compared to PBS (see Figure B). Importantly, combination therapy with VCN-01 + nal-IRI at either dose displayed significantly reduced tumor growth compared to each treatment alone. qPCR analyses performed on tumors collected at end of study confirmed the presence of viral genomes and E1A mRNA suggesting intratumoral viral replication and indicating ongoing transcriptional activity of VCN-01. Preliminary analyses suggest that combination therapy with VCN-01 + nal-IRI has a tolerable toxicity profile. These data are consistent with the hypothesis that topo1 inhibitors increase cellular E2F1 protein expression resulting in upregulation of the modified VCN-01 E1A promoter thereby enhancing viral gene expression and improving anti-tumor efficacy. The combination of VCN-01 + topo1 inhibitors, such as Nal-IRI, may demonstrate improved efficacy in the treatment of human pancreatic cancer.



A. In vitro mRNA analyses. E2F1. Human pancreatic cancer NP-18 cells were treated with 100x IC_{50} of the indicated topo1 inhibitors, SN-38 (0.100 μM), IRI (200 μM), topo (2.00 μM) for 24 hrs. RNA was isolated and qPCR specific to E2F1 performed. **E1A**. NP-18 cells were infected with VCN-01 (MOI 1) and 24 hrs later treated with 100x IC_{50} of the indicated topo1 inhibitors for 24 hrs. RNA was isolated and qPCR specific for E1A was performed. The dotted lines mark the baseline mRNA levels; untreated cells for E2F1 and VCN-01-infected cells for E1A. **B. Anti-tumor efficacy.** NP-18 cells ($4x10^6$) were implanted into each flank of nude mice (n=8 mice). After tumors reached 150 mm^3, VCN-01 ($4x10^{10}$ vp) was delivered IV where indicated (day 1). Animals were treated with PBS or Nal-IRI (IP) at two doses, 5 mg/kg (Nal-IRI low) or 10 mg/kg (Nal-IRI high), on days 3, 10, and 17. Tumor volume was monitored until Day 37. Each treatment alone, VCN-01 or Nal-IRI, significantly inhibited tumor growth compared to PBS, while combination therapy, Combo low and Combo high, significantly reduced tumor growth compared to each treatment alone.

1761. Investigating the Potential of Oncolytic Adenovirus-Mediated Immunotherapy in the Neoadjuvant and Metastatic Settings for Pancreatic Cancer

Kazuho Inoko, Shuhei Shinoda, Kari Jacobsen, Masato Yamamoto, Julia Davydova

Department of Surgery, University of Minnesota, Minneapolis, MN

Background: Pancreatic ductal adenocarcinoma (PDAC) remains one of the most common and lethal cancers. Even in patients with localized and resectable PDAC, upfront surgery often fails to eradicate this disease mainly due to micro-metastases. Accordingly, neoadjuvant therapy (non-curative treatments before surgery) is becoming the de facto standard of care for this population with the intent of reducing postoperative recurrence. On the other hand,

patients with metastatic PDAC are ineligible for surgery, and instead receive systemic chemotherapy to prolong survival. However, these efforts have thus far not led to major improvements in survival. Furthermore, despite breakthroughs in cancer immunotherapy, its efficacy is limited in PDAC. In the current study, we investigated the potential of a replication competent oncolytic adenovirus (OAd) as an immunotherapeutic strategy for PDAC in both neoadjuvant and metastatic settings. **Methods and Results:** We used the immunocompetent Syrian hamster model, which is permissive to OAd replication and provides an advantage in simulating OAds behavior in humans. We employed a type 5 adenovirus with the RGD-fiber and no therapeutic transgenes (Ad5-RGD) to better investigate the intrinsic ability of OAd to induce immunogenic cell death (ICD). We first evaluated the cytotoxicity and immunostimulatory responses induced by Ad5-RGD in a hamster PDAC cell line, PGHAM-1, which harbors a KRAS mutation (G12D), the most common oncogene in PDAC. Ad5-RGD showed efficient replication and cytotoxicity in hamster PDAC cells, while increasing the level of extracellular ATP, suggesting the potential to induce ICD. We then evaluated *in vivo* immunomodulatory activities of Ad5-RGD in the neoadjuvant setting. Hamsters were subcutaneously inoculated with PGHAM-1 cells. After tumor establishment, the tumors were simply resected in the upfront surgery (control) group. In the neoadjuvant OAd group, the tumors were treated with intratumoral injection of Ad5-RGD ($2.5x10^9$ pfu/injection) every two days for a total of three injections and were surgically resected three days after the final injection. After 31 days from the initial treatments, the hamsters in both groups were rechallenged with the same PDAC cells, mimicking micro-metastasis after surgery. Compared to the upfront surgery group, the neoadjuvant OAd treatment showed significant tumor growth inhibition in the rechallenged tumors, resulting in complete tumor regression in 33% of animals and significantly improved survival. We further evaluated *in vivo* systemic efficacy of Ad5-RGD. Hamsters bearing subcutaneous PGHAM-1 tumors on both flanks were treated intratumorally with PBS or Ad5-RGD as above in one of the two tumors. Compared to the PBS group, localized OAd treatment showed significant tumor growth inhibition not only in the injected tumors, but also in the non-injected distant sites, resulting in complete tumor regression in 60% of animals and significantly improved survival. Notably, all the surviving animals from both settings completely controlled the further tumor challenges over > 300 days. The cytotoxicity assay with splenocytes collected from these long survivors showed significantly higher killing of PGHAM-1 target cells compared with splenocytes from naïve hamsters, indicating the establishment of a long-lasting antitumor immune memory response. **Conclusion:**This is the first study to demonstrate the potential of OAd to induce systemic, long-lasting antitumor immune responses in replication-permissive, immunocompetent PDAC models in both neoadjuvant and metastatic settings. These findings provide far-reaching implications for the use of OAd for patients with PDAC at all stages.

1762. Inhibition of c-MYC in Hepatocellular Carcinoma via AAV8 Mediated Gene Transfer

Isaiah L. Reeves[1,2], Stephen M. Winston[1,2], Yunyu Spence[1], Dongli Hu[1], Chris Morton[1], Andrew M. Davidoff[1,2]

[1]Department of Surgery, St. Jude Children's Research Hospital, Memphis, TN,[2]Graduate School of Biomedical Sciences, St. Jude Children's Research Hospital, Memphis, TN

Inhibiting the key transcription factor MYC has long been a goal of cancer therapy due to the central role of MYC in human cancers, although, it has yet to be accomplished clinically. Hepatocellular carcinoma (HCC) is the most common liver malignancy in adults and the second most common liver tumor in children and is often associated with MYC deregulation. The mini protein Omomyc has been shown to induce tumor regression in a variety of cancer types by preventing the heterodimerization of c-MYC with its obligate binding partner MAX, thus preventing its transcriptional activation. but questions about in vivo peptide toxicity and efficacy remain. Due to the natural strong liver tropism of AAV8, we hypothesized that targeted delivery of Omomyc using the AAV platform will be much more efficacious and limit off target toxicity. Expression of Omomyc in HCC cell lines *in vitro* by transient transfection and doxycycline induced expression induced an 8-20 fold inhibition of proliferation depending on cell type, including G2 cell cycle arrest (about 50%) in the high c-MYC expressing cell line Hep3B. We have also demonstrated a 2-fold induction of autophagy, measured via mCherry-GFP-LC3 levels in HCC cell lines with a direct correlation to the amount of c-MYC expressed in these cell lines. Autophagosome formation was also observed via electron microscopy and immunofluorescent microscopy. When combined with conventional therapeutics like the multi-kinase inhibitor sorafenib, Omomyc induces autophagy another 20%. Additionally, Omomyc results in significant levels of cell death (20-60%), in direct correlation with the level of c-MYC protein expression. Our preliminary data suggests that this could be due to induction of autophagic cell death, with high levels of a necrotic phenotype observed via Annexin-V staining. Treatment of HCC with recombinant AAV8 with vector genomes encoding Omomyc results in a profound cytostatic effect (2-7 fold lower proliferation rate), and very strong cytotoxicity when combined with sorafenib, leading to high levels of cell death in Hep3B cells. We have also demonstrated a potential synergistic effect of combining autophagy inducing compounds like sorafenib and mTOR inhibitors being able to increase transduction efficiency by approximately 50-60% in our HCC cells lines as well as enhanced cytotoxicity. We have also demonstrated the efficacy of recombinant AAV delivered Omomyc *in vivo* by inhibiting tumor growth and prolonging mouse survival when combined with sorafenib. Taken together, our data suggest that our novel AAV8 delivery of Omomyc has the ability to inhibit cellular proliferation, induce autophagy, and induce cell death in HCC which may prove to be a novel therapeutic approach for the treatment of HCC, especially when combined with conventional therapeutics.

1763. Treatment Effect of CD133-Targeted Oncolytic Adenovirus for Colorectal Peritoneal Metastases in a Preclinical Model

Mizuho Sato-Dahlman, Praveensingh B. Hajeri, Kari Jacobsen, Brandon Hitchcock, Masato Yamamoto
University of Minnesota, Minneapolis, MN

Introduction: Colorectal cancer (CRC) is the third most common cause of cancer-related death in the US. While the death rate has been gradually decreasing for the last 20 years owing to advances in screening and treatments, its local recurrence and distant metastasis rates are still disappointingly high. Peritoneal metastases (PM) are a common type of metastatic spread in CRC, and occurs in approximately 10 to 15% of patients with CRC. From a patient's perspective, the presence of PM is not considered curable unlike resectable liver or lung metastases. Despite significant progress in the development of new chemotherapies, the survival of patients with PM remains poor. Therefore, development of novel therapeutic approach for PM is critically important to improve the prognosis of patients with advanced CRC. A growing body of evidence suggests that expression of CD133 is reported to highly correlate with metastasis, recurrence, and treatment resistance in CRC patients. We therefore hypothesize that development of novel approach that eliminating CD133-positive cells leads to suppression of metastasis and recurrence, and provide more effective treatment of PM in CRC patients. **Methods:** We used CD133-targeted oncolytic adenovirus to eliminate CD133-positive cells. We generated CD133-targeted OAd (CD133-OAd) by using our adenovirus-based library screening system. We evaluated the target specificity of the virus *in vitro* and *in vivo* analysis. For *in vitro* analysis, we used binding assay and cell killing assay with CD133-positive or CD133-negative CRC cell lines. As a proof of concept, the *in vivo* therapeutic potential of the CD33-OAd was investigated in a mouse peritoneally metastatic colon cancer model with luciferase-labeled CD133-positive human CRC cells (HCT116-Luc). HCT116-Luc cells were inoculated intraperitoneally into mice, and treatment started 5 days post cell inoculation. On day 6, mice with tumors were divided into 4 groups: PBS intraperitoneal (i.p.) injection (200ul, every week for 3 weeks), wild-type Adenovirus type 5 (Ad5-WT), CD133-OAd, and integrin-targeted OAd (RGD-OAd) i.p. injection (1×10^{10}vp/200ul, every week for 3 weeks). All mice were monitored by IVIS imaging once a week to confirm and track tumor growth. **Results:** Binding and cell killing assay confirmed that the CD133-OAd selectively infected CD133-positive cells and lysed them efficiently. *In vivo* anti-tumor assay demonstrated that CD133-OAd significantly suppressed the peritoneal metastasis of HCT116-Luc cells on day 28 after initial treatment compared to administration of PBS or RGD-OAd. The survival of mice with PM of HCT116-Luc cells was significantly increased by treatment with CD133-OAd compares with PBS, Ad5-WT, or RGD-OAd treatment. Interestingly, Adenovirus type 5 (Ad5-WT) showed anti-tumor activity, and its level was comparable with CD133-OAd. However, Ad5-WT injected mice started to show severe toxicity and die from around 21 days after initial viral injection. At necropsy of Ad5-WT injected mice, all of the mice showed abdominal adhesion. H&E staining of liver indicated that leukocyte infiltration into liver parenchyma and especially around blood vessels. Further analysis of toxicity of Ad5-WT is ongoing and will be presented. **Conclusions:** Intraperitoneal administration of CD133-OAd has therapeutic potential to suppress the peritoneal metastasis in mouse model without significant toxicity, resulting in prolonged survival.

1764. Harnessing Viral-Mediated NIS Expression to Enable Imaging of Colorectal Cancer Tumors

Stacey Wilber, Sacha Robert, Margarita V. Romanenko, Kazuho Inoko, Julia Davydova, Christopher J. LaRocca
University of Minnesota, Minneapolis, MN

Introduction: Patients with advanced and metastatic colorectal cancers often have limited therapeutic options, especially if they do not respond to conventional treatments such as chemotherapy or surgery. In these scenarios, new therapeutic approaches are needed, and oncolytic viruses represent novel agents that have the potential to enable innovative treatment regimens. Methods: Our group has generated an oncolytic adenovirus which expresses the sodium iodide symporter (OAd-NIS) as a function of viral replication. The vector was thoroughly tested *in vitro* across multiple human colorectal cancer cell lines (LoVo, SK-CO-1, WiDr) to evaluate transgene expression and cytocidal effects. Additionally, to evaluate the ability of the OAd-NIS virus to facilitate imaging of human colorectal tumors, small animal PET/CT was utilized to visualize radiotracer uptake. Results: The OAd-NIS virus is based upon the ΔE3 NIS backbone structure (that has been previously described) and also employs the Ad 5/3 chimeric fiber to optimize binding and transduction in human colorectal cancer cells. Our prior work in pancreatic cancer demonstrated that viral constructs with deletions of the Adenovirus Death Protein (ADP) provide for increased *in vitro* NIS expression. In all three colorectal cancer cell lines, we compared vectors both with and without ADP and anti-human NIS antibody staining demonstrated much stronger NIS expression in ADP-deleted vectors. LoVo cells were utilized to establish subcutaneous tumors in the flanks of athymic nude mice, which were subsequently inoculated with the OAd-NIS virus ($5x10^{10}$ pfu / tumor). Radiotracer (^{124}I, 0.25 mCi) was administered via intraperitoneal injection and PET/CT images were acquired with the Sofie G8 system. Evaluation of the cross-sectional images demonstrated avidity within the flank tumors, thereby confirming the ability of the OAd-NIS vector to enable transgene expression in human colorectal cancer tumors. After harvesting the tumors, viral copy number analysis and NIS staining confirmed strong *in vivo* viral replication and NIS expression within the tumors. Conclusion: The OAd-NIS virus demonstrates transgene expression across multiple colorectal cancer cell lines and in murine models. It highlights the potential of viral-mediated NIS expression to enable radionuclide-based imaging for colorectal cancer. Future studies will examine the potential therapeutic effects of combining the OAd-NIS virus with radioactive iodine (^{131}I) in colorectal cancer models. This preliminary data highlights the potential of OAd-NIS to serve as a theranostic (*thera*peutic and dia*gnostic*) agent for colorectal cancer and supports the pursuit of additional testing toward clinical translation.

1765. High Potential of a Novel oHSV-1 Vector Encoding Immune Factors for Cancer Therapy

Junmei Zhang[1], Wei Ma[1], Zhao Gao[2], Gang Zou[2], Jim Zhen Wu[2], Qiong Zhou[1]

[1]WuXi AppTec, Shanghai, China,[2]Ark Biopharmaceutical, Shanghai, China

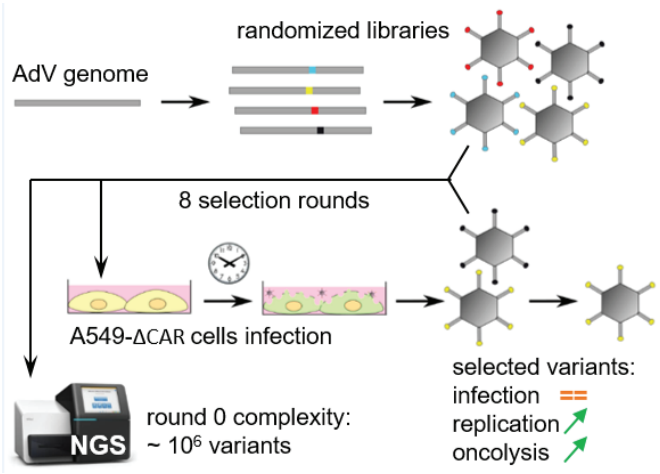
Oncolytic immunotherapy, a virus-mediated therapeutic modality, is emerging as a novel and promising anticancer therapy, especially for patients with cancers resistance to other therapeutics. Oncolytic viruses can directly lyse cancer cells and indirectly elicit antitumor immunity in the tumor microenviroment. Recently, oncolytic herpes simplex virus (oHSV) has been extensively explored and widely used in clinical. oHSV has exhibited great potential in anticancer applications with its advantages of natural tumor affinity and high capacity for insertion of a therapeutic gene(s). However, there are significant technical challenges in the genetic manipulation of the large virus genome, such as the complexity of the viral genome, potential impacts on viral fitness and replication, and unpredictable effects on the host immune responses. To facilitate the genetic manipulation of the oHSV vector, we have established bacterial artificial chromosome (BAC) and CRISPR systems for genetic modifications of oHSV. The BAC and CRISPR methods can complement each other and may be applied to different genetic modifications. The two-method approach can be used for accelerating the development of novel oHSV-1 and other large genome virus vectors. Using the BAC and CRISPR systems, we generated a novel oHSV-1 vector with the deletion of viral neurovirulence factor ICP34.5 for improving the safety while maintaining the robust virus replication capacity. We also introduced an immunostimulatory factor and an immune checkpoint antibody into the oHSV-1 vector at a novel insertion site for enhancing the antitumor immunity. The recombinant oHSV-1 had the same level of replication efficiency as the parental virus and exhibited greater than 90% killing effect on SK-MEL-28, HepG2 and LN-Cap cell lines at 96 h post infection in cell-based assays. The recombinant oHSV-1 was further evaluated in mouse tumor models. In the mouse model of human melanoma SK-MEL-28 cell the recombinant oHSV-1 displayed the inhibition of tumor growth in a dose dependent manner, and almost completely eliminated tumor at a dose of 1×10^7 pfu/mouse. The available results indicate that the oHSV-1 vector has high potential in exploring genetically modified oHSV-1 products for the treatment of cancer. Currently we are conducting additional studies for further improvement and characterization of the novel oHSV-1 vector.

1766. Development of Enhanced Oncolytic Vectors by Adenovirus Directed EVOlution (ADEVO)

Erwan Sallard[1], Julian Fischer[2], Nissai Beaude[1], Arsalène Affes[1], Eric Ehrke-Schulz[1], Wenli Zhang[1], Katrin Schröer[1], Adrian Westhaus[3], Marti Cabanes-Creus[4], Leszek Lisowski[4], Zsolt Ruszics[2], Anja Ehrhardt[1]

[1]Department of Human Medicine, Center for Biomedical Education and Research (ZBAF), Witten/Herdecke University, Witten, Germany,[2]Faculty of Medicine, University of Freiburg, Freiburg, Germany,[3]GENETHON, Evry, France,[4]Children's Medical Research Institute, Westmead, Australia

Directed evolution is a vector development method consisting in the generation of random vector variants libraries, followed by the selection of improved variants fitting selection criteria. Directed evolution yielded few successes in Adenovirus vector (AdV) development until now, mainly due to difficulties in constructing large random libraries. Meanwhile, AdVs clinical applications are still hampered by the predetermined tropism of natural adenoviruses. To overcome this challenge, we hypothesized that validated vector retargeting technologies like peptide insertions can be incorporated into the AdV toolbox. Here we designed novel Adenovirus Directed EVOlution (ADEVO) protocols for AdV retargeting, based on random peptides insertion in the exposed HI loop of the fiber knob domain, and used them to increase the oncolytic efficiency of several AdVs. As a proof-of-concept, AdV genome libraries were constructed by recombination of random oligonucleotides in the fiber gene, following three distinct protocols. Vector libraries were generated following genome libraries transfection in HEK293 cells, and selected by serial passaging on A549-ΔCAR cells that are non-permissive for natural human adenovirus type 5 (Ad5), with the goal of identifying variants able to infect and lyse these cells efficiently. Library complexities were estimated by NGS variant calling and capture-recapture population size modeling. Crystal violet was used to stain live cells in oncolysis assays. All three ADEVO protocols enabled the construction of high complexity libraries, with up to 8.7e5, 9.6e5 and 1.7e5 unique variants in each initial vector libraries, which is an approximate 100-fold improvement compared to previously published AdV libraries. After 8 rounds of selection by serial passaging of the replication-competent vectors, the non-functional variants containing stop codons had been eliminated, and the most highly enriched variants were recloned and purified. Interestingly, they did not display enhanced or more specific transduction but intensified and accelerated replication and cell lysis in all tested tumor cell lines. This is consistent with the selection criteria given, that was the efficient completion of full replication cycles. The mechanisms of this so far unsuspected involvement of the fiber protein in stages of the AdV replication cycle other than receptor binding are currently being investigated. Finally, the selected inserts were applied to the fiber proteins of Ad34 and Ad35-based replication-selective oncolytic vectors to increase their therapeutic index. ADEVO, our novel directed evolution workflow for Adenoviruses, facilitates the generation of highly diverse variant libraries and the selection of improved vectors with user-friendly protocols. Besides the development of enhanced oncolytic AdVs, we expect that this technology can be applied to other vector features and contribute to a wide range of clinical applications.



1768. Genetically Engineered Oncolytic Measles Virus with Pro-Apoptotic Gene of Human Origin: Its Mechanistic Basis for Selective Killing of Triple Negative Breast Cancer Cells

Alok Kumar[1], Subbarao Naidu[2], Maitreyi Syamala Rajala[1]

[1]School of Biotechnology, Jawaharlal Nehru University, New Delhi, India,[2]School of Computational and Integrative Sciences, Jawaharlal Nehru University, New Delhi, India

Breast cancer is the second most leading cause of cancer related deaths worldwide including India. Types of breast cancer include ductal carcinoma *in situ*, invasive ductal carcinoma, inflammatory breast cancer, and metastatic breast cancer. Triple negative breast cancer (TNBC), a subtype comprises of 15-20% of all breast cancers is an aggressive one with poor prognosis. Currently available treatments such as tamoxifen and trastuzumab based therapies are targeted towards estrogen receptor positive (ER+) and human epidermal growth factor receptor 2 (Her2/neu) positive tumours. TNBC being negative for ER, PR (progesterone receptor) and Her2/neu is insensitive to endocrine therapy and targeted molecular therapies leading to recurrence and metastasis. Thus, new therapies are being developed to target TNBC; one such therapy is the use of engineered viruses which is rapidly evolving and holds great promise as a potential treatment for various cancers including breast cancer. Our earlier studies reported the development of a genetically engineered measles virus armed with BNiP3, a pro-apoptotic gene of human origin and its selective anti-proliferative effects in TNBC cells. In this study, we sought to understand the mechanistic insights of oncolytic measles virus induced selective killing of TNBC cells using bioinformatic tools and experimental studies. Upon comparison of TNBC *vs*. non-TNBC cells and TNBC *vs*. non-TNBC tumor tissues using the available data bioinformatically, five hub genes were identified to be involved in tumor development and progression in both the data sets. Of which, β-catenine was observed to be the most significant one. As β-catenine is known to be over expressed in invasive tumors and its correlation with poor prognosis, the regulatory role of oncolytic measles virus on β-catenine pathway was investigated. The results had shown reduced expression of β-catenine in TNBC cells compared to non-TNBC cells following oncolytic virus infection at transcript and protein level by real time PCR and western blot analysis respectively. Further experiments had demonstrated the significant inhibition of its downstream targets Cyclin D1, matrix metalloprotease 7, c-JUN, CD44 and Ki67 which are involved in cell proliferation and invasion. Second reason for selectively was observed to be an efficient internalization of virus into TNBC than non-TNBC cells as the expression of CD46 receptor being used by the engineered virus for internalization is high in TNBC cells. This led to the increased expression of BNiP3 protein as well in TNBC cell. Our bioinformatic studies inclduing protein-protein docking had shown the strong interaction between BNiP3 and anti-apoptotic protein BCL2 speculating that this interaction may release BCL2 bound pro-apoptotic protein Bax to induce apoptosis. Third possible reason for the selectively was observed to be the interaction between one of the virus proteins, 'V' protein with Stat1/2, a transcriptional factor required for transactivation of interferon stimulatory genes. As the virus 'V' protein expression would be more in TNBC cells following infection, its interaction with Stat1/2 may inhibit the expression of antiviral interferon stimulatory genes facilitating efficient replication of oncolytic measles virus subsequently induction of apoptosis in TNBC cells. Further studies are ongoing; based on our preliminary results, the above three mechanisms are likely to be regulated by oncolytic measles virus for selective killing of TNBC cells signifying its potential to be a novel therapeutic agent to selectively target TNBC.

1769. A Novel Virotherapy Vector Engineered for Tumor Selective Transgene Delivery Following Intravenous Administration: TROCEPT

Rahul C. Khanolkar[1], James A. Davies[1,2], Alexander T. Baker[1,3], Thomas A. Webb[1], Somayeh Shahreza[1], Hanni K. Uusi-Kerttula[2], Lucy M. Williams[1], Rejina Gurung[1], Emma Henderson[1], Samantha L. Moore[1], Johanne M. Pentier[1], Bent K. Jakobsen[1], Alan L. Parker[1,2], David K. Cole[1,2], Andrew B. Gerry[1]

[1]Accession Therapeutics, Oxford, United Kingdom,[2]Cardiff University, Cardiff, United Kingdom,[3]Division of Cancer and Genetics, School of Medicine, Cardiff University, Cardiff, United Kingdom

TROCEPT is a novel tumor selective, lytic, virotherapy vector engineered to remove all natural tissue tropisms and reach the tumor following intravenous (IV) delivery. This addresses the main limitation of other viral vectored therapies: sequestration in normal tissues and rapid clearance by the liver which reduces the amount of therapy which is delivered to the point of need. This results in the requirement for local delivery to the tumor. TROCEPT has been further engineered to specifically infect cancerous tissues positive for αvβ6 integrin, a molecule expressed on most carcinomas. The TROCEPT platform can be loaded with transgenes to enable *in-tumor* production of powerful therapeutic drugs. The lead program, TROCEPT-01 (ATTR-01), enables tumor-localized expression of high concentrations of an immune checkpoint inhibitor. Herein, we demonstrate that TROCEPT-01 is safe and tumor selective in pre-clinical models, *in vitro* and *in vivo,* and that αvβ6 integrin is a tumor

selective target in human tissues. Our studies show that TROCEPT-01 is selectively infectious for tissues which are αvβ6 integrin$^+$ and does not replicate outside of cancerous cells. *In vivo* models support a favorable, tumor specific, biodistribution of the TROCEPT vector. We go on to demonstrate delivery of TROCEPT-01 to the tumor following intravenous administration. Finally, we show that infection with TROCEPT-01 results in expression of functional immune checkpoint inhibitors. TROCEPT-01's tumor selective transgene delivery and *in-tumor* production of immune checkpoint inhibitors enables high concentration immunotherapy doses, specifically in the tumor. This will address undesirable off-target toxicities associated with checkpoint therapy, potentially increasing efficacy, and patient tolerance. Additionally, several pre-clinical studies have demonstrated that oncolytic viruses can induce anti-viral immunity against infected tumor cells, recruiting cytotoxic T cells and other pro-inflammatory cell types. Thus, TROCEPT enabled delivery of checkpoint inhibitors has the potential to generate a synergistic effect, first attracting and activating T cells, and then enabling tumor-localized production of checkpoint inhibitors to boost anti-tumor T cell responses and increase response rates. TROCEPT has the potential to deliver new and powerful therapeutic drugs for in-tumor treatment of cancer following intravenous deliver. TROCEPT-01 is currently undergoing IND/CTA-enabling studies and is expected to enter First In Human studies in late 2024 in multiple solid tumor indications.

1770. Preclinical Toxicology Assessment of Intracranial Administration of an Oncolytic Measles Virus Strain Armed with *H. pylori* Neutrophil-Activating Protein Prior to Phase I Trial in Glioblastoma Patients

Kim Viker[1], Susanna C. Concilio[1], Susan M. Clark[2], Steven Robinson[3], Roy Dyer[4], Naomi Gades[5], Jenny Pattengill[6], Ianko D. Iankov[2], Evanthia Galanis[3]

[1]Molecular Medicine, Mayo Clinic, Rochester, MN,[2]Mayo Clinic, Rochester, MN,[3]Oncology, Mayo Clinic, Rochester, MN,[4]Immunochemical Core Laboratory, Mayo Clinic, Rochester, MN,[5]Comparative Medicine, Mayo Clinic, Scottsdale, AZ,[6]Animal Histology Research Core, Mayo Clinic, Scottsdale, AZ

Introduction: Glioblastoma (GBM) is the most common malignant brain tumor in adults and has a dismal prognosis with a median survival of 14-18 months despite multimodality treatment. Engineered measles virus (MV) strains based on the attenuated MV Edmonston (MV-Edm) vaccine platform have demonstrated significant efficacy against GBM models. A recently completed first-in-human clinical trial using a carcinoembryonic antigen expressing MV strain (MV-CEA) in recurrent GBM has demonstrated safety and biologic activity. To further increase the immunomodulatory potential of oncolytic cell death, we engineered MV to express an immunostimulatory transgene, the secretory form of the neutrophil-activating protein (NAP) of *H. pylori*. MV-s-NAP resulted in curative anti-tumor effect in syngeneic orthotopic murine GBM models, which can be enhanced by the addition of immune checkpoint inhibitors. This study was designed to evaluate the safety of intracranial administration of MV-s-NAP in MV-replication susceptible transgenic IFNAR KO-CD46Ge mice in preparation for clinical translation.**Methods:** Forty-eight (24 males and 24 females) 5-to-6-week-old measles-immunized IFNAR KO-CD46Ge mice were used in this study. MV immunization status in this toxicology study mimicked the patient population in the proposed trial. Mice were stratified into 3 groups with 16 mice per cohort. Treatment with MV-s-NAP at "Low Dose" (1 x 10^4 $TCID_{50}$) or "High Dose" (1 x 10^5 $TCID_{50}$) or vehicle control on day 1 and repeated on day 5.The highest proposed total virus dose administered to the mice in this study was 2 x 10^5 $TCID_{50}$ MV-s-NAP (divided in two equal doses). By direct brain weight conversion, this would be approximately equivalent to 7 x $10^8$$TCID_{50}$ in an adult human, which exceeds the maximum proposed dose in the human trial. Body weight and clinical signs of toxicity were monitored daily. Hematology, plasma chemistry, plasma cytokines, gross pathology, and histopathology of major organs were analyzed on days 4, 8 (early timepoints), 28(intermediate timepoint), or 56 (late timepoint). Biodistribution of MV-s-NAP genomes was assessed by qRT-PCR. **Results:** Intracranial treatment with MV-s-NAP did not impact body weight in either males or females and none of the mice experienced toxicity or clinically adverse health effects. No difference between control and treated animals was observed in complete blood counts, plasma chemistry values (including liver and kidney function), or proinflammatory cytokines at any time point. MV genomes were only detected by qRT-PCR on day 4 in the brain tissue of two animals that received a single high dose injection: these findings were not associated with histopathologic changes and were deemed to be residual from Day 1 administration. All remaining tissues tested at all time points for animals that received either one or two injections of either Low Dose or High Dose MV-s-NAP were negative for MV. Histopathologic findings for all tissues were within normal limits. Of note, no cytopathic effect, inflammation, or other abnormality was observed in the histologic examination of the injected mouse brains. **Conclusion:** Intracranial delivery of MV-s-NAP was well tolerated with no significant toxicity being observed following direct administration in the brains of MV-replication susceptible transgenic mice. This outcome supports the safety of the MV-s-NAP platform for clinical oncolytic virotherapy of GBM. This study was supported by NIH grant RO1CA258239.

1771. A Novel Immunocompetent, Adenovirus-Permissive Model to Analyze the Oncolytic Potential and Anti-Tumor Immunity of Human Adenovirus Against Metastatic Breast Cancer

Sacha Robert[1], Natasha Ivelisse Roman[2], Kazuho Inoko[1], Margarita V. Romanenko[1], J. Michael Mathis[3], Julie Ostrander[4], Julia Davydova[1]

[1]Surgery, University of Minnesota, Minneapolis, MN,[2]Pharmacology, University of Minnesota, Minneapolis, MN,[3]Health Science Center, University of North Texas, Fort Worth, TX,[4]Medicine, University of Minnesota, Minneapolis, MN

Breast cancer remains a formidable challenge necessitating innovative therapeutic approaches. This study addresses the imperative for new breast cancer therapeutics and focuses on the potential of human adenovirus (Ad) in this context. Recognizing the limitation of predictive immunocompetent animal models due to the inability of human adenovirus to replicate in them, we introduce a novel hamster

syngeneic model developed to assess the oncolytic potential and anti-tumor immunity of human adenovirus against metastatic breast cancer. Our "Mammary INtra Ductal" (MIND) model, utilizing the HMAM5 cell line, a chemically-induced hamster luminal breast cancer cell line, offers a unique combination of orthotopic, immunocompetent, and human Ad-replication-permissive features. This surpasses existing non-Ad-permissive immunodeficient rodent models, enhancing relevance and utility. To enable comprehensive tracking of tumor growth and metastasis, Luc-ZsGreen-HMAM5 cells were employed for bioluminescence imaging using the IVIS® Spectrum *in vivo* imaging system. Real-time visualization 24 hours post-cell injection validated successful tumor establishment within the model. Tumor progression, dependent on injected cell quantity, revealed a 50% and 100% tumor engraftment rate with 200,000 and 800,000 HMAM5 cells, respectively. The rate of tumor growth was notably faster in hamsters injected with 800,000 cells as opposed to those injected with 200,000 cells. Histological analyses confirmed the connection of tumors to the mammary fat pad, with estrogen receptor-positive staining validating the luminal molecular classification of the tumors. Metastatic potential was observed post-mortal by enlarged lymph nodes (including para-aortic and inguinal nodes), indicating the need for further histological characterization to distinguish between inflammation and metastasis. *In vitro* analysis of binding and oncolysis assays revealed the enhanced responsiveness of HMAM5 cells to human Ad modified with an RGD-fiber, contrary to Ad5 and Ad5/3 counterparts. Subsequent pilot *in vivo* experiments with repeated Ad5-RGD-WT injections demonstrated significant tumor growth inhibition compared to untreated tumors, showcasing the efficacy of human adenovirus to replicate in this model. Following analyses will confirm viral spread and replication within tumors and validate cell trafficking in lymphatic nodes using Luc-ZsGreen-HMAM5 cells. Upcoming plans include immunological profiling, combinatorial therapy investigations with adenovirus and estrogen therapy, such as Tamoxifen, and abscopal effect experiments. Overall, the success of establishing this orthotopic syngeneic breast cancer hamster model, underscored by its demonstrated Ad-replication permissiveness with RGD-fiber, provides a platform for comprehensive safety and efficacy evaluations of human adenovirus, contributing to advancements in breast cancer therapeutics.

1772. An RNA-Based Platform to Facilitate Arming of Oncolytic Picornaviruses

Kyla A. Boyd, Caitessa G. Venables, Velia Penza, Rebecca Nace, Autumn J. Schulze

Mayo Clinic, Rochester, MN

Oncolytic virotherapy is the use of viruses to treat cancer. Clinical data indicates that oncolytic vectors have limited therapeutic efficacy compared to preclinical studies. This has led to arming viruses via the addition of transgenes largely focused on immune stimulation or chemotherapeutic combination as well as the use of repeat dosing regimens. However, genetic arming is not ideal for all viruses and systemic repeat dosing may be hindered by development of neutralizing antibodies. Picornaviruses are small positive-sense RNA viruses with a limited packaging capacity and cannot be efficiently armed with many therapeutically relevant genes, such as cytokines, via gene insertion. To date, several picornaviruses have been explored in clinical trials, and have resulted in a modest boost to overall survival in patients treated. Immune-related signatures have been observed in some responders versus non-responders providing prospective therapeutic combinations. Therefore, we hypothesize that rational arming of picornaviruses will boost their therapeutic effect and further improve patient outcomes. We have developed an RNA-based oncolytic platform for mengovirus, a murine picornavirus, based on the delivery of synthetic RNA genomes (iRNA) via lipid nanoparticles (LNPs), which may facilitate repeat intravenous dosing while expanding opportunities for targeting based on nanocarrier modification. This platform also augments our capacity to explore novel arming mechanisms. Here we describe an optimized microRNA-targeted Mengovirus iRNA construct, MC_0R, deliverable via LNP. The specific infectivity, translation, and cytotoxicity of the iRNA were comparable to an untargeted construct *in vitro*. To assess therapeutic efficacy, mice bearing MPC-11 subcutaneous tumors were treated with vehicle, empty LNPs or LNPs containing mengovirus iRNAs and tumor volume was monitored. All mice in the MC_0R-LNP treated group showed tumor regression, significantly improved overall survival, and viremia. Further confirmatory *in vivo* studies are ongoing. Overall, we observed that mengovirus iRNAs delivered by non-viral vectors are safe and can have a therapeutic effect. This capsid independent delivery opens opportunities for initial and repeat dosing that can bypass toxicity and immune neutralization. Our results support the continued development of MC_0R as an RNA-based oncolytic virus platform and allow exploration of combining RNA-based therapeutics with our oncolytic iRNA formulation, studies that are currently ongoing.

Friday Posters: Cancer - Targeted Gene and Cell Therapy

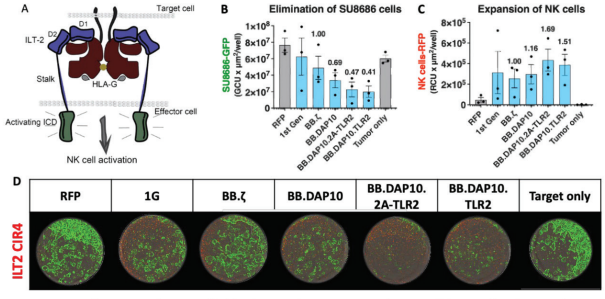
1773. Chimeric ILT Receptors (CIR) Coupled with Novel and Potent Costimulatory Domains Efficiently Direct Natural Killer Cells to Eliminate HLA-G Positive AML and Solid Tumors

MyLinh T. Duong, Jihyun Park, Raphael Ognar, Simon Wain-Hobson, Joseph Henri Bayle

NKILT Therapeutics, Houston, TX

Background Human leukocyte antigen (HLA)-G directs immunosuppression by binding to ILT2 in lymphoid cells and ILT4 in myeloid cells via ITIM signaling motifs. HLA-G expression is low or absent in normal tissues, except for the cytotrophoblasts in the placenta during pregnancy. However, about 50% of cancers derepress HLA-G as a mechanism to evade immune attack. To effectively target the multiple structural isoforms of HLA-G, we developed an approach utilizing ILT2 and ILT4 as engagers and linking them with intracellular signaling domains to create chimeric ILT receptors or CIRs that drive activation of Natural Killer (NK) cells. *Methods* $CD56^+$ NK cells isolated from PBMCs (up to 5 donors) were activated with IL-15, IL-2 and a feeder-free cocktail of an immobilized cytokine and activating ligand and transduced with γ-retroviruses directing expression of various CIR-containing vectors together with soluble IL-15 and a ΔCD19 marker. After 8 days of expansion, CIR- or CAR-NK cells were

cocultured with GFPffluc-expressing tumor cell lines with or without transgenic HLA-G G1, G2 and G5 isoforms or primary human cell types. Cytotoxicity of CIR-NK cells was determined by reduction of GFP fluorescence or luciferase activity and supernatants were analyzed for IFN-γand TNF-α release. Transduced NK cells were labeled with nuclear-RFP for measurement of expansion in coculture. *Results* CIR (ILT2 or ILT4.4-1BB.ζ) constructs were efficiently and stably expressed in NK cells (>80% $CD19^{+}$) and induced HLA-G-specific killing of AML and solid tumor cells relative to mock-transduced cells. In cocultures against THP1 and KG1 target cells expressing exogenous G1, G2 or G5 isoforms, CIR-NK cells displayed enhanced target cell killing in 2 and 7-day assays relative to mock-transduced effector cells (Mock = 1.47E6 ± 4.9E5 vs. CIR = 5.7E5 ± 6.0E5 in coculture against KG1-HLA-G1). To improve cytotoxicity and persistence of CIR-NK cells, a screen of >45 novel intracellular costimulatory molecules to replace 4-1BB.ζ resulted in several potent candidates. Notably, ILT2 or ILT4 CIR fused to 4-1BB.DAP10 and TLR2 constructs achieved approximately 70% greater tumor cell killing and 2-fold greater expansion over 4-1BB.ζ counterparts. Further, in serially repeated cocultures in which tumor cells were added up to 4 times every 2 days, the ILT4.4-1BB.DAP10-2A.TLR2 CIR-NK cells eliminated more than 60% of Molm13 tumor cells (4-1BB.ζ = 3.96E7 ± 5.37E6 vs. 4.1BB.DAP10.2A-TLR2 = 1.45E7 ± 3.34E6), expanded more than 2-fold relative to CIR-NK cells containing 4-1BB.ζ (4-1BB.ζ= 1.49E5 ± 5.72E3 vs. 4.1BB.DAP10.2A-TLR2 = 3.26E5 ± 5.1E4) and was accompanied by 2-fold higher IFN-γ secretion (4-1BB.ζ = 15.3 pg/ml ± 1.1 vs. 4.1BB.DAP10.2A-TLR2 = 35.5 pg/ml ± 3.58). We validated the safety profile of ILT2 and ILT4 CIR-NK cells against more than 15 primary human cell types populations from PBMCs and CIR expression did not induce targeting against normal tissues relative to mock-transduced NK cells. *Conclusions* The use of natural receptors (ILT2/4) to target HLA-G permits pairing of the ILT receptors with the multiple immunosuppressive HLA-G isoforms thereby circumventing a mechanism for target escape often observed in CAR-directed therapy. We demonstrated that engineered CIR-NK cells, coupled with potent intracellular costimulatory domains, can convert HLA-G expression in cancer cells from an inhibitor of immune surveillance into a practical target for anti-cancer therapy.



Cytotoxicity and Expansion of CIR-NK cells. (**A**) Schematic of a CIR comprising the extracellular D1 and D2 domains of ILT2, a CD8α-derived stalk and transmembrane domain, and intracellular costimulatory domain (ICD) driving NK cell activation. (**B**) Elimination of SU8686 pancreatic cancer cells and (**C**) Expansion of CIR-NK cells in 7-day coculture. Fold change relative to ILT2.4-1BB.ζ. (**D**) Images of coculture in B & C taken by the Incucyte. Green = SU8686-GFPFluc, Red = NK-RFP.

1774. Adeno-Associated Virus-Mediated Expression of Rituximab for the Treatment of Central Nervous System Lymphoma

Marcela S. Werner, Otto D. Cerqueira, Lama Abdullah, Jillian Roamer, Jenny A. Greig, James M. Wilson

Gene Therapy Program, University of Pennsylvania, Philadelphia, PA

Introduction: Central nervous system lymphoma (CNSL) is a group of rare incurable malignancies that originate in the brain or spinal cord (primary CNSL) or from systemic disease that metastasizes to the CNS (secondary CNSL). Up to 95% of CNSL cases arise from B-cell non-Hodgkin lymphoma (NHL). Although there is a lack of consensus, the 5-year survival rate for patients diagnosed with CNSL is <30%, and the overall survival from diagnosis is estimated to be <7 months for patients aged 70 years or older. Rituximab, a human/murine chimeric anti-CD20 monoclonal antibody, is part of the standard of care regimens for the treatment of indolent and aggressive forms of B-cell NHL. However, rituximab has low bioavailability in the CNS because of the intrinsic properties of the blood-brain barrier. In this study, we established a model for rituximab expression mediated by adeno-associated virus (AAV) vectors (AAV.Rituximab) for direct CNS delivery to bypass the blood-brain barrier and maximize the effectiveness of rituximab against B-cell CNSL. Methods and Results: We evaluated four different codon-optimized versions of AAV.Rituximab in healthy *Rag1* knockout (*Rag1*$^{-/-}$) mice following intracerebroventricular (ICV) delivery of $1x10^{11}$ genome copies (GC) of vector per animal. Treatment was well tolerated, and no signs of toxicity were observed. We selected the top codon-optimized version of AAV.Rituximab based on enzyme-linked immunosorbent assay results, which showed an average expression of 102 ng rituximab/mg total protein in saline-perfused brain tissue homogenates and 815 µg rituximab/mL in serum by day 30 after treatment. Rituximab identity was confirmed via mass spectrometry analysis, and heavy- and light-chain expression was confirmed via Western blot using brain tissue homogenates from ICV-treated mice. We evaluated the DNA and RNA biodistribution in brain and liver tissues of these animals by quantitative PCR; results confirmed successful CNS transduction and transgene expression. Because of vector leakage into the systemic circulation, vector transduction and transgene expression also occurred in the liver of *Rag1*$^{-/-}$ mice injected ICV with AAV.Rituximab. Tumor challenge experiments using human lymphoma cell lines (Ramos, Oci-ly3, and Daudi) implanted into the brain parenchyma of adult *Rag1*$^{-/-}$ mice demonstrated that ICV treatment with $1x10^{11}$ GC/mouse of the lead codon-optimized AAV.Rituximab candidate delayed tumor progression and increased overall survival in all xenograft models tested compared with control groups injected with AAV.Isotype antibody vector control. Conclusions: These preclinical findings highlight the potential of direct CNS delivery of AAV-based gene therapy to achieve sustained expression of therapeutic monoclonal antibodies such as rituximab for the treatment of B-cell CNSL.

1775. Gene-Edited Hematopoietic Stem Cells to Enable Next-Generation CAR-T Cell Therapy for the Treatment of AML

Julia Etchin[1], Yonina Keschner[1], Mariana Silva[2], Hillary Hoyt[2], Matthew Ung[3], Nathan Manalo[4], Juliana Xavier-Ferrucio[4], Amanda Halfond[1], Julia DiFazio[1], Michelle Lin[4], Julian Scherer[2], John R. Lydeard[1], Tirtha Chakraborty[5]

[1]Discovery and Molecular Engineering, Vor Biopharma, Cambridge, MA,[2]Cancer Immunotherapy, Vor Biopharma, Cambridge, MA,[3]Quantitative Biology, Vor Biopharma, Cambridge, MA,[4]Preclinical and Translational Biology, Vor Biopharma, Cambridge, MA,[5]Vor Biopharma, Cambridge, MA

Acute myeloid leukemia (AML) is a hematologic malignancy characterized by the abnormal clonal expansion of leukemic blasts. The lack of cancer specific antigens (Ags) coupled with heterogenous expression of the disease has made the treatment of AML difficult. Targeted therapies, such as CAR-Ts and the CD33-directed therapy gemtuzumab ozogamicin have shown significant anti-leukemic activity in both preclinical and clinical studies of AML but are hampered by severe myelotoxicity due to on-target, off-tumor activity. We developed an approach of genetically ablating biologically dispensable target Ags from hematopoietic stem cell grafts, thereby restricting the Ag expression to AML cells and enabling subsequent targeted therapy without risk of on-target off-tumor toxicities. Here, to identify and prioritize AML target Ags to address the challenge of heterogeneous AML expression we created a comprehensive multi-modal single cell expression atlas quantifying the intensity and (co)expression patterns of leukemic blast cells. In an assessment of 28 paired diagnosis and relapse patient bone marrows (BMs), we profiled ~80 candidate Ags (by applying RNA sequencing with surface Ag feature barcoding (>450,000 total cells)). Among the top target Ags based on ranking of average surface expression across all blasts in each patient are CD33 (currently under clinical evaluation (NCT05984199, NCT04849910)) and CLL-1. A promising AML target candidate, ADGRE2/EMR2, is among the top 25 highest surface expressors in both diagnosis and relapsed samples. Encouraged with the consistent expression levels, we developed ADGRE2-directed CAR-T cells. Novel ADGRE2-specific binders were identified and activation, cytokine secretion and potent killing against the ADGRE2$^+$ MOLM13 AML cells but not ADGRE2-lacking cells was confirmed. However, healthy BM and peripheral blood flow cytometric (FC) analysis showed that ADGRE2 is also expressed on hematopoietic stem and progenitor cells (HSPCs) and myeloid lineages, suggesting that editing may be necessary to protect healthy graft from subsequent off-tumor targeting. Human genetic data of ADGRE2 null individuals provide compelling evidence for biological dispensability (https://gnomad.broadinstitute.org/). To validate these null natural variants (NVs), we utilized a HEK293T-based system. Protein quantification of 7 predicted NVs showed surface and complete or partial intracellular protein loss in 5 NVs. Of those validated, two NVs that exhibited surface loss with reduced intracellular protein levels are found in conserved isoforms. We next utilized the adenine base editing (ABE) approach to knock out *ADGRE2* in HSPCs, observing >90% editing with a corresponding surface protein loss. Pharmacology studies using NSG mice showed no impact of ADGRE2 deletion in HSPCs on long-term 16-week engraftment and multilineage differentiation as compared with unedited cells and maintained editing. To enable combinatorial targeting of ADGRE2, we simultaneously ablated *ADGRE2* and *CD33* in HSPCs, observing >90% editing at both loci with consequent protein loss of both targets and no impact on myeloid cell differentiation. In conclusion, our data provide a discovery framework for prioritizing AML targets based on expression patterns throughout disease progression. Paired with multiplex genome editing to reduce the toxicity to healthy donor graft, this may enable administration of combinatorial therapeutics with reduced on-target, off-tumor toxicity for AML patients.

1776. Pegenzileukin, a Selectively Pegylated IL-2 Analog, Enhances Anti-Tumor Function of NK Cells without Activating Regulatory T Cells

Anna Oja[1], Nada-Katarina Kasic[1], Ieva Cesonyte[1], Katarzyna Franciszkiewicz[2], Loreley Calvet[2], Sukhvinder Sidhu[2], Andre Kunert[1], Robert Igarashi[1]

[1]Oncology Research, Sanofi, Amsterdam, Netherlands,[2]Oncology Research, Sanofi, Vitry, France

Adoptive cell therapy using natural killer (NK) cells for cancer treatment is attractive due to their anti-tumor activity, their high safety profile, and feasibility of using NK cells "off-the-shelf". K-NK cells, NK cells expanded using plasma membrane particles (PM21) from K562-mb21-41BBL cell line, have high anti-tumor activity against a range of tumor types. IL-2 promotes NK cell proliferation and survival. While use of IL-2 showed early promise in oncological clinical studies, the high toxicities and non-specific engagement, particularly to promote Treg cell activation, has limited its clinical use. Pegenzileukin (SAR444245) is a synthetic IL-2 that is selectively pegylated and preferentially binds the IL-2 receptor β/γ complex, but not the α receptor. This allows for selective activation of effector CD8+ T cells and NK cells with diminished Treg cell activation. Thus, pegenzileukin shows reduced activation of Treg cells and limits their deleterious effects as compared to IL-2. PBMCs and K-NK cells were stimulated with pegenzileukin to investigate the activation of the different immune cell populations. K-NK and CD8+ T cells can be selectively activated by pegenzileukin to cause activation and proliferation. Stimulation of K-NK cells with pegenzileukin maintains their viability for at least 2 weeks in vitro and increases K-NK cell numbers in vivo in mice. Importantly, pegenzileukin does not cause aberrant cytokine production by K-NK cells in the absence of tumor cells, and likely would not increase the risk of graft-vs-host or host-vs-graft when used as a clinical combination, albeit a clinical study is required to demonstrate this hypothesis. Furthermore, pegenzileukin enhances K-NK cell cytotoxicity up to 13-fold compared to no cytokine treatment. In vivo administration of pegenzileukin together with K-NK cells improves tumor control and correlates with higher numbers of K-NK cells in peripheral blood. Thus, a combination therapy of adoptive K-NK cells and pegenzileukin has the potential to significantly improve pharmacokinetics of K-NK cells and enhance pharmacodynamics for cancer therapy.

1777. Exploring BAFF-R as a Novel Target for Chimeric Antigen Receptor T-Cell Therapy in Chronic Lymphocytic Leukemia

Yan Luo[1,2], Yaqing Qie[1], Martha Gadd[1], Shuhua Li[1], Tanya Hundal[1], Hemant Murthy[3,4], Roxana Dronca[3], Mohamed A. Kharfan-Dabaja[3,4], Hong Qin[1,2,3]

[1]Regenerative Immunotherapy and CAR T- Translational Research Program, Mayo Clinic, Jacksonville, FL,[2]Department of Cancer Biology, Mayo Clinic, Jacksonville, FL,[3]Department of Internal Medicine, Mayo Clinic, Jacksonville, FL,[4]Blood and Marrow Transplantation and Cellular Therapy Program, Mayo Clinic, Jacksonville, FL

Tremendous benefits have been reported by a significant number of patients who have received chimeric antigen receptor (CAR) T-cell therapy against B-cell malignancies. With the passage of time and accumulation of surveillance data, a new set of challenges have been identified with the observations of antigen-escape relapse and of variability in patient responses to the treatment. Chronic lymphocytic leukemia (CLL) is an example of a refractory disease in which CD19 CAR T-cell therapeutic success has not been realized. In this study, we explored the use of B-cell activating factor receptor (BAFF-R) targeting CAR T-cells as an alternative immunotherapy for CLL. BAFF-R-mediated signaling regulates B lymphocyte development and survival and is expressed almost exclusively on B cells and most human B-cell lymphomas and leukemias. The finding of universal BAFF-R expression on CLL positions it as an ideal target for developing CAR T-cell therapy against CLL. Our data demonstrated that BAFF-R CAR T-cells elicited antigen-specific cytotoxicity against both CLL cell lines and primary CLL tumors isolated from patients. Given that CD19 antigen-escape is a major factor leading to release after CAR T-cell treatment, we explored the potential of using the BAFF-R CAR T-cell therapy to address this unmet need. A CD19-deficient CLL model was generated by genetically knocking out CD19 expression on the MEC-1 human CLL cell line. As expected, CD19-deficient MEC-1 cells showed resistance to CD19 CAR T-cells; however, BAFF-R CAR T-cells demonstrated potent cytotoxicity against these tumor cells, supporting our hypothesis that BAFF-R can be targeted for CD19 negative tumors. Finally, we validated the therapeutic efficacy of BAFF-R CAR T-cells in an autologous experiment setting using CLL patient samples. We successfully generated patient-derived BAFF-R CAR T-cells using a limited amount of blood samples from three CLL patients and demonstrated the cytotoxicity of these CAR T-cells against autologous CLL tumors. All our proof-of-principle data highlight BAFF-R-targeting CAR T-cell therapy as a novel therapeutic approach for CLL patients who have failed all options including CD19 CAR T-cell therapy.

1778. Molecular Shielding of CD52 Retains Expression, Anti-Phagocytic Don't Eat Me Function and Protects from Alemtuzumab-Mediated Depletion

Anna Camus[1], Emiel Ten Buren[1], Laura Garcia-Prat[1], Brault Julie[1], Valentin Do Sacramento[1], Sophie Lackner[1], Rosalba Lepore[2], Benedetta Romeo[1], Mathilde Testut[1], Klin Daniel[1], Izabela Durzynska[3], Saniye Yumlu[1], Stefanie Urlinger[1]

[1]Cimeio Therapeutics, Basel, Switzerland,[2]University Hospital and University of Basel, Basel, Switzerland,[3]Ridgeline Discovery, Basel, Switzerland

Targeted immunotherapies such as monoclonal antibodies aim to eliminate transformed or dysregulated cells expressing a specific target antigen and are highly effective in the treatment of various hematologic diseases. E.g., Alemtuzumab is approved for treatment of CD52+ malignancies as well as multiple sclerosis and is clinically assessed in reduced intensity conditioning and for lymphodepletion prior to administration of CAR-T cells. These novel applications raised the interest in engineered cell products with resistance to Alemtuzumab, which is usually achieved by CD52 knock-out (KO). CD52 is found on most cells of the lymphoid lineage as well as on monocytes and macrophages. Mature CD52 is a highly glycosylated 12-mer peptide, which is GPI-anchored to the cell surface and acts as a regulator suppressing inflammatory responses. This implies that CD52 KO on T cells will not be functionally inert and thus influence the activity and exhaustion of CD52 KO lymphocytes. Here, we shield CD52 from Alemtuzumab binding by introducing by a single amino acid (aa) substitution. This minimally invasive approach combined with careful selection of the substitution preserved expression and function of the engineered protein. From the determination of the Alemtuzumab epitope through a comprehensive mutagenesis screen three key residues emerged, which were predicted to retain GPI anchoring and glycosylation. By detailed binding assays and glycosylation profiling we identified two aa substitutions fully shielding CD52 from Alemtuzumab binding with limited impact on the glycosylation pattern. Primary T cells, HPSCs and cell lines were genetically engineered using base editing for both shielding variants. Engineered CD52 variants lose binding to Alemtuzumab, while binding of a control CD52 antibody remains unchanged, demonstrating expression levels comparable to unedited cells (Fig. 1a). We confirmed shielding of CD52 variants in antibody-dependent cytotoxicity (ADCC) assays with cells overexpressing either WT or CD52 variants. Due to similar processing of CD52 and CD24, we speculated that CD52 also inhibit phagocytosis. Indeed, CD52 KO primary T cells are phagocytosed at significantly increased levels when exposed to macrophages compared to CD52 WT T cells. Importantly, this was not observed with T cells expressing CD52 shielding variants (Fig. 1b), which also showed expression of phenotypic T cell markers and activation capabilities comparable to WT T cells. In analogy to what we have shown for targeted immunotherapies paired with engineered CD123[1], CD117 and CD45, we successfully shielded CD52 from Alemtuzumab binding by single aa substitutions while maintaining expression and biochemical properties. Moreover, our data is the first indication that CD52 can act as a novel don't eat me signal and single aa substitutions in CD52 retain this anti-phagocytic function. Further studies need to verify

increased in vivo persistence of engineered lymphocytes as compared to CD52 KO cells and thus being a superior alternative to CD52 KO approaches. 1 Marone, R. et al., J Exp Med. 2023

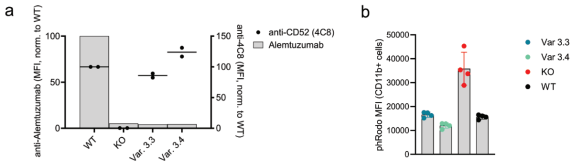


Figure1: Characterisation of shielded primary human T cells. T cells were either base edited cells for the shielding variants or to generate knock outs and sorter for loss of Alemtuzumab binding by low cytometry.
a) Shielded variants completely loose Alemtuzumab binding while binding of the control CD52 antibody 4C8 remains unchanged. This demonstrates expression levels comparable to unedited cells. b) Median fluorescence intensity of phRodo dye in the macrophage (CD11b+) population was measured by flow cytometry analysis after 2 h incubation with CD52 WT, KO, Var 3.3 or Var 3.4 edited primary T cells previously stained with phRodo dye.

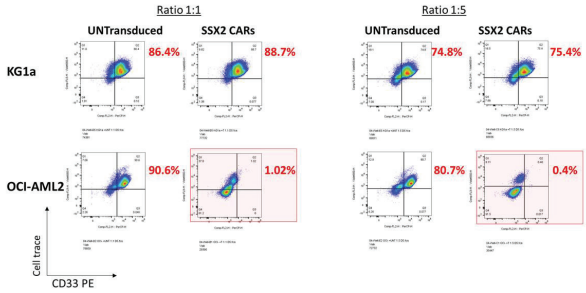
1779. Development of a Novel Cell Therapy Targeting SSX2 for Acute Myeloid Leukemia

Achouak A. Achour

Cancer Research Center, Children National Hospital, Washington, DC

Background: Acute Myeloid Leukemia (AML) is the most common leukemia in adults and the second most common leukemia of childhood. Disease relapse is still a leading cause of death in these patients. With the success of CD19-CAR-T cell therapies for Acute Lymphoblastic Leukemia (ALL), novel T-cell therapeutics are also being explored for AML. Hence, we hypothesize that gene engineering T cells to target the tumor associated antigen Synovial Sarcoma X Breakpoint 2 (SSX2) will elicit potent anti-leukemia effects. **Objective:** To determine whether a novel T-cell product engineered to express a chimeric antigen receptor (CAR) targeting the HLA A2-restricted SSX2 epitope 41-49 will have anti-leukemia activity in vitro and in vivo. **Methods:** To achieve this goal, we developed a novel CAR which recognizes the 41-49 epitope of SSX2 in an HLA-A*0201 restricted manner, similar to the recognition of a TCR. To confirm a lack of off-target specificity of the CAR, we screened $SSX2_{41-49}$ peptide expression in HLA-A2+ healthy tissues and primary AML blasts by flow cytometry and immunochemistry (using bone marrow biopsy sections from AML patients with healthy donor control peripheral blood samples). We also tested the functionality of SSX2-CAR-T cells against AML cell lines by performing a co-culture assay using HLA A2-positive (OCI-AML2) and HLA A2-negative (KG1a) AML cell lines as targets. $SSX2_{41-49}$ CAR T versus control unmodified T cells were evaluated in this model in the absence of cytokines. Subsequently, to assess the anti-tumor activity of $SSX2_{41-49}$ CAR T *in vivo* we established an AML model in NSG mice. Mice were intravenously injected with HLA-A2 + (OCI-AML2) and HLA-A2 - (KG1a) AML cell lines expressing FFLuc. Upon confirming tumor engraftment by bioluminescence imaging (BLI), mice received a dose of $10x10^6$ cells/mouse of $SSX2_{41-49}$ CAR T-cells or control unmodified T-cells at day 7 after tumor injection. Leukemia burden was monitored weekly using BLI. Peripheral blood and splenocytes were analyzed every 2 weeks to track the CAR-T. **Results/discussion:** Screening of SSX2 expression confirmed high $SSX2_{41-49}$ peptide expression in HLA-A2 positive AML cells only. *In vitro*, $SSX2_{41-49}$ CAR-T demonstrated enhanced cytolytic activity, at both 1:1 and 1:5 effector: target ratios, exclusively against the HLA-A2 + OCI-AML2 cells (range 80-98%), In contrast, no cytolytic activity was observed against the HLA-A2 - KG1a cells (range 1-20% cytotoxicity) (**Figure**1). Our *in vitro* studies confirmed robust specificity and efficacy of $SSX2_{41-49}$ CAR-T cells. In a xenograft model, $SSX2_{41-49}$ CAR-T demonstrated modest anti-leukemic activity (after 3 weeks of tumor injection) with no acute infusion-related toxicities. To increase potency, combination therapy with the DNA methyltransferase (DNMT) inhibitor, 5′-azacitidine (AZA) was explored. Early study showed increase expression of $SSX2_{41-49}$ in HLA-A2 + OCI-AML2 cells (from 10% to 35% after 5-AZA treatment). **Future studies** will combine $SSX2_{41-49}$ CAR T with 5 AZA treatment one week after tumor injection to enhance $SSX2_{41-49}$ expression and tumor kill. Ultimately results from these studies will complete the IND-enabling studies needed to translate this novel treatment to the clinic.

Figure1. T cells expressing a $SSX2_{41-49}$ CAR eliminate exclusively Cell trace+ CD33+ THP-1 cells *in vitro*.



1780. Engineered Receptor Provides Inducible Signal 2 and Signal 3 Costimulation to Augment TCR-Based Cell Therapies of Cancer

Mansi Narula, Johannes Englisch, Azlann Arnett, Tadahiro Honda, Maksim Mamonkin, Norihiro Watanabe

Center for Cell and Gene Therapy, Baylor College of Medicine, Houston, TX

Cellular immunotherapy using natural and engineered TCR-based T cells produces versatile and highly sensitive tumor targeting in preclinical models. However, their activity in a clinical setting is limited due to the lack of costimulatory signaling and cytokine, which reduces functional persistence of T cells and their clinical benefit. Engineered constitutive cytokine signaling helps boost T-cell persistence but comes with a risk of accelerated T-cell exhaustion or uncontrolled proliferation. We *hypothesized* TCR-based therapies can be enhanced by integrating physiologic costimulatory Signal 2 and a conditional Signal 3 resembling natural cytokine signaling. To test this hypothesis, we created a panel of engineered receptors with an extracellular domain derived from a 4-1BB ligand (4-1BBL) and intracellular domains emanating signaling from cytokine receptors. Upon antigen recognition and TCR stimulation in T-cells, these dual-stimulatory receptors engage 4-1BB, which is transiently upregulated on T-cell surface, thus producing Signal 2 with physiologic intensity and timing. Additionally, 4-1BB engagement activates intracellular signaling of the engineered receptor producing temporary Signal 3 to further aid in costimulation and survival of activated T-cells. We found a specific costimulatory receptor configuration comprising 4-1BBL linked with an intracellular domain of cMPL (a thrombopoietin receptor) produced highest activity in our models. When tested in T cells expressing a chimeric T-cell receptor (cTCR) targeting a leukemic antigen, co-expression of our engineered receptor on these cells produced 25-fold higher expansion than unarmed cTCR T cells,

in a 9-day tumor coculture assay. Similarly, when expressed on T cells redirected with leukemia-specific T-cell engager, the costimulatory receptor improved cell expansion by 35-fold, indicating the benefit is not limited to engineered TCRs. Mechanistically, the engineered costimulatory receptor induced strong STAT3 and STAT5 activation downstream of cMPL, concomitant to 4-1BB expression in activated T cells. Finally, a single injection of the engineered receptor armed cTCR T-cells mounted robust expansion and durable persistence in the absence of exogenous cytokines in a mouse xenograft model of human leukemia, in which unarmed cTCR T cells had only transient anti-tumor activity. These results suggest the application of the newly developed costimulatory receptor could be extended to diverse TCR-based therapies, to overcome the limited cell persistence.

1781. Targeting TdT for Highly Selective Therapy of Acute Lymphoblastic Leukemia

Pan Yin[1], Norihiro Watanabe[1], Zhang Ding[2], Xinya Liu[3], Mark Yarmarkovich[3], Alexey Stepanov[2], Maksim Mamonkin[1]

[1]Baylor College of Medicine, Houston, TX,[2]The Scripps Research Institute, San Diego, CA,[3]NYU Grossman School of Medicine, New York, NY

CAR T cells targeting pan-B cell antigens produce robust responses in patients with B-cell acute lymphoblastic leukemia (B-ALL) but often induce concurrent B-cell aplasia which requires additional management. Similar targeting of pan-T cell antigens to treat T-ALL could induce dangerous T-cell aplasia with associated immunodeficiency that is harder to compensate than the loss of B-cells. Targeting antigens highly restricted to leukemic cells with minimal presence on normal tissues can help overcome unwanted ablation of the critical immune cells. Terminal Deoxynucleotidyl Transferase (TdT) is expressed in the majority (>80%) of T-cell and B-cell ALL and is commonly used as a diagnostic marker. Normal expression of TdT is restricted to lymphocyte progenitors undergoing somatic rearrangement of antigen receptors and is absent in mature lymphocytes or primitive and committed progenitors. A recent study showed TdT-derived peptide on HLA-A2 can be recognized by a TCR which effectively redirects CD8+ T cells to eliminate leukemia. We hypothesized TdT/HLA-A2 targeting can be improved by utilizing a specific single-chain variable fragment (scFv) which could be used instead of a TCR to activate both CD4+ and CD8+ T cells for optimal anti-tumor effect. Using phage display, we identified a human scFv that selectively recognizes a TdT peptide complexed with HLA-A2. Structural modeling of the scFv-HLA/TdT interaction showed three potential hydrogen bonds formed between CDR regions of both heavy and light chains of the scFv and the TdT-derived peptide in the HLA-A2 groove. Notably, due to low antigen presentation, T-cells expressing an scFv-based CAR produced limited cytotoxicity against leukemic cells. However, a chimeric TCR (cTCR) with a TdT-specific scFv replacing either alpha or beta variable region enabled strong cytolysis of HLA-A2+ TdT+ cell lines NALM-6, BV173 and TALL-1. As expected, TdT cTCR T cells showed no activity against TdT+ HLA-A2- cell line CCRF-CEM and TdT- HLA-A2+ cells THP-1, GDM-1, and Loucy. Disruption of surface HLA Class I expression in BV173 by CRISPR/Cas9 editing abrogated their recognition by TdT cTCR T cells. Further, TdT cTCR T cells elicited no detectable cytotoxicity against normal autologous HLA-A2+ peripheral blood T- and B-cells unlike control CD19 CAR T cells that produced robust killing of peripheral B cells. In a mouse xenograft model of human B-ALL (BV173), a single injection of TdT cTCR co-expressing a costimulatory receptor produced complete elimination of detectable leukemia with 100% survival of all animals for the duration of the experiment (>60 days). Finally, we created a bispecific T-cell engager (BiTE) using the TdT/HLA-A2 specific scFv. Normal T-cells were effectively redirected to recognize and eliminate B-ALL and T-ALL cell lines BV173 and TALL-1 in the presence of soluble TdT BiTE. These results suggest TdT+ B-ALL and T-ALL can be effectively targeted by both cellular and biologic immunotherapy without ablating normal lymphocytes.

1782. Engineered *ortho*IL2-CAR T Cells Generated Using Viral Vector-Free, Three-in-One Nonviral CRISPR-Based Technology Demonstrate Enhanced Antitumor Activity in a Murine Model of Leukemia

Qian Zhang[1], Yihao Wu[1], Jingyi Yang[1], Eric Nigel Ebenezer Anand Manoharan[1], Tyra Wentz[1], Alvin Yu[1], Kevin Zhou[1], Morgan Hresko[1], Leon Su[2], Daniel Ryan[3], Christopher Garcia[2], Michael Milone[1]

[1]Upenn, Philadelphia, PA,[2]Stanford University, Stanford, CA,[3]Agilent Research Labs, Santa Clara, CA

Our previous studies have shown that an orthogonal IL-2 and IL-2Rβ system offers a potent approach to enhance CAR-T therapy. *Ortho*CAR T cells can be produced by transferring CAR and oIL2Rβ genes into T cells through a viral gene delivery system like lentivirus or retrovirus. However, this process carries with it the risks of variable transgene expression and oncogenesis due to random integration of transferred genes into the T cell genome. To address these challenges, we engineered *ortho*CAR T cells by combining multigenic-editing strategies, CRISPR/Cas9-mediated gene knockout (KO) and HDR-mediated gene knock-in (KI) to direct CAR integration into the IL2 locus. The IL2 gene locus was selected as the CAR target site to disrupt expression of the native IL2, which is functionally replaced by the orthogonal system and would be expected to also prevent the effects of CAR T-cell-derived IL2 on endogenous Tregs that depend upon this cytokine. IL2 KO and CAR KI were achieved with an efficiency of 46% (range 30 - 65%) using a 2nd generation, anti-CD19 CAR with 4-1BB and CD3ζ signaling domains in a donor DNA containing a CBh promoter with 650 bp homology arms flanking the CRISPR/Cas9 cut site within the IL2 gene. Sequencing confirmed the expected knock-in of the transgene as well as > 96% IL2 KO efficiency that was also associated with reductions in IL-2 production by the edited CAR T cells following antigen stimulation. The Prime Editing 3 (PE3) system was used to convert endogenous wildtype IL2Rβ to orthogonal IL2Rβ using a transfection of a PE3-encoding in vitro transcribed mRNA, Prime Editing guide RNA (pegRNA), and nicking sgRNA (ngRNA). This approach was able to achieve an editing efficiency of 71% (range 54-89%). This tandem engineering strategy not only results in more uniform CAR T expression, but also demonstrated *ortho*IL2-enhanced antitumor activity in vivo using immunodeficient NSG mice xenografted with NALM-6 (leukemia) model. Unexpectedly, control T cells bearing the IL2 KO with CAR KI were also highly functional in vitro and in vivo with activity that more rapid and potent than

conventional CAR T cells made by lentiviral transduction. This was associated with reduced tonic CAR signaling in the edited IL2-CAR19 T cells compared to the conventional CAR19 T cells. Our results indicate that the IL2 gene is dispensible for CAR T cells, and a virus-free tri-genome editing workflow has the potential to enable rapid and flexible manufacturing of precisely engineered CAR T cell products to advance CAR T cell immunotherapy.

1783. SynNotch-Gated CARs Enhance Specificity, Promote Persistence, and Reduce Toxicity in AML

Shawn Barman, Samy Leon Jambon, Jainping Sun, Sakunthala Muthugounder, Yong-Mi Kim, Shahab Asgharzadeh, Chintan Parekh, Babak Moghimi
CHLA/USC, Los Angeles, CA

Background and significance: Despite advances in modern chemotherapy and bone marrow transplantation, the outcome for patients with relapsed and refractory pediatric acute myeloid leukemia (AML) remains very poor. While CAR-T immunotherapy has proven extraordinarily effective in treating lymphoblastic leukemia, its success in treating AML is yet to be realized. A lack of tumor-specific antigens in AML and the risk of toxicity have hindered the development of AML-specific CAR-T cells. Additionally, current AML CARs in clinical trials often lack T-cell persistence and have failed to demonstrate effectiveness. We have engineered logical gated CAR-T cells targeting a combination of CD33 and CD123 in AML to address the lack of tumor-specific antigens. These gated CAR-T cells are designed to deliver precise killing against dual-positive tumor cells while sparing normal tissues expressing individual targets. **Methods**: We engineered gated CAR-T cells to express CD123 through a CD33 synthetic receptor and used in vitro coculture, cytokine release, and cytotoxicity assays to evaluate their effectiveness against AML cell lines and patient-derived xenografts (PDXs). Subsequently, we employed immunocompromised murine models of AML to assess their efficacy in vivo and utilized humanized mouse models to evaluate hematopoietic toxicity. **Results**: Our findings demonstrate that CD33-gated CD123 CAR-T cells effectively eliminate AML in vitro and in vivo, particularly against AML cell lines and in PDXs. Unlike constitutively expressed CD123 CARs, the CD123 CAR expressed via the CD33 SynNotch circuit preserves hematopoietic stem cells (HSCs), reducing the risk of on-target off-tumor hematopoietic toxicity. These gated CAR-T cells also show enhanced resilience to exhaustion, an improved memory profile, and increased proliferation potential. Notably, the moderated CAR signaling within this circuit further prevent cytokine release syndrome, addressing a major limitation in CAR-T dose escalation and enhancing overall efficacy. **Conclusion**: Precision SynNotch gated CAR-T cells targeting a combination of CD33 and CD123 in AML that prevent hematopoietic toxicity and demonstrate superior T-cell phenotype, are positioned to overcome significant shortcomings of current AML CAR-T cell therapies.

Friday Posters: Challenges to Immunological Responses to Therapeutic Interventions

1784. Reducing Cas9-Specific Immune Responses Through the Self-Inactivating CRISPR (SiC) System in the Canine DMD Model

Bryan Hu[1], Yongping Yue[1], Xiufang Pan[1], Matthew J. Burke[1], Keqing Zhang[1], Jin Han[1], James A. Teixiera[1], Sandeep R. P. Kumar[2], Roland W. Herzog[3], Dongsheng Duan[1,4,5]
[1]Department of Molecular Microbiology and Immunology, School of Medicine, University of Missouri, Columbia, MO,[2]Department of Pediatrics, Indiana University, Indianapolis, IN,[3]Department of Pediatrics, IU School of Medicine, Indianapolis, IN,[4]Department of Chemical and Biomedical Engineering, College of Engineering, University of Missouri, Columbia, MO,[5]Department of Neurology, School of Medicine, University of Missouri, Columbia, MO

Adeno-associated virus (AAV)-mediated CRISPR Cas9 editing holds great promise in treating Duchenne muscular dystrophy (DMD). However, previous studies have shown that Cas9 immunity drastically reduces the number of edited muscle fibers, impeding successful treatment. Thus, there is an urgent need to minimize Cas9-specific immune responses for AAV CRISPR therapy. One approach is to impose a transient expression of the Cas9 protein. This can be achieved through a self-inactivating CRISPR (SiC) system. Co-delivery of a gRNA that specifically targets the Cas9 expression cassette can abrogate the expression of the Cas9 protein, thereby becoming self-inactivating. Many groups have generated different self-inactivating CRISPR systems to reduce off-target editing. However, it is unclear whether this approach can be purposed to reduce Cas9-specific immune responses. Here, we evaluated dystrophin restoration and Cas9-specific immune responses using the SiC system in Welsh corgi muscular dystrophy (WCMD) and labrador retriever muscular dystrophy (LRMD) dog models of DMD. WCMD and LRMD dogs carry intronic insertions in the *DMD* gene. We previously designed and selected the best mutation-specific gRNAs for each model. We adopted the SiC-V2-Cas9G7 gRNA that targets a sequence in the SpCas9 genome from Kelkar et al. (Mol Ther 28:29-41, 2020). The self-inactivating gRNA was packaged either within an alkaline phosphatase (AP) reporter cassette or included as part of the therapeutic gRNA cassette. We produced AAV8 vectors and co-injected intramuscularly in one WCMD dog and two LRMD dogs. For the WCMD dog study, we co-injected the CK8.SpCas9 vector, the WCMD gRNA vector, and the AP.SiC gRNA vector at the dose of 1 x 10^{13} vg/muscle/vector. For the LRMD dog study, we co-injected one dog with the CK8.SpCas9 vector, the LRMD gRNA vector, and the AP.SiC gRNA vector at the dose of 1x10^{13} vg/muscle/vector. We co-injected the other LRMD dog with the CK8.SpCas9 vector, the LRMD gRNA. SiC gRNA vector, and an AP reporter vector at the dose of 1x10^{13} vg/muscle/vector. All vector mixtures were injected into three muscles of each dog. All injected dogs were placed under a twelve-week high-dose prednisolone immune suppression as previously published. The WCMD dog was euthanized at 3 weeks post-injection. LRMD dogs

were biopsied at 3 and 6 weeks post-injection and euthanized at 12 weeks post-injection. Plasma, serum, and PBMCs were collected every week from one day before injection till termination. We are currently processing and evaluating the tissues collected from the study. We will report our results at the annual conference (Supported by NIH).

1785. Transgene-Directed Immunological Investigations into Immune-Mediated Myositis Following Delandistrogene Moxeparvovec Gene Therapy

Sohrab Khan[1], Hélène Haegel[2], Andreas Hollenstein[2], Christoph M. Wandel[2], Damon R. Asher[1], Danielle A. Griffin[1], Emanuel Palatinsky[1], Stefanie Mason[1], Rachael A. Potter[1], Ida Moeller[1], Susan T. Iannaccone[3], Craig M. Zaidman[4], Louise R. Rodino- Klapac[1]

[1]Sarepta Therapeutics, Inc., Cambridge, MA,[2]F. Hoffmann-La Roche Ltd, Basel, Switzerland,[3]University of Texas Southwestern Medical Center and Children's Health, Dallas, TX,[4]Department of Neurology, Washington University in St. Louis, St. Louis, MO

Introduction: Delandistrogene moxeparvovec is a recombinant adeno-associated virus rhesus isolate serotype 74 vector-based gene transfer therapy, designed to compensate for absent functional dystrophin in Duchenne muscular dystrophy (DMD) by delivering a transgene encoding delandistrogene moxeparvovec micro-dystrophin, an engineered protein retaining key functional domains of wild-type dystrophin. As of January 2024, delandistrogene moxeparvovec is approved in the USA, UAE, and Qatar for the treatment of ambulatory pediatric patients aged 4 through 5 years with DMD with a confirmed mutation in the *DMD* gene. Two cases of immune-mediated myositis (IMM) were reported in ENDEAVOR (SRP-9001-103; NCT04626674), an open-label, Phase 1b, multi-cohort study of delandistrogene moxeparvovec. Case 1 occurred in a 9-year-old patient with a deletion of exons 3-43 of the *DMD* gene, 35 days post-dosing, and Case 2 occurred in a 7-year-old patient with a deletion of exons 8 and 9 of the *DMD* gene, 29 days post-dosing. Both patients experienced muscle weakness and received immunosuppressive treatment, including high-dose corticosteroids and tacrolimus. We conducted a clinical and immunological investigation to better understand the antigenic determinants of IMM events in these two patients. **Methods:** An interferon-γ enzyme-linked immunosorbent spot (ELISpot) assay was used to detect T-cell responses to delandistrogene moxeparvovec micro-dystrophin peptide pools. An *in silico* tool was used to determine micro-dystrophin peptide binding with human leukocyte antigen class I (HLA-I) or human leukocyte antigen class II (HLA-II) molecules corresponding to the patients' specific HLA allele combinations. **Results:** ELISpot analysis suggested that IMM resulted from T cell-mediated responses directed against specific delandistrogene moxeparvovec micro-dystrophin peptides corresponding to exons 8 and 9 (Case 1) and exon 8 (Case 2) of the *DMD* gene. *In silico* analysis of HLA presentation suggested a high risk of immunogenicity for peptides derived from exons 8 and 9 of the *DMD* gene. **Conclusions:** These results are consistent with clinical trials of other investigational DMD gene therapies suggesting that patients with deletions in regions of the *DMD* gene overlapping those expressed in a given microdystrophin may be at an increased risk of IMM following gene therapy. Future work may provide a better understanding of risk factors to mitigate safety concerns in patients with potentially higher-risk *DMD* mutations.

1786. A Novel Quantitative Methodology for Profiling Anti-AAV Antibodies: Insights from a Pilot Study in SMA Type I Patients Receiving Zolgensma

Tomas Baldwin[1], Ivan Doykov[1], Pierpaolo Ala[2], Nikki Cornell[2,3], Mariacristina Scoto[2,3], Giovanni Baranello[2,3], Haiyan Zhou[1], Francesco Muntoni[2,3], Kevin Mills[1], Wendy Heywood[1]

[1]Genetics and Genomic Medicine, University College London - Institute of Child Health, London, United Kingdom,[2]Developmental Neurosciences, University College London - Institute of Child Health, London, United Kingdom,[3]Great Ormond Street Hospital, London, United Kingdom

Adeno-associated virus (AAV)-based gene therapies have emerged as a promising strategy the treatment of rare diseases. However, a significant hurdle remains in the form of the common prevalence of anti-AAV antibodies in the population. There is currently no consensus in methodologies for the assessment of anti-AAV antibodies, with options ranging from semi-quantitative ELISAs to *in vitro* and *in vivo* neutralisation assays. Whilst informative, there is significant variability between outputs, leading to a call for a more quantitative approach. In this study, we present an innovative fully quantitative methodology designed to assess anti-AAV antibody response using multiplexed targeted proteomics. Through a pilot investigation involving 20 spinal muscular atrophy (SMA) patients and 10 healthy controls, we evaluated changes in the immune complex binding to AAV9 capsid (VP1-3) both pre and post AAV9-based gene therapy and correlate our findings with reported adverse events. Our findings reveal longitudinal shifts in the composition of the immune complex binding AAV9, characterized by a significant increase in the prevalence of IgG1 binding ($p<0.0001$), with IgG1 titer increasing for up to 6 months post infusion in some patients. Additionally, alterations in IgG2 binding were observed ($p<0.001$), highlighting the dynamic nature of the immune response to AAV9-based interventions. Additionally, varying levels of seroprevalence of anti-AAV9 antibodies was identified in 40% of the healthy control group ($p=0.0025$), emphasizing the significance of understanding baseline immunity in the general population. The implications of our methodology extend beyond the specific patient cohort studied, providing a valuable tool for enhancing our understanding of AAV seroprevalence in diverse populations. Crucially, the quantitative nature of our methodology allows quantitative isotype specific cutoff values before the AAV administration, and the monitoring of patients' immune responses over time, allowing . These quantitative determination could be important to assess the relevance of "below" qualitative threshold AAV determination for the long term efficacy and toxicity, and could provide a very sensitive methodology for assessing the impact of immunosuppression for re-administration of AAV-based gene therapies.

1787. Impact of Serotype Switching on Intra-Articular Administration of High Capacity Adenovirus Vectors on Transgene Expression

Jiansen Yan[1], Camilla Majano[1], Zelong Dou[1], Yangjin Bae[1], Masataka Suzuki[2], Amanda Rosewell[2], Racel Cela[1], Carolina Leynes[1], Ming-Ming Jiang[1], Philip Ng[1], Brendan Lee[1]

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX,[2]Center for Cell and Gene Therapy, Baylor College of Medicine, Texas Children's Hospital, Houston, TX

High Capacity Adenovirus (HCAd) vector-based intra-articular gene therapy has completed phase I clinical trial in osteoarthritis (NCT04119687). However, the impact of pre-existing Anti-Ad5 adaptive immunity resulting from natural infection on joint transduction and transgene expression remains unknown. Given the chronic nature of osteoarthritis progressing over many decades, multiple administrations of vectors may be required to sustain therapeutic benefits, potentially inducing new anti-vector immunity after the initial intra-articular administration. To address this issue and circumvent the preexisting immunity, we explored the the use of adenovirus vectors with different capsid serotypes. First, we evaluated the ability of different serotype vectors to mediate joint transduction in wild-type FVB/NJ mice through intra-articular injection of high capacity adenovirus 5 (HCAd5), HCAd1, HCAd2, and HCAd6 expressing luciferase. Consecutive detection of luciferase activity by IVIS demonstrated effective transduction in joint tissues and constant transgene expression over time for all HDAd serotypes. Intra-articular re-injection with HCAd5-LacZ at 16 weeks after the first injection in wild-type mice showed decreased transduction when using the same serotype vector, whereas re-administration of different serotypes of HCAd improved transduction as demonstrated by LacZ staining of knee joints. To mimic natural immunization, wild-type FVB/NJ mice were immunized with two intramuscular injections of first-generation serotype 5 Ad, followed by intra-articular administration of HCAd5, HCAd1, HCAd2, and HCAd6 expressing LacZ. Our findings revealed that preexisting Anti-Ad5 immunity resulting from immunization reduced HCAd5 vector transduction and transgene expression after intra-articular administration, while switching the serotypes to HCAd2 and HCAd1 improved transgene expression. These findings offer valuable insights into strategies for overcoming immunity against adenoviruses, potentially improving the effectiveness of HCAd vector-based intra-articular gene therapy for knee joint diseases such as in post-traumatic osteoarthritis.

1788. Rituximab Extends Duration of Efficacy of AAV-hLDLR in Reducing LDL-C in Cynomolgus Monkeys

Sijia Huang[1], Zhikui Gao[1], Simian Li[1], Yiting Liu[2], Guang Qu[1,2], Lixin Jiang[1,2]

[1]NGGT (Suzhou) Biotechnology Co., Ltd., Suzhou, Jiangsu, China,[2]NGGT, Inc, Walnut Creek, CA

Introduction: Familial hypercholesterolemia (FH) is mostly caused by a defect in the gene encoding low-density lipoprotein receptor (LDLR). Massive elevations in serum cholesterol levels are observed in homozygous FH patients (HoFH). When left untreated, this genetic disorder of cholesterol metabolism can lead to premature atherosclerosis, cardiovascular disease (CVD) and often death at a very young age. We developed NGGT006, a liver-targeting AAV-hLDLR vector and demonstrated that it restored the uptake of LDL-cholesterol (LDL-C) from peripheral blood by expressing WT human LDLR (hLDLR) protein on liver cell surface. However, in WT cynomolgus monkeys, a single injection of NGGT006 reduced serum LDL-C level for a short time. Then serum LDL-C returned to the baseline level. Associated with the declined effect in LDL-C reduction, antibodies against hLDLR were detected, as well as the significant increases of liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), suggesting the activation of immunity and liver toxicity. This study explored the effect of immunosuppression mediated by Rituximab, an anti-CD20+ B-cell monoclonal antibody in WT cynomolgus monkeys with NGGT006 administration. **Method/Result:** Four WT AAV8 neutralizing antibody (NAb) negative cynomolgus monkeys were randomly divided into NGGT006 group and combination of Rituximab/NGGT006 group, with 2 animals in each group. Rituximab was administered by intravenous injections at 20mg/kg at day -3, 18, and 71, followed by NGGT006 at 5×10^{13}vg/kg at day1. Levels of liver enzymes, AST and ALT, serum LDL-C, lymphocyte subsets (CD45+CD19+) and anti-hLDLR antibodies were monitored. In NGGT006 only group, a transit reduction of serum LDL-C level was observed for 5 weeks. In contrast, treatment of NGGT006 combined with Rituximab maintained the reduced LDL-C level for at least 24 weeks till the anatomy point. No significant increase in AST or ALT was detected. Consistent with the profound depletion of CD45+ CD19+ B-cells, no increase in anti-hLDLR antibody was observed. **Conclusion:** These data show that immunosuppression by Rituximab may result in a prolonged effect of LDL-C reduction and decrease hepatotoxicity mediated by AAV-hLDLR in non-human primates (NHPs). This study supports the clinical deployment of Rituximab in the setting of AAV-mediated LDLR gene transfer for treating HoFH.

1789. Characterization of the Immune Response to Adeno-Associated Virus Vectors in the Central Nervous System of an Ovine Model

Nathan K. Yingling, Ashley Harkins, Hector Ribeiro Benatti, Meghan Blackwood, Motahareh Arjomandnejad, Toloo Taghian, Sarah Nath, Darcy Reil, Heather L. Gray-Edwards, Allison M. Keeler-Klunk

UMass Chan Medical School, Worcester, MA

Background: Adeno-associated virus (AAV) stands out as a prominent gene delivery system. Despite AAV's relatively low immunogenicity, host immune responses remain an impediment to widespread AAV application in gene therapies. These challenges require special consideration when targeting the central nervous system (CNS) due to its unique immunologic nature. As such, research into understanding the immune response to AAV in the CNS is warranted. In an ongoing CNS-directed AAV clinical trial, the induction of systemic capsid-specific immune responses and concurrent increases in the inflammatory cytokine, CXCL10, within the cerebral spinal fluid (CSF) were observed suggesting the recruitment of inflammatory cells to the CNS. To further understand these findings, we investigated the

immune response to AAV gene therapy after CNS-directed delivery in a sheep model. **Methods:** Lambs were assigned to either, an AAVrh32.33 conjugated to secreted embryonic alkaline phosphatase (SEAP) (n=2) or a PBS control (n=1). Bilateral thalamic injections were performed, with treated lambs receiving 4.8E12 vg in 175ul per thalami and control sheep receiving 175ul of PBS per thalami. Baseline assessments included physical and neurological exams. Blood and CSF collection were performed before vector administration. Animals were then monitored for 6 weeks by clinical and neurological exams, and blood and CSF analysis. Animals were terminated at 6 weeks, and tissues were collected for immunohistopathological and vector genome analyses. **Results:** Treated lambs exhibited neurological signs, including ocular strabismus, hind limb weakness and disordered proprioception. One treated lamb was noted to have eating disturbance, leading to weight loss and termination on week 4. No neurological signs were noted in the control animal. Treated lambs showed decreases in body mass by their endpoint. As expected of growing lambs, the control increased its weight by 25%. Liver function tests were unremarkable, except for a transient increase in AST levels observed postoperatively in all animals, which normalized by week 1. Analyses in both serum and CSF of treated lambs demonstrated early and sustained increases in SEAP transgene expression, with treated lambs showing a 4.3 to 5.6-fold increase in average relative light units (RLU) in the CSF when compared to serum levels at endpoint. No transgene expression was seen in the control lamb. Serum CXCL-10 analyses showed no differences between AAVrh32.33 lambs and the PBS control, however, CSF CXCL-10 concentrations increased on weeks 3 and 5 in the treated lambs and remained elevated when compared to the control. One treated animal had a positive ELISPOT response at endpoint. **Conclusions:** These findings provide a model for understanding responses observed in clinical trials and developing immune-modulating strategies to allow for safer and more effective therapies. Our results underscore the complexities of AAV immunogenicity in the CNS and could help us further understand both local and systemic immune responses to AAV gene therapy in the CNS. Forthcoming studies will encompass flow cytometry from peripheral mononuclear blood cells isolated from peripheral blood and whole brain tissue, immunohistochemical analysis, AAV vector biodistribution, IFN-γ and PDL-1 concentrations of the CSF and serum.

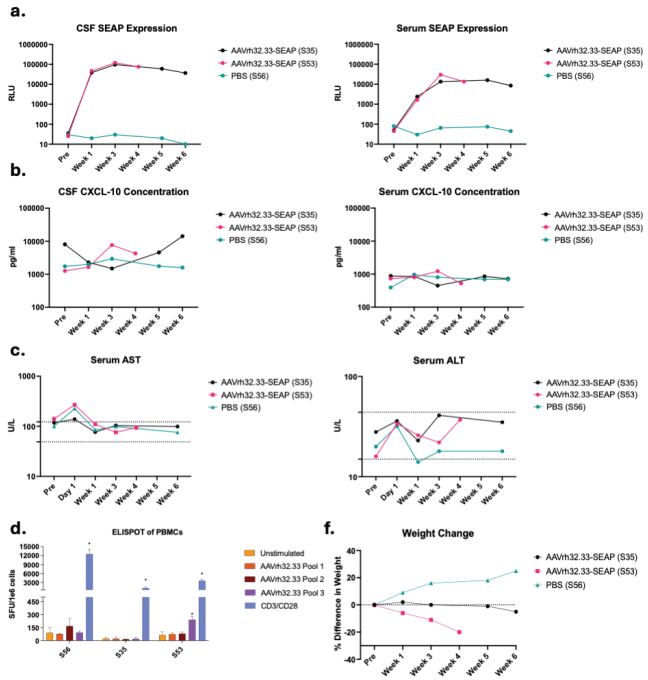


Figure 1. (a) ELISA results of CSF & Serum SEAP transgene expression. (b) CXCL-10 assay results in CSF & Serum. (c) AST and ALT lab results. (d) ELISPOT results from PBMCs collected at endpoint (* indicates significant response). (f) Changes in weights, interpreted percent change from "pre" timepoint baseline.

1790. Safety and Feasibility of Parenchymal Re-Dosing of AAV2-hAADC into Non-Human Primate

Anitvir S. Taunque, Matthew T. Rocco, Allison Clark, Akhila Dandamudi, Kaya Ceyhan, Meika Travis, Vikas Munjal, Krystof Bankiewicz, Lluis Samaranch

Neurological Surgery, The Ohio State University, Columbus, OH

Gene therapy has shown exciting and promising results in multiple clinical trials across the globe. However, the current paradigm in the field portrays it to be a one-time administration; several studies report a significant post-injection adeno-associated virus (AAV) vector-specific immune response. While this holds true for most systems within the body following systemic delivery, the brain is an immune-privileged organ with the potential to circumvent inflammatory responses depending on the delivery platform. In this study, three non-human primates (NHP) received an initial injection of AAV2-hAADC (human aromatic L-amino acid decarboxylase) within the left thalamus. After 7 months, an identical second dose was given in the contralateral thalamus. Animals were necropsied four months after the second injection and brains were processed for histology. Serum, CSF, and PBMC samples were collected periodically to assess humoral immunity, while brain sections were stained to analyze vector distribution, transgene expression, and cell-mediated responses. No significant cage-side findings were observed in any of the animals during periodic in-life neurological assessment. Whole blood chemistry showed no hematological abnormalities or signs of liver, kidney, or electrolyte dysfunction at any of the examined time

points. Immunohistochemistry assessment of brain sections showed transgene expression in both infused thalami, suggesting no AAV2 or transgene neutralization. Histopathologic analysis showed no brain edema, perivascular cuffing, interstitial cellular infiltration, or cytotoxic response, supporting the absence of an ongoing active cellular immune response. IgG and IgM serum levels remained near the baseline levels for the duration of the study. CSF and serum neurofilament light chain levels also support the same conclusion. We believe this data supports the hypothesis that a second brain parenchymal dose of AAV2 is well-tolerated and opens the opportunity to explore the clinical implications of brain parenchymal AAV2 redosing.

1791. A Study of the Immunogenicity of Cas9 in the Developing and Mature Mouse Central Nervous System

Robert Duba-Kiss[1], David R. Hampson[1,2]

[1]Department of Pharmacology & Toxicology, University of Toronto, Toronto, ON, Canada,[2]Faculty of Pharmacy, University of Toronto, Toronto, ON, Canada

Adeno-associated virus (AAV) gene therapies are being assessed as potential treatments for a variety of neurological genetic disorders. Some AAV therapies in development rely on the expression of proteins which are not endogenously produced by the patient, such as Cas9 nucleases used in gene editing. A drawback of such strategies is the risk of triggering an inflammatory immune response directed against the exogenous polypeptide, in this case a prokaryotic protein. AAV therapies for some neurodevelopmental disorders may require the treatment of newborns or infants who have immature immune systems; it is generally thought that they exhibit weaker immune responses compared to older children and adults. Inflammatory responses against foreign transgenes might therefore be mitigated by early postnatal vector administration. This study seeks to elucidate differences in the immune response to Cas9 expressed in the CNS between newborn and mature mice, with the goal of determining whether early postnatal expression of Cas9 results in lower inflammation and toxicity compared to adults. AAV-Cas9 was administered to the CNS of early postnatal mice via intracerebroventricular injection and to adult mice via intra-cortical injection; the degree of neurotoxicity and inflammation elicited in each cohort was quantified one-month post-treatment. Several key controls were included in our study. Analyses of neuroinflammation were also performed three months after neonatal treatment to assess whether any degree of immune tolerance to Cas9 observed at the 30-day post-injection time point is maintained after full maturation of the mouse brain and immune system. The results have implications for the use of non-self proteins in gene therapies for neurodevelopmental and other disorders. This research is supported by the Canadian Institutes of Health Research.

1792. Immunocompetent hiPSC-Based 3D Models to Study Innate Immune Response to Gene Therapy Vectors

Catarina Monteiro Gomes[1,2], Gabriela Silva[1], Stephan Holtkamp[3], Mark Trautwein[3], Daniel Simão[1], Paula Marques Alves[1], Catarina Brito[1,2]

[1]IBET, Oeiras, Portugal,[2]Instituto de Tecnologia Química e Biológica António Xavier, Oeiras, Portugal,[3]Bayer AG, Wuppertal, Germany

Background and novelty: Recombinant AAV (rAAVs)-based gene therapies for central nervous system (CNS) disorders face significant challenges due to host immune responses. Astrocytes and microglia initiate innate immune responses in the CNS, eliciting neuroinflammation. Nonetheless, a thorough understanding of the molecular mechanisms driving and sustaining glial cell activation remains elusive, particularly due to the scarcity of human cell models depicting the neuro-immune axis. Here, we present an innate immunocompetent 3D cell model that recapitulates neuroinflammatory hallmarks and propose it as a platform for the preclinical development of viral vector-based therapies. **Experimental approach:** hiPSC-derived neural progenitors were cultured as neurospheres in perfusion stirred-tank bioreactors (STB, 200 mL) and differentiated into neurospheroids (iNSpheroids) composed of neurons, astrocytes, and oligodendrocytes, as previously reported. hiPSC-derived microglia cells (iMGLs) were co-cultured with iNSpheroids, optimizing the medium composition and cell ratios. To assess rAAV immunogenicity and increase throughput, a miniaturized and parallelized STB system (15 mL, Ambr15) was used. iNSpheroids were challenged with inflammatory factors (e.g., TNF-α, LPS), and rAAV2 and rAAV9, candidates for gene therapy due to broad tropism and ability to cross the blood-brain barrier, respectively. **Results and discussion:** iMGL in iNSpheroids displayed morphofunctional plasticity (ameboid to ramified) and expression of microglia proteins (e.g., TMEM119 and P2RY12). Detection of phagolysosome-like structures containing MAP2-positive neurite fragments suggested physiological synaptic pruning. Upon exposure to proinflammatory stimuli, TIC (TNF-α, IL-1α, and C1q) or LPS plus IFNγ, iNSpheroids upregulated neuroinflammatory genes (e.g., IL-6 and CXCL8) and secreted inflammatory cytokines/ chemokines (e.g., CXCL8, and CCL2), all hallmarks of neuroinflammation. iNSpheroids exposed to rAAV2- and -9 had 20-40 % eGFP-positive cells, with negligible contribution from microglia and differences in neuron/ astrocyte tropism. Neuroinflammatory responses to rAAV9 were milder than to prototypical stimuli. iNSpheroids upregulated IL-6, CXCL8, and TNF. Notably, iMGL-iNSpheroids displayed downregulation of these inflammatory mediators, suggesting a protective effect of iMGL. Altogether, we report the human iNSpheroids model as a useful preclinical model to dissect CNS-specific neuroinflammatory mechanisms and viral vector immunogenicity, paving the way to accelerate the development of gene therapies.

1793. A Half-Life Extended IgG Protease with Reduced Immunogenicity to Enable Efficient *In Vivo* Gene Transfer and Vector Readministration

Maximilian M. Sauer, Benjamin Dutzar, Emily Frazier, Kui K. Chan, Daniel Farrell, Gavin Robertson, Andrew Jerome, Yifan Song, Eric Tarcha, Erik Procko

Cyrus Biotechnology, Seattle, WA

Adeno-associated virus (AAV) neutralizing antibodies (nAbs) are prevalent in humans and block AAV vector-mediated *in vivo* gene transfer and vector re-administration. Hence, AAV nAbs remain a major challenge for AAV-mediated gene therapy. Strategies to overcome this challenge often include immunosuppressives or plasmapheresis and are not effective for efficient nAb removal. The immunoglobulin G (IgG) degrading enzyme IdeS from Streptococcus pyogenes is clinically approved for transplantation patients and can cleave AAV nAbs. However, the immunogenicity of this bacterial enzyme and its short *in vivo* half-life limit its efficacy. Here, we employed computational protein design to engineer IdeS variants with reduced immunogenicity and extended *in vivo* half-life. First, in a cell culture assay, IdeS was processed by antigen-presenting cells and IdeS peptides displayed on HLA class II (HLA-II) were isolated and identified by mass spectrometry. Using machine learning algorithms trained on large data sets of HLA-II-bound peptides, mutations predicted to decrease HLA-II affinity were then introduced into the major HLA-II epitopes that we had identified. The mutations were validated biochemically to reduce peptide affinity for HLA-II and thus reduce T cell immunogenicity. Second, we introduced N-glycosylation sites into the IdeS sequence to shield B cell surface epitopes, creating hyperglycosylated drug candidate CYR241. In addition to reducing reactivity against anti-IdeS antibodies in serum from donors with recent strep infections, IdeS glycosylation also resulted in an extended *in vivo* half-life, which we further improved by generating a human serum albumin (HSA) fusion variant (drug candidate CYR212). CYR241 and CYR212 demonstrate similar endopeptidase activities and specificities as wild-type IdeS. Pharmacokinetic-pharmacodynamic studies in rabbits reveal that CYR241 and CYR212 deplete IgG for more than 5 and 12 days respectively, in contrast to short-lived, wild-type IdeS (2 days), resulting in a significantly improved therapeutic window in which antigen-binding IgG fragments have been cleared but full-length IgG has yet to rebound. In ongoing experiments, CYR241 or CYR212 administration in mice passively immunized with human intravenous Ig (IVIg), which inhibits gene therapy due to anti-AAV nAbs, enables efficient liver gene transfer by AAV vectors. In conclusion, our results provide a framework to overcome the challenge of pre-existing nAbs to AAV gene therapy allowing repetitive gene transfers with high efficiency.

1794. A Simple, Rapid and Robust Bioluminescent Assay for Detecting Anti-AAV Neutralizing Antibodies in Serum Samples

Brock F. Binkowski[1], Morten Seirup[1], Trish Hoang[2], Marjeta Urh[1]

[1]Promega Corporation, Fitchburg, WI,[2]Promega, Fitchburg, WI

Adeno-associated viruses (AAVs) are widely used vectors to deliver transgenes for gene therapy. Individuals with pre-existing immunity to AAVs are less likely to benefit from gene therapy owing to the presence of neutralizing antibodies (NAbs) that reduce cellular uptake. Widespread, pre-existing immunity in human and animal subjects increases the need for sensitive assays to detect NAb activity in serum samples, allowing drug sponsors to identify candidates for enrollment in clinical trials or patient eligibility. Here we describe a cell-based transduction inhibition (TI) assay utilizing NanoLuc® luciferase, an enzyme engineered to be extremely bright and structurally stable. The superior brightness of NanoLuc® (~150-fold brighter than firefly luciferase) enables the sensitive detection of NAb activity using low MOI values (100 - 1,000) and short incubation times (≤24 hours). The assay is tolerant to high levels of human serum, allowing dilutions as low as 1:3, and luminescence is measured following addition of an add-mix-read (homogeneous) detection reagent (Bio-Glo-AAV). We screened 1:3, 1:10 and 1:20 dilutions of human serum samples using low MOI values to demonstrate the influence of sample dilution on hit rate. We also compared head-to-head with the analogous approach using firefly luciferase as the reporter, and we validated use of thaw-and-use HEK293 cells. Taken together, this rapid, highly sensitive and reliable assay enabled by NanoLuc® AAV reporter technology precisely measures NAbs against AAVs in both human and animal serum.

Friday Posters: Immune Targeting and Approaches with Genetically-Modified Cells and Cell Therapies

1795. Dual Targeting of Drug-Resistant Liver Cancer: Integrating HSP90 Inhibition with Multikinase Inhibitor Chemotherapy and GRP78-CAR-NK Immunotherapy

Youssef Sabha, Sajjan Rajpoot, Chih-Hsiung Chen, Xuening Wang, Dongfang Liu

Pathology and Laboratory Medicine, Rutgers University-New Jersey Medical School, Newark, NJ

Drug resistance represents a major obstacle for cancer therapy, which makes hepatocellular carcinoma (HCC) difficult to treat. Drug resistance emerges when tumors have genetic changes leading to tumor evolution. The mutations in the genome cause drug-resistant cancers to have a growing number of misfolded proteins that make the proteome unstable. Tumors utilize heat shock proteins such as Heat Shock Protein 90 (HSP90) or Glucose Regulated Protein 78(GRP78), to stabilize misfolded proteins allowing cells to survive and evolve in drug-resistant cancers. By inhibiting HSP90, we make the tumor cells susceptible to drug therapies (i.e., multikinase inhibitors). Strikingly, when HSP90 is inhibited, surface expression of GRP78 increase on tumor cells allowing for precise targeting as a tumor antigen. Futhermore, HSP90 inhibition has strong effects on both the intracellular proteome through regulation of VEGFR2 signaling and surface proteome by regulation of the Heat Shock Response. Immunotherapies are growing in popularity because of harnessing the power of immunity systems instead of intracellular pathways that are targeted using chemotherapies, radiotherapy, and surgery. Natural Killer (NK) cells, are soldiers that survey the body,

killing tumor cells, dysfunctional cells, aged cells, or virally infected cells. However, tumor cells can thwart NK action through immune evasion. Our lab has recently developed Chimeric Antigen Receptor-Natural Killer (CAR-NK) therapies to target liver cancer by precise tumor detection and killing. While CAR-NK therapies have shown success in blood cancers, challenges persist in treating solid tumors such as HCC and pancreatic cancers, resulting in suboptimal outcomes. This study proposes a unique approach to enhance specific CAR-NK immunotherapy with a combination of HSP90 inhibitor chemotherapy to target the drug-resistant HCC. We used a novel "kick and kill" model whereby we "kick" the tumor cells to be sensitive to immune cells and then "kill" the tumor using CAR-NK cell therapy with enhanced cancer detection and killing. We hypothesize that inhibition of HSP90 function will improve precise GRP78-CAR-NK targeting and killing of drug-resistant HCC. To achieve this, we used high-throughput assays to evaluate the "kick and kill" model on cells derived from tumors, followed by testing efficacy of the combination therapy in various mouse models. We found combination chemotherapy and GRP78 targeting CAR-NK suppressed HCC growth in vivo and in vitro. Ultimately, this study uncovers a novel role for HSP90 in NK cell killing of tumors and how that enhances CAR-NK therapy treatment of solid tumors. This study also pushes the boundaries of traditional clinical targeting of HSP90 to synergize with novel CAR-NK immunotherapies.

1796. Unlocking CAR T Cell Activity Against Metastatic Osteosarcoma Using RNA-Based Nanoparticle Vaccination

Paul Castillo[1], Ruixuan Liu[1], Elizabeth Ogando-Rivas[1], Arnav Barpujari[1], Dingpeng Zhang[1], Hector Mendez-Gomez[1], Xiaojie Ma[1], John Ligon[1], Duane Mitchell[1], Jianping Huang[2], Elias Sayour[2]

[1]Pediatrics, University of Florida, Gainesville, FL,[2]University of Florida, Gainesville, FL

Background: Metastatic osteosarcoma (OSA) has a dismal prognosis despite novel targeted therapies, including CAR T cells. Unleashing CAR T cell therapy against poorly immunogenic cancers requires new technologies that activate the tumor microenvironment (TME), while inducing CAR T cell migration, proliferation, and persistence into tumor to sustain tumor-specific immunity. We have developed tumor RNA-loaded nanoparticle (CD70-encoding RNA-NP) vaccines to overcome CD70-specific CAR T cell limitations in OSA. **Methods:** Using two murine solid tumor models (K7M2 osteosarcoma and B16F0 melanoma, in Balb/c and C57Bl/6 background) highly metastatic to lungs, we implant K7M2 and B16F0 intravenously (i.v.). We subsequently generated NPs complexed with RNA encoding for CD70 antigen and control vaccine (GFP RNA). RNA-NPs are administered intravenously beginning on day 5 post-tumor implantation and day 7, 14 & 21. Mouse CD70-specific CAR T cells are manufactured after activation of T cells with CD3 and CD28 antibodies for 2 days. CAR T cells are ready for use in downstream experiments 2 days after pMSGV8-mCAR transduction and cells were injected (i.v.) on day 6. **Results:** In preliminary data, we show that intravenous administration of CD70-encoding RNA-NPs rapidly recruit CD70-directed CAR T cells into tumor stroma unlocking their activity in two immunocompetent aggressive lung metastatic tumor models. Interestingly, regression of CD70-expressing tumors associates with CAR T cell mobilization out of peripheral blood and into reticuloendothelial (RES) tissues (i.e., lung, and spleen), a phenomenon that correlates with upregulation of vaccine-induced type I interferon (IFN) and activation of CCR2 pathway. In addition to the CAR T-mediated antitumor killing, an antigen spreading phenomenon was observed. The long-term survival benefit (150 days) provided by this approach reached 70-80% in both models compared with 0-10% of control groups. **Conclusions:** Based on these findings, we hypothesize that tumor trafficking and persistence of CD70 CAR T cells depend on coordinated cytokine/chemokine signaling that can be ignited through intravenous RNA-NP vaccination. RNA-NPs in combination with CAR T cells prolong long-term survival of mice bearing these aggressive tumor models which was not only mediated by CAR T cells but also by activation of endogenous adaptive immunity. Future studies include the evaluation of this approach in large animal models prior to translation into first-in-human clinical trials.

1797. Generation of Monocytes and Macrophages from CD34+ Cord Blood Cells for Cell Therapy

Katherine Brempelis, Jared Pudiwitr, Carrie Stoltzman, Erika von Euw, Colleen Delaney

Deverra Therapeutics, Seattle, WA

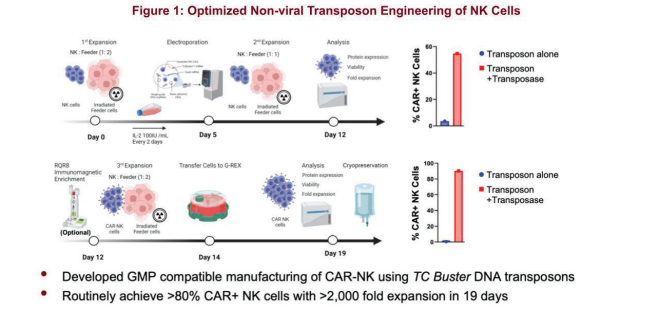
Introduction: Solid tumors are challenging targets for existing cell therapies due to poor infiltration of the tumor mass by immune cells and immunosuppression in the tumor microenvironment (TME). Current approaches to overcome these challenges have had limited success and there are still no FDA-approved cell therapies for solid tumors. In addition, autologous cell therapies are expensive, patient-dependent, and can require weeks to produce limited cell numbers. To address these issues, we developed an *ex vivo* process to expand and differentiate CD34+ cells into monocytes and macrophages, cell types that traffic to and persist in solid tumors. Furthermore, these cells can be engineered to resist immunosuppression in the TME and to recruit immune effector cells, providing an off-the-shelf cell therapy option with the potential to revolutionize the treatment of solid tumors. **Methods & Results:** We optimized three consecutive phases of *ex vivo* culture conditions to generate macrophages from pooled cord blood-derived CD34+ hematopoietic stem and progenitor cells (HSPCs): 1) expansion of CD34+ HPSCs using a proprietary culture process, 2) continued expansion and differentiation of cells into monocytes, and 3) terminal differentiation of cells into macrophages. Monocytes and macrophages were phenotypically characterized using flow cytometry and functional *in vitro* assays, including cytokine release and phagocytosis assays. From each starting CD34+ HSPC, we generate up to 400,000 differentiated progeny cells which are ≥80% CD14+CD11b+ monocytes (at the end of Phase 2) or macrophages (at the end of Phase 3). These myeloid cells phagocytose *Staphylococcus aureus* bioparticles and release cytokines in response to LPS stimulation, and our macrophages express canonical markers including CD40, CD86, HLA-DR, and CD206. **Conclusions:** Our proprietary *ex vivo* culture platform produces therapeutically relevant numbers of functional monocytes and macrophages for use as allogeneic off-the-shelf cell therapies. We are currently assessing the effects of cryopreservation as well as targets and strategies to genetically engineer these cells to drive anti-tumor immune responses while resisting the immunosuppressive TME.

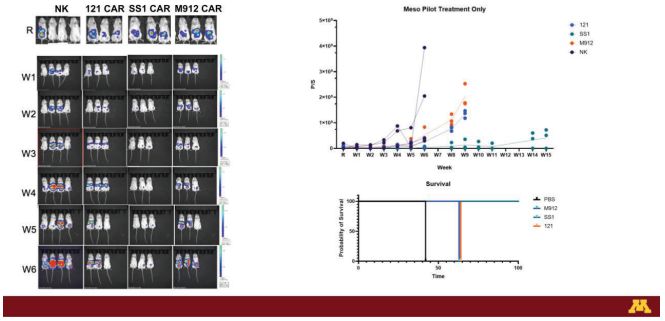
1798. Armored CAR-NK Cell Therapy for Ovarian Cancer

Joshua B. Krueger, Jae Woong Chang, Young Vue, Alexandria Gilkey, Timothy Folsom, Ethan Niemeyer, Joseph Skeate, Beau Webber, Branden S. Moriarity

University of Minnesota, Minneapolis, MN

Immunotherapy with chimeric antigen receptor (CAR) expressing NK cells engineered via viral vectors has shown exceptional efficacy against hematologic cancers in clinical trials. This method, however, is limited in cargo capacity and large-scale manufacturing for clinical use is cost-prohibitive. We have addressed these shortcomings by engineering CAR-NK cells using non-viral transposon engineering that can be paired with CRISPR/Cas9 editing simultaneously. We believe that this approach avails opportunities that will allow researchers to address some of the more difficult solid tumor cancer types. Here, we developed a CAR-NK therapy with specific advantages to killing mesothelin positive ovarian cancers which tend to form solid tumors, a new area for CAR NK cells. We started by testing 3 different mesothelin binders named SS1, 121, and M912. Our transposon vector also encodes for secretion of the cytokine IL15 for increased persistence of the CAR NK cells. The SS1, 121, and M912 CAR NK cells with soluble IL15 (Armored-NK) were tested in vitro and in vivo. No CAR variant stood out as a clear winner during the in vitro testing so all three proceeded into a pilot in vivo study. In this experiment, NSG mice were engrafted with a mesothelin high tumor cell line, A1847, engineered to expressLuciferase. A1847 cells were injected 14 days prior to the Armored CAR NK injection. Both tumor cells and Armored CAR NK cells were delivered via intraperitoneal (IP) injection.At week 6, control mice, 121 treated, and m912 treated mice had tumors beginning to grow exponentially. The SS1 CAR seemed to be controlling tumor growth. This trend continued for 3 more weeks as the SS1 treated mice did not see significant tumor growth while all other mice in the study had to be euthanized due to tumor burden. SS1 mice survived long term with the only detectable tumor being a mass that grew at the injection site, outside of the IP space. With these results we have decided to continue with the SS1 CAR and plan to test additional edits to these cells before attempting a phase 1 clinical trial. The two other modifications that were described in our pre-IND filing is a knockdown or knockout of the CISH gene, an immune checkpoint inhibitor as well as expression of a TGFb dominant negative (DN) receptor. The TGFbDN receptor is able to sequester TGFb, a cytokine often in the tumor microenvironment, which normally suppresses immune function. The binding of this cytokine to a dominant negative receptor will prevent its normal activation on NK cells. We believe these edits will push NK cells into being a viable treatment for solid tumors.





1799. Iterative Engineering of Polarization-State Responsive Synthetic Promoters for Autonomous Control of Macrophage Polarization Logic

Frances Liu[1], Michelle Hung[1], Assen Roguev[1], Magdalena Cichewicz[1], Yin Yin Chong[1], Cesar Juarez[1], William Hendriks[2], Jessica Haverkamp[2], Mark Tomishima[3], Timothy K. Lu[4], Russell Gordley[1], Philip Lee[5]

[1]GeneFab, LLC, South San Francisco, CA,[2]Blue Rock Therapeutics LP, Cambridge, MA,[3]BlueRock Therapeutics LP, New York, NY,[4]Senti Biosciences, Inc, South San Francisco, CA,[5]GeneFab, LLC, Alameda, CA

Introduction Macrophages are plastic cells capable of sensing microenvironmental cues and are generally polarized to either a pro-inflammatory (M1-like) or anti-inflammatory (M2-like) state. Macrophages can contribute to disease progression in both autoimmune disorders and cancer when polarized to a pathological state (M1 or M2, respectively). Here, we leveraged macrophages' innate ability to sense extracellular anti-inflammatory signals to design and build highly discriminatory M2 polarization-state specific promoters. We then paired these promoters with candidate polarization state master regulators for autonomous control of macrophage polarization logic. **Methods** Five rounds of promoter engineering were performed to optimize for the best M2-polarization state-specific promoters. This included 1) bioinformatic mining of native enhancer sequences from ATAC-Seq data, 2) minimal promoter engineering, 3) functional mapping of regulatory regions with mutational variants, 4) rational redesign of native enhancer elements, and 5) combinatorial rearrangement of elements from multiple enhancer families. Synthetic

promoters were paired with fluorescent reporters in lentiviral vectors and individually transduced into primary human derived macrophages and then polarized. State-specific promoter activity was assessed by fluorescent activity compared to the elongation factor 1 alpha short promoter (EFS) as a constitutive control promoter. Top M2 state-specific promoter candidates were then paired with candidate master regulators to create synthetic gene circuits to "sense" polarization state and "respond" by driving macrophage phenotype. Macrophage polarization phenotype was assessed using multi-color flow cytometry and cytokine expression. **Results** We designed synthetic enhancer-based promoters with >10-fold M2-specificity over the M1 state and >100-fold specificity over the M0 state, but they were <15% of the strength of EFS. To increase promoter strength, we reengineered the core promoter which increased promoter strength by >10-fold while maintaining high levels of M2-state specificity. From functional mapping of regulatory regions, we redesigned the synthetic promoters to achieve >800% of the strength of EFS with >150-fold M2 specificity over the M1 state and >40-fold specificity over the M0 state. By combining regulatory elements from multiple enhancer sequences, we achieved >100-fold M2 specificity over both M0 and M1 states with promoter strength ranging from >200-400% of EFS. When pairing an M2-state specific promoter with a cytokine-based M2 master regulator, we engineered an autocrine positive feedback loop thereby locking the cells into an M2-like state. We alternatively used these same M2 state-specific promoters to drive M1-associated transcription-factor based master regulators. In this case, the cells switched towards a pro-inflammatory phenotype in the presence of immunosuppressive cues. **Conclusions** We have built an iterative promoter discovery platform capable of being applied to the discovery of novel extracellular cue-responsive promoters in many cell types. We used this platform to discover promoters specific to the M2 polarization state in macrophages. We also demonstrated modularity of these state-specific promoters with multiple payload types (e.g., cytokines and transcription factors) which enabled us to build gene circuits that control macrophage polarization phenotype to either "lock" the cells in a pre-determined state or "switch" it from one polarization state to another.

1800. PSMA/NKG2DL Tandem CAR T-Cells to Overcome Antigen Heterogeneity and Improve Anti-Tumor Efficacy Against Prostate Cancer

Jennifer Bolsée, Benjamin Violle, Thuy Nguyen, Céline Jacques-Hespel, Eytan Breman

R&D, Celyad Oncology, Mont-Saint-Guibert, Belgium

Background: Chimeric antigen receptor (CAR) T-cell therapies have shown remarkable success in treating hematological malignancies. However, demonstrations of efficacy in solid cancer indications have been limited so far, largely due to antigen heterogeneity, antigen loss or modulation, and inadequate T-cell function. Hence, design of multispecific CAR T-cells, targeting several cancer antigens simultaneously, might be envisaged as one of the best options to overcome antigen heterogeneity in solid cancers. NKG2D ligands (NKG2DL), are a group of 8 stress-induced ligands expressed by virtually all cancer types and absent from normal healthy cells. These therefore represent an attractive approach for multispecific CAR T-cells in large panel of cancer indications, including prostate cancer. Methods: To improve CAR T-cell efficacy against prostate cancer, we designed a prostate specific membrane antigen (PSMA)/NKG2DL tandem CAR T-cell candidate that encompasses the extracellular domain of the natural NKG2D receptor fused to an anti-PSMA scFv. Functionality of the PSMA/NKG2DL tandem CAR T-cells was evaluated in cocultures with cells expressing the main antigen (i.e. PSMA and NKG2DL) or not (only NKG2DL) and was compared to the functionality of single-targeting CAR T-cells. Results: We show here that PSMA/NKG2DL tandem receptor was highly expressed and that T-cells expressing this tandem were able to secrete IFN-γ against prostate cancer cell lines expressing, (LNCAP cells and PC-3 PSMA, PSMA+) or not (PC-3 WT cells, PSMA-) the principal target antigen. In addition, our results demonstrate that PSMA/NKG2DL tandem CAR-T cells proliferated and eliminated PSMA+ and PSMA- prostate cancer cells *in vitro*. Finally, upon chronic antigen stimulation with HeLa cells overexpressing PSMA, PSMA/NKG2DL tandem CAR T-cells were able to control tumor cell growth and proliferate as efficiently as PSMA single-targeting CAR T-cells. Conclusions: Our *in vitro* data indicate that PSMA/NKG2DL tandem CAR T-cells are an attractive approach to overcome antigen heterogeneity and improve anti-tumor efficacy in prostate cancer. In addition, by targeting stress-induced targets rather than lineage specific targets, the issues with antigenic heterogeneity/modulation may be avoided. Additional *in vivo* studies will be performed to validate *in vitro* results obtained so far.

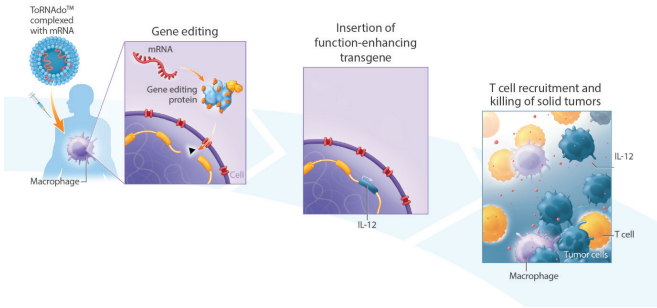
1801. iPSC-Derived Macrophages Engineered to Express IL-12 Enhance Cancer Cell Killing of a Multi-Cell Type Therapeutic Platform

Ian Hay, Abigail Blatchford, Claire Aibel, Christopher B. Rohde, Matthew Angel

Factor Bioscience Inc., Cambridge, MA

The immunologically cold microenvironment of solid tumors limits the ability of immune cells to infiltrate and attack cancer cells. The systemic injection of potent cytokines such as interferon gamma (IFNγ) and interleukin 12 (IL-12) have shown tumor shrinkage in mouse models, but have faced challenges in human clinical trials due to dose-limiting toxicities. Myeloid cells, including macrophages, can deliver cargo to the solid tumor microenvironment. However, autologous macrophages are limited by their resistance to genetic engineering, limited expansion potential, cost of characterization and the limited quantity of cells attainable via leukapheresis. Bioreactor-based differentiation of induced pluripotent stem cells (iPSCs) into macrophages can overcome the limitations of autologous macrophages, enabling the generation of large numbers of well characterized, genetically identical, engineered macrophages. Here, we present iPSC-derived macrophages engineered to overexpress IL-12 for delivery to the tumor microenvironment. Bi-allelic knockout of the beta-2-microglobulin gene ($B2M^{-/-}$) is designed to reduce alloreactivity of transplanted iPSC-derived macrophages. $B2M^{-/-}$ iPSC-macrophages transfected with mRNA encoding both IL-12 subunits joined by a self-cleaving peptide (IL-12 P2A) or a flexible linker (IL-12 fusion) resulted in the production of 20 and 40 ng/mL of the IL-12 p70 heterodimer, respectively, as assessed via ELISA 24 hours after transfection. Both IL-12 mRNA constructs resulted in similar levels of IFNγ production when co-cultured with PBMC-derived T cells (17 ng/mL for IL-12 fusion and 15 ng/mL for IL-12 P2A) with no detectable IFNγ in the absence of IL-12 mRNA or T cell co-culture, indicating functional bioactivity. The co-culture

of B2M$^{-/-}$ iPSC-derived macrophages with allogeneic PBMC-derived T cells did not upregulate T-cell activation markers CD25 or CD69, as assessed via flow cytometry, suggesting a lack of host-vs-graft-type immunoreactivity of the B2M$^{-/-}$ iPSC-derived macrophages, a feature of importance for durable adoptive cell therapies. When IL-12-mRNA transfected iPSC-derived macrophages were co-cultured with PBMC-derived T-cells and a GFP-expressing ovarian adenocarcinoma cell line SK-OV-3 at a 10:1 effector to target ratio for 72 hours, cancer cell killing was significantly greater when compared to non-transfected iPSC-derived macrophages, as assessed by real-time fluorescence imaging (p=0.03). Engineered iPSC-derived macrophages offer a path to repolarize the tumor microenvironment and translate the clinical success of T cell immunotherapies targeting hematologic cancers to solid tumors, which comprise 90% of cancer cases. Here we show a scalable platform for producing iPSC-derived macrophages engineered to express the potent immunostimulatory protein IL-12. Engineered iPSC-derived macrophages may thus prove useful for the development of therapies designed to deliver biologics previously limited by toxicity or bioavailability directly to the tumor microenvironment.



1802. A Dermal Fibroblast Derived Human Induced Pluripotent Stem Cell (hiPSC) Line That Efficiently Differentiates into NK Cells

Vivek Chandra, Lindsay Bailey Steinitz, James Pierpoint, Jenny Yang, Garrett Wong, Erik Willems

Thermo Fisher Scientific, Carlsbad, CA

Natural killer (NK) cells are specialized cells of the immune system that play critical roles in innate and adaptive immunity. Their ability to target and kill tumor cells have made them a promising candidate for the next generation of cell-based immunotherapies. NK cells, like T cells, can be engineered to express chimeric antigen receptors (CARs) to improve targeting the cancer cell of interest. NK cell therapies have the potential to use as an allogenic therapeutic with main advantages being reduced cost and greater accessibility to patients. The use of primary NK cells to reach expansion requirements for manufacturing has proved difficult, and their genetic engineering is more challenging compared to other immune cell types. However, human induced pluripotent stem cells can be utilized to generate a nearly endless supply of NK cells and their genomes can be engineered easily. Here, we report the generation of a human induced pluripotent stem cell (hiPSC) lines with the intent to identify iPSC lines that are amenable to NK differentiation. We identified that one of the hiPSC lines derived from human dermal fibroblasts, which was normal in a variety of pluripotency and genomic stability assays, efficiently differentiates into NK cells. These NK cells express functional NK cell markers, and we also demonstrate that these hiPSC differentiated NK cells are functional by different cell-based assays like target cell killing and cytokine release assays. In summary, we have identified iPSC lines and a differentiation workflow that enable efficient generation of functional iPSC-derived NK cells.

1803. Nanobody-Based CAR Targeting of an Endogenous Retroviral Envelope for the Treatment of Glioblastoma

Saliha Majdoul[1], Wenxue Li[2], Kevon Sampson[2], Kari Wilson[1], Deepa Seetharam[3], Anandani Nellan[1], Ashish H. Shah[3], Avindra Nath[2], Naomi Taylor[1]

[1]National Cancer Institute, Center for Cancer Research, Pediatric Oncology Branch, National Institutes of Health, Bethesda, MD,[2]National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD,[3]Department of Neurosurgery, University of Miami School of Medicine, Miami, FL

Glioblastoma multiforme (GBM), identified as grade IV astrocytoma, is the most common malignant brain tumor and is highly aggressive. Despite surgical resection, radiotherapy, and chemotherapy it has a poor prognosis with a median survival time of 14-16 months. Thus, it is critical to develop new therapeutic strategies for these patients. We recently showed that the human endogenous retrovirus HERV-K (subtype HML-2), normally silenced in non-malignant cells, is reactivated in GBM and contributes to the maintenance of glioblastoma stem cells, enabling tumor regrowth. Furthermore, the HML-2- envelope (Env) is expressed at high levels at the surface of GBM tumors compared to healthy brain tissue, making it an attractive target for the development of a chimeric antigen receptor (CAR)-based immunotherapy strategy. We first developed an HML-2 Env-specific nanobody through the screening of a synthetic humanized VHH phage display library and used epitope fingerprinting to map binding to a non-linear epitope in the Env surface unit domain. This nanobody was then incorporated into CAR constructs harboring either 4-1BB or CD28 costimulatory domains. Notably, HML-2 Env-targeting CARs were efficiently expressed at the surface of transduced primary T cells and exhibited high induction of both IFN-γ and IL-2 cytokines in response to HML-2 Env+ GBM at multiple effector:target ratios. Moreover, HML-2 Env CAR-T demonstrated high *in vitro* cytotoxicity, as assessed by cellular impedance assays. These promising results highlight the strong potential for a HML-2 Env-targeting CAR T-cell therapeutic approach for the treatment of GBM. S.M. and W.L. are co-first authors. A.H.S., A.N. and N.T. are co-senior authors.

1804. Repeated Antigen Stimulation Mediated Exhaustion, Not Fratricide, Drives B7H3 CAR-NK Cell Dysfunction in Glioblastoma Multiforme

Kajal Chaudhry[1], Nethaji Muniraj[1], Joshua L. Terao[2], Ashley Geiger[1], Ehsan Dowlati[3], Haili Lang[1], Eric S. Yvon[2], Matthias Holdhoff[4], Rick Jones[4], Barbara Savoldo[5], Michael D. Keller[1], Conrad Russell Y. Cruz[1], Catherine M. Bollard[6]

[1]Children's National Hospital, Washington, DC,[2]The George Washington University, Washington, DC,[3]Department of Neurosurgery, Georgetown University Medical Center, Washington, DC,[4]Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD,[5]UNC Lineberger Comprehensive Cancer Center, Chapel Hill, NC,[6]The George Washington University, Bethesda, MD

Glioblastoma multiforme (GBM) is a devastating brain cancer with a uniform poor prognosis for patients with relapsed/refractory disease. We have evaluated allogeneic "off the shelf" NK-cell-based immunotherapies against GBM in pre-clinical models. We hypothesized that redirecting NK-cell specificity to the tumor-associated antigen B7H3 using a chimeric antigen receptor (CAR) would enhance the potency of NK-cell products against GBM. We showed that B7H3-CAR-NK products elicited robust killing against GBM cells (89.73±2.44 % @20:1 E:T ratio) in vitro. However, CAR-NK products failed to show a significant effect on the survival in the U87 MG *in vivo* model compared to untransduced NK cells (P>0.05). Hence, we interrogated multiple mechanisms, including B7H3 antigen upregulation/trogocytosis, TGF-β mediated suppression, and NK cell exhaustion. First, we observed that CAR-NK and untransduced NK cells (Un-NK) cells upregulated B7H3 antigen on activation. We knocked out (KO) the B7H3 antigen on NK cells to avoid B7H3 antigen upregulation and associated fratricide, however, we failed to see any significant fratricide in KO-CAR-NK cells compared to control CAR-NK products. Second, we confirmed that trogocytosis-mediated transfer of B7H3 antigen to CAR-NK also did not mediate significant fratricide in Trog+ve CAR-NK compared to Trog -ve CAR-NK cells. Third, we identified that TGF-β secreted by GBM rendered CAR-NK cells dysfunctional with significant downregulation of activation receptors, including NKG2D (68.14%±6.92% downregulation; p<0.05), which markedly reduced NK cell cytolytic ability. To overcome TGF-β immune suppression, we co-transduced NK-cells to express the B7H3-CAR and the TGF-β-DNR (72.21% ± 23.05%). Co-expression of TGF-β-DNR and B7H3-CAR on NK cells preserved their cytolytic function and activating receptor expression in the presence of TGF-β in vitro. Co-transduced DNR.B7H3-CAR-NK-cells lysed 85.45 %±3.05% of U87-MG cells in the presence of TGF-β in comparison to 80.89% ±14.84% in its absence (E:T ratio 20:1; p>0.05). However, DNR.B7H3-CAR-NK cells failed to enhance murine survival in the U87 MG *in vivo* model. To understand the CAR-NK product failure we used a repetitive tumor stimulation method, which recapitulates the in vivo model better. Using this model, we confirmed that Un-NK and B7H3 CAR-NK cells have limited serial killing ability compared to B7H3 CAR-T cells. NK cells showed functional exhaustion post stim 2 with significantly decreased proliferation, reduced IFN-γ and TNF-α secretion, and diminished cytolytic activity (p<0.05) compared to B7H3 CAR-T cells. Therefore, we next evaluated whether supplementing with IL-15 could rescue CAR-NK cells from exhaustion. We observed that by adding IL-15, CAR-NK and Un-NK serial killing were comparable to B7H3 CAR-T cells. In sum, we conclude that B7H3 CAR-NK cell dysfunction is related to NK cell exhaustion in response to repetitive antigen stimulation, which can be overcome with local IL-15 delivery. Our next step is to validate this approach in vivo, If successful, we will evaluate CAR-NK products engineered to resist TGF-β and secrete IL-15 in the clinical setting for patients with relapsed/refractory GBM.

1805. Novel Costimulatory Domain Improves CAR-M Activity Against Tumors

Sumiti Jain, Kevin Carbajal, Kilsoo Jeon, Anastasya Birger, Grace Reynolds, Prasanna Ekambaram, Beatrice Breckheimer, Jessica Gorczynski, Warren Anderson, Adam Pecoraro, Matthew Esquivel, Max Richter, Matthias Schroff

Inceptor Bio, Raleigh, NC

Myeloid cells, unlike other immune cells such as T cells or NK cells, are known residents in the solid tumor microenvironment (TME). In the absence of checkpoints and in proinflammatory conditions, myeloid cells (particularly M1 macrophages) are known to be capable of direct phagocytosis of tumor cells and can present tumor-associated antigens to the host immune system. However, the immunosuppressive conditions within the TME restrict and limit the anti-tumor response of tumor associated macrophages (TAMs), including their ability to recruit and activate other immune cells against the tumor. Engineered CAR-Monocytes can serve a unique function in cell therapy by bridging a key gap in the treatment of solid tumors. We are developing an autologous engineered CAR-M cell therapy product targeting Glypican-3 (GPC3) to treat hepatocellular carcinoma (HCC). The CAR serves a dual purpose as a homing 'GPS' signal for trafficking directly to the tumor site, and for directing phagocytosis specifically at targeted tumor cells. We have designed a proprietary CAR molecule (M83 CAR) which contains a macrophage-specific costimulatory domain that significantly increases the phagocytosis function of the CAR-M cells. Furthermore, we have demonstrated that the M83 CAR-M cells are not inhibited by the prevalent CD47 checkpoint ('do not eat me' signal expressed on tumor cells), which is known to restrict myeloid function in the TME. Effective phagocytosis of engineered CAR-M cells is central to subsequent mechanisms of actions that can elicit robust anti-tumor immunity against patient-specific neoantigens. However, the first barrier to overcome is effective infiltration of engineered CAR-M into the tumor from the periphery. We have demonstrated that our engineered CAR-Monocyte drug product can successfully home to the targeted tumor specifically, from the periphery. Subsequent *in situ* differentiation of CAR-monocytes into CAR-macrophages (M1) enables robust tumor cell phagocytosis, the central mechanism that can result in the following series of events: 1) proinflammatory shift in the TME milieu, 2) recruitment of innate and adaptive immune cells, 3) activation of T-cells against tumor neo-antigens. This is a unique outcome of the CAR-M mechanism of action that is not capitulated by CAR-T or CAR-NK cells. Leveraging and further enhancing CAR-M function could lead to the rejection of the tumor and its metastases, particularly in combination with other immune-modulating therapies.

1806. Evaluating mRNA-Based Multi-Antigen Targeting CAR T Cell Therapy Against Glioblastoma

Oula Dagher[1], Avery D. Posey[1,2]

[1]Systems Pharmacology and Translational Therapeutics, University of Pennsylvania, Philadelphia, PA,[2]Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA

Glioblastoma multiforme (GBM) is the most common and aggressive primary brain malignancy, characterized by heterogenous antigen expression and very low survival rates post standard therapy. Chimeric antigen receptor (CAR) T cell therapies are immune cells engineered *ex-vivo* via viral transduction, gene-editing and integration, transposition, or mRNA incorporation and exert potent target-specific anti-tumor cytotoxicity. Due to the complex tumor heterogeneity and self-renewal capacity of glioma stem cells, localized intracranial delivery of CAR T cells engineered to target multiple GBM associated antigens have been investigated both preclinically and clinically and have demonstrated better remission profiles as compared to single antigen targeting CAR T cells. However, most anti-glioma CAR T cells investigated have been engineered via viral transduction for constitutive expression of CAR molecules, which introduces risks of off-tumor toxicities and the perpetuation of neurotoxicity in GBM patients. Here, we hypothesize that the combination of surgical resection with multivariant targeting of glioma antigens via mRNA-based CAR T cells would provide a safe and efficacious translational platform for treating GBM patients. Using two separate mRNA incorporation strategies, we have utilized a "one-day" CAR T cells production protocol and evaluated whether treatment of tumors derived from U87-GM cells with pooled mRNA-based CAR T cells (CARPool) or mRNA-based CAR T cells expressing multiple CARs (MVCAR) targeting IL13Rα2, HER2, and/or EphA2 would elicit effective anti-tumor efficacy. We utilized real-time eSight imaging and impedance analysis, flow-cytometry based analysis, and ELISA to compare best-in-class combination of multi-antigen targeting CAR T cells against a heterogenous pool of single-antigen null U87 glioma cells (U87KO-pool) *in vitro*. The efficiency of our single-scFv mRNA-based CAR T cells was initially validated by the target-specific cytotoxicity of U87 cells that express the target glioma antigen as well as increased CAR T cell proliferation and IFNγ secretion, and lack of cytotoxicity of target-null U87 cells. Notably, triple-targeting CARPool or MVCAR T cells elicited superior efficacy against U87KO-pool as compared to single- or dual-targeting CAR T cells, at low E:T ratios *in vitro*. We utilized glioma xenograft mouse models in NSG mice undergoing partial tumor resection to evaluate the efficacy of localized delivery of triple-targeting mRNA-based MVCAR or CARPool. We created a resection cutoff criterion that maintains only minimal residual disease and used this criterion to subdivide mice within each group based on resection intensity (desired or undesired). Our preliminary data from the resection model shows that CAR T cell treated mice undergoing "desired resection" had higher survival rates as compared to their counterparts in "undesired resection" subgroups. Significance: Multivariant targeting of glioma associated antigens with mRNA-based CAR T cell therapies provide an easily translational platform for the management of GBM patients undergoing surgical resection and help counteract solid tumor evasion mechanisms. Additionally, the one-day protocol for transient CAR expression in mRNA-based CAR T cells is expected to generate a safer and faster alternative to traditional CAR T cells undergoing standard manufacturing.

1807. cGMP-Grade MAD7 TARGATT™ Master iPSCs for Allogenic Cell Therapies

Ruby Tsai[1], Alfonso Farruggio[2], Simon Wu[3], Lin Jiang[4]

[1]CEO and President, Applied StemCell, Inc., Milpitas, CA,[2]Head of Research and Development, Applied StemCell, Inc., Milpitas, CA,[3]Associate Director, Project Management, Applied StemCell, Inc., Milpitas, CA,[4]Production/GMP Cell Products, Applied StemCell, Inc., Milpitas, CA

Induced pluripotent cells (iPSCs) for site-specific DNA-fragment knock-in are essential for developing and manufacturing the next generation of cell therapeutic products. Traditional genome editing technologies, such as CRISPR/Cas9 systems, can be used to establish stable knock-in cell lines; however, transgene size can be limited, and substantial licensing fees are required to move the engineered cell line into product development and manufacturing. To generate a ready-to-use cGMP-grade iPSC line for site-specific gene of interest (GOI) knock-in, we adapted the TARGATT system by integrating an attB landing pad into the Hipp11 locus (H11) by MAD7 in a cGMP-grade iPSC line, and isolated desired clones harboring the landing pad, which then were confirmed by Sanger Sequencing. With an established cGMP-grade TARGATT iPSC line, we then inserted a single copy of RFP construct at the H11 locus to verify efficiency of knock-in (insertion) as well as expression robustness and the ability of engineered iPSCs to be differentiated into natural killer (NK) cells (iPSC-derived NK cells). Our data demonstrates that the TARGATT system can be particularly useful for cancer immunotherapy applications. For example, unique therapeutic genes (such as CAR constructs) can be inserted in the cGMP TARGATT Master iPSC line for differentiating into NK cells or T cells that could potentially be utilized to fight various serious human diseases.

1808. Exploring Adenovirus Type 35 as Oncolytic Virus in Combination with NK-Cell Therapy as a Novel Concept in Cancer Treatment in Respiratory Disease

Luca Scarsella[1], Wenli Zhang[2], Serge C. Thal[3], Anja Ehrhardt[1], Malik Aydin[4]

[1]Witten/Herdecke University, Witten, Germany,[2]Witten/Herdecke, Witten, Germany,[3]Helios University Hospital Wuppertal, Wuppertal, Germany,[4]Faculty of Health, Department of Human Medicine, Witten/Herdecke University, Witten, Germany

Oncolytic therapy using adenoviruses (Ad) presents a promising approach for the treatment of solid tumors, as evidenced by multiple clinical trials. Besides oncolytic viruses, also Natural killer (NK) cells are becoming a central role in CAR-based cellular immunotherapy against malignancies. However, the potential synergistic implications of combining adenoviral oncolytic therapy with NK cell-mediated immunity remain insufficiently studied, although NK cells demonstrated robust anti-viral and -tumor activity. While there are indications in the literature that Ad5-based vectors carrying Ad35 or Ad37 fibers may transduce NK cells, a comprehensive analysis of a broad spectrum of

human Ads on primary NK cells is lacking. Therefore, our primary aim was to identify optimal Ad vector candidates and investigate the role of NK cells in the context of Ad transduction. To address this objective, we investigated different recombinant Ad vectors (n=11) from different species containing a dual-reporter cassette in the E3 region of the Ad genome, encoding a green-fluorescent protein, a nano-Luciferase, and a neomycin resistance gene. To monitor the transduction efficiency in cells following 24 hours of cultivation, we conducted luciferase assays and quantitative PCR to measure transduction efficiencies and viral progeny in the supernatant. PBMCs were isolated through centrifugation and subsequently NK cells were isolated. A co-culture model involving Ad35-infected A549 cells and primary NK cells was established to analyze the impact of oncolytic therapy on NK cell cytotoxicity. Our investigations revealed that Ad35 from species B using CD46 as its primary cell entry receptor, and Ad37, derived from species D using GD1a glycan as an entry receptor, exhibited the highest transduction efficiencies in PBMCs obtained from healthy donors. Subsequently, we identified Ad types with optimal tropism for NK cells, with Ad35 as the most suitable for viral transduction using 2000 and 5000 viral particles per cell (vpc) for NK cell infection (p=0.0286). Furthermore, Ad35 demonstrated potent oncolytic activity in A549 cells, resulting in robust cell lysis 3 days post-infection (p=0.0001). To understand the role of NK cells during Ad transduction, co-culture experiments using primary NK cells were performed. These experiments revealed the presence of Ad virion in the culture medium produced by infected A549 cells and we detected infected NK cells. We hypothesize that the infection of NK cells was the result of viral progeny derived from infected A549 cells, which led to infection of NK cells. Moreover, an LDH assay indicated an increase in LDH secretion (20 %) as a marker of cell toxicity in the co-culture medium, particularly when A549 cells were pre-transduced with Ad35, which was in sharp contrast to uninfected A549 cells. Flow cytometry analyses of Ad-specific surface receptors on NK cells post-infection revealed a decrease of CD46 expression (18 %) in comparison to uninfected NK cells, while CAR expression remained constant. In conclusion, our findings demonstrate that Ad35 effectively transduces NK cells and exerts a rapid oncolytic effect in A549 cells, making it a promising candidate for both oncolytic and cell-based viral therapies. In the subsequent steps, we will focus on human lung tumor samples and the molecular analysis of alterations in NK cells after Ad35 vector application.

1809. Characterization of Allogeneic Engineered Human "Tissue-Tregs" with Immune Suppression and Tissue Homing Capabilities for Treatment of Acute Inflammatory Diseases

Yash Agarwal, Priya Saikumar, Maegan E. Hoover, Guoping Deng, Madison Milaszewski, Carlos E. Frias, Abigail Doherty, Scott Hussell, Rebecca Brunette, Marko Repic, Tiffany F. Chen, Thomas Wickham, Payam Zarin

GentiBio, Cambridge, MA

FoxP3+ Regulatory T cells (Tregs) orchestrate immune tolerance, thus preventing life-threatening autoimmunity, while maintaining tissue homeostasis and promoting repair following tissue damage. Tissue-resident Tregs appear to have a highly specialized gene expression program that enables the simultaneous activation of the anti-inflammatory and pro-repair functions. Adoptive transfer of allogeneic tissue Tregs could prove effective in patients with acute inflammatory conditions such as Acute Respiratory Distress Syndrome, acute kidney injury, or acute ischemic stroke where only a swift intervention could be useful, necessitating an "off-the-shelf" therapeutic that could ameliorate disease within hours. However, while the repair program has been well characterized in murine models, the understanding of tissue Tregs in humans is limited. Here, we engineer tissue Tregs from bulk CD4+ T cells with enhanced tissue-homing and suppression. Further, we demonstrate the effectiveness of allogeneic Tregs when transferred into a mouse model of acute lung injury. Engineered human regulatory T cells (EngTregs) were generated from CD4+ T cells using CRISPR-Cas9 to knock-in a strong promoter at the *FOXP3* gene locus to stabilize *FOXP3* expression and express a chemically inducible IL-2 signaling complex (CISC). This promotes rapamycin-activated IL-2-like signaling only in the engineered cells, thus enabling enrichment of FoxP3+ cells in the culture process. To impart a tissue-Treg phenotype, we overexpressed a 'tissue-Treg' associated protein in EngTregs. We found that the final product (Tissue EngTregs) had a robust Treg phenotype as measured by high expression of key Treg-associated proteins such as FoxP3, CD25, CD39, HLA-DR, TNFRII and more, and increased suppression of proliferation of a polyclonal pool of effector T cells relative to control mock-engineered cells. Tissue EngTregs also upregulated several chemokine receptors (CCR4, CCR5, and CCR8) that would enable chemokine-dependent homing to inflamed tissues and demonstrated modulation of tissue repair. When exposed to an acute inflammatory cytokine milieu, tissue EngTregs also respond by rapid sequestration of several inflammatory molecules (e.g. TNFα, IL-2, IL-4, IL-33) *in vitro*. Therefore, Tissue EngTregs have significant potential for the treatment of acute inflammatory diseases. As proof-of-principle we also evaluated the efficacy of allogeneic surrogate murine Engineered Tregs (mEngTregs) in an LPS induced model of acute lung injury. mEngTreg treated animals showed significantly improved disease outcome and lower levels of inflammatory cytokines such as IFNγ and TNFα in the bronchoalveolar lavage (BAL). Also, mEngTregs were found to effectively home to the site of pulmonary inflammation, and resemble endogenous Tregs in expression of key markers such as FoxP3, CD25, ST2 and CCR4. Overall, these data are supportive of the potential for developing a potent off-the-shelf engineered Treg cellular therapy for human acute inflammatory disease.

1810. Preclinical Development of KYV-201, an Investigational Allogeneic Anti-CD19 CAR T-Cell Therapy for Autoimmune Diseases

Ashley Mahne[1], Brandon Kwong[1], Joseph Cheng[1], Jessica Wang[1], Jesus Banuelos[1], Peter Starokadomskyy[1], Soo Park[1], Candice Gibson[1], Shouvonik Sengupta[1], Simone Sandoval[1], Jazmin Bravo[1], Jeanne Flandez[1], Shairaz Shah[1], Amanda Goodsell[1], Yong Zhang[2], Ian Miller[2], Birgit Schultes[2], Tom Van Blarcom[1]

[1]Kyverna Therapeutics, Emeryville, CA,[2]Intellia Therapeutics, Cambridge, MA

Background: Autologous anti-CD19 CAR T cells show early clinical evidence of safety and efficacy for the treatment of numerous autoimmune diseases (Schett G. *Lancet*. 2023). Allogeneic CAR T cells have the potential to further address drug supply for the substantial B-cell driven autoimmune disease population with advantages that include scalability, removal of the need for patient apheresis, and reduced treatment wait times. Emerging data in autoimmune disease trials suggests long-term CAR T-cell persistence is not required as patients have achieved long-term remissions (>1 year) despite B-cell compartment reconstitution within months (Schett G. ACR 2023). This suggests persistence-associated limitations of allogeneic CAR T cells observed in oncology (DiNofia AM. *Nat Rev Clin Oncol.* 2021) may not apply to the same extent to autoimmune disease. KYV-201 is an investigational allogeneic anti-CD19 CAR T-cell therapy for B-cell-driven autoimmune diseases. KYV-201 combines the same fully human anti-CD19 CAR construct as KYV-101, an autologous CAR T-cell therapy being studied in lupus nephritis, systemic sclerosis, myasthenia gravis, and multiple sclerosis, with a differentiated allogeneic platform developed by Intellia Therapeutics involving selected CRISPR/Cas9-mediated gene edits (Blarcom TV, *CAR TCR Summit. 2023;* Taubmann J. EULAR 2023*)*. These edits intend to avoid GvHD by removing the endogenous TCR and provide immune evasion to prolong CAR T-cell persistence by removing key cell surface proteins involved in host recognition of an allogeneic cell product. *Objectives*: To generate preclinical data supporting advancement of KYV-201 to first-in-human clinical studies. *Methods*: CAR-mediated and target-dependent activity of KYV-201 against both CD19$^+$ target cell lines and primary B cells was demonstrated with in vitro cytotoxicity, cytokine production, and proliferation studies, as well as with an in vivo xenograft model. Functionality of the immune evasion edits was demonstrated by in vitro assays modeling host T and NK-cell-mediated rejection mechanisms. Genotoxicity concerns of the product were assessed through chromosomal translocation, karyotyping, and cytokine independent growth assays. *Results*: KYV-201 demonstrated CAR-mediated and CD19-dependent cytotoxicity, cytokine production and proliferation against the human CD19$^+$ NALM6 cell line. In vivo, KYV-201 effectively controlled growth of a CD19$^+$ NALM6 xenograft in a dose-dependent manner. In co-culture with allogeneic T and NK cells, KYV-201 mitigated allogeneic recognition and rejection responses. In co-culture with allogeneic healthy donor PBMCs, KYV-201 eliminated primary B cells and proliferated in a CAR-dependent manner (Figure 1), avoiding rejection by alloreactive T cells and NK cells. Genotoxicity assays revealed minimal levels of translocations or chromosomal abnormalities by ddPCR and karyotyping, and a lack of transformation by cytokine independent growth assays. *Conclusion*: The preclinical data generated for KYV-201 demonstrates the functionality and safety necessary to advance to first-in-human studies, bringing the promise of allogeneic CD19 CAR T cells for autoimmune diseases closer to fruition.

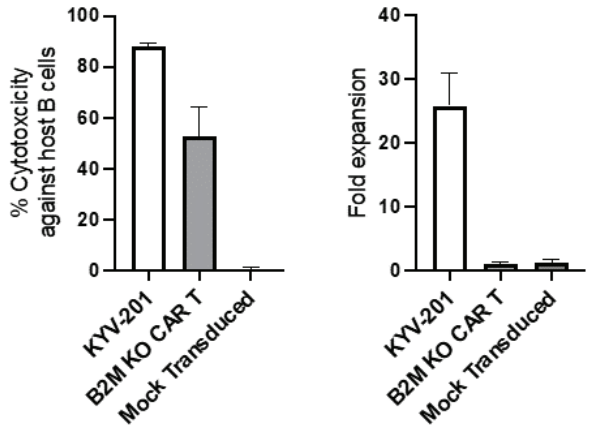


Figure 1. KYV-201, CAR T cells lacking HLA Class I (B2M KO CAR T cells), or mock transduced T cells were co-cultured with allogeneic PBMCs for 6 days at a (CAR) T cell:PBMC ratio of 1:30. KYV-201 demonstrated CAR-mediated cytotoxicity against primary B cells that was higher than that seen with B2M KO CAR T cells (left panel). KYV-201 expanded following CAR-mediated activation, in contrast to B2M KO CAR T cells that were mostly eliminated by the end of co-culture (right panel).

1811. A Novel Approach to Target Autoreactive B Cells Using ADAMTS13-Derived Chimeric Auto-Antigen Receptors (CAARs) T Cells in the Context of Immune-Mediated Thrombotic Thrombocytopenic Purpura (iTTP)

Roman H. Khadka[1], Kalpana Parvathaneni[2], Jennifer Hoffmann[1], Ezgi Tezer[1], Morgan Hresko[1], Don L. Siegel[1], Vijay Bhoj[2]

[1]Center for Cellular Immunotherapy, University of Pennsylvania, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, PA

Background: Immune-mediated thrombotic thrombocytopenic purpura (iTTP) is a severe form of thrombotic microangiopathy caused by autoantibodies directed against ADAMTS13 (disintegrin and metalloproteinase with thrombospondin motifs) which functions as von Willebrand factor (vWF) cleaving protease. Accumulation of ultralarge vWF during iTTP is characterized by microvascular thrombi, focal neurological symptoms and ischemic multi-organ injury leading to high degree of fatality in absence of interventions. While existing strategies to manage iTTP including plasma exchange offer transient benefit, there is frequent relapse in patients highlighting the need for more robust and sustained therapies to tackle both the autoantibodies and contributing autoreactive B cells. **Objective:** To address this gap, we utilized a novel approach to target ADAMTS13-specific B cells using chimeric auto-antigen receptors (CAAR) T cells as therapeutic approach for iTTP. **Methods:** We generated CAARs employing different combinations of ADAMTS13 domains as extracellular targeting domains. We also created a panel of target B cell-like Nalm6 cell lines expressing iTTP patient-derived autoantibodies. ADAMTS13-CAAR T cells were tested for efficacy and specificity using co-culture assay with Nalm6 target cells *in-vitro*. In vivo experiments utilized Nalm6 targets in a murine xenograft model to test the efficacy of ADAMTS13-CAAR T cells compared to a non-specific CD19-targeted CAR T

(CART-19). We further assessed the ability of ADAMTS13-CAARs to target polyclonal patient B cell using a serum adsorption assay. **Results:** ADAMTS13-CAAR T cells exhibited specific cytotoxicity against Nalm6 cells-expressing human iTTP autoantibodies. We additionally observed robust IFN-gamma secretion and degranulation as measured by CD107a expression in ADAMTS13-derived CAAR T cells. Importantly, we demonstrate using xenograft murine model that CAAR T cells are capable of persisting and targeting B cells expressing autoantibody specific to human-ADAMTS13. Adsorption of iTTP sera with ADAMTS13-CAAR T cells significantly depleted autoantibodies compared to adsorption with control non-CAAR T cells ($p<0.05$, t-test). **Conclusions:** We have successfully demonstrated ADAMTS13-based CAAR T cells can eliminate pathogenic B cells. Hence, CAAR-based T cell approach has significance and potential applications to ameliorate thrombotic microangiopathy in iTTP

1812. Engineering Tregs with a CAR, Phenotype Lock™ and Synthetic Cytokine Signaling Improves Their Potential as a Cellular Therapy for Various Immune Conditions

Sim Tung, Bethany McCloskey, Somayya Manzoor, Mahnoor Irfan, Julieta Karegli, Joanne Anstee, Marco Romano, Yasaman Shahrabi, Amy Billman, Evanthia Nikolopoulou, Jenny McGovern, Daniela Penston, Nathalie Belmonte, Marc Martinez-Llordella, Tracey Lodie

Quell Therapeutics, London, United Kingdom

Regulatory T cell (Treg) therapy is a promising new approach to induce immune tolerance in organ transplantation and autoimmunity. Some of the limitations of polyclonal Treg cell therapy include lack of specific targeting, the loss of stability and limited durability. Chimeric antigen receptors (CARs) are recombinant fusion receptors that can redirect Tregs to recognize desired target antigens. FoxP3 is a hallmark marker and an essential master transcription factor of Tregs which controls numerous genes responsible for Treg phenotype, stability and function. Tregs require the crucial IL-2 cytokine for their generation, survival, expansion and stability. IL-2 promotes high phosphorylated STAT5 (pSTAT5) levels within Tregs which cascades signaling events downstream to induce pro-survival protein production amongst other cellular events to sustain Treg viability and expansion. We have designed and engineered multiple novel modules which can provide Tregs with specific targeting via CAR molecules, a Phenotype Lock™ which maintains high FoxP3 expression and a synthetic receptor to trigger survival signals. Our results demonstrate that Tregs engineered with a CAR recognise and respond to the desired target antigen by upregulating an immune activation response. Tregs bestowed with the Phenotype Lock™ technology expressed and maintained high levels of FoxP3 expression along with other Treg-associated markers, supporting their suppressive function. The synthetic cytokine receptors developed utilise the pSTAT5 signalling pathway and mediate a constitutive signal with equivalent downstream transcriptional effects to the IL-2 signalling pathway. We have developed a toolbox of synthetic cytokine signaling technologies which consist of a tethered cytokine and a receptor dimerization approach. These approaches can be selected for engineering into the cell depending on the strength of pSTAT5 signalling required. The synthetic cytokine signaling allowed the engineered Tregs to extend their survival in vitro, importantly without secretion of pro-inflammatory cytokines. Gene expression analysis revealed that the CAR Tregs with the engineered modules expressed high levels of Treg stability and survival related genes, which supports the in vitro functional readouts. Furthermore, CAR Tregs equipped with the synthetic cytokine signaling demonstrated therapeutic capabilities and survived longer in murine models. In conclusion, we have demonstrated that our CAR, Phenotype Lock™ and synthetic cytokine signaling technologies can be used to engineer and enhance Tregs as a way to promote their specific targeting and durability whilst maintaining their suppressive function and phenotypic stability.

1813. Development of a Novel Allogeneic γδ-T Platform for Off-the-Shelf Adoptive Cell Therapy for Oncology and Autoimmune Diseases

Arash Nanbakhsh, Lukasz Wlodarek, Kevin Nguyen, Weijie Lan

Overland Pharma, South San Francisco, CA

Introduction: Despite significant clinical benefit for treating hematological malignancies and recent exciting development for autoimmune diseases, autologous cell therapy products face meaningful challenges in terms of expansion to broad patient population, including manufacture scale-up, high cost and certain severe adverse effects. Owing to their innate and MHC-independent cell killing capacities, γδ-T cells have gained increasing interest as a differentiated allogeneic cell type. Establishing robust expansion protocols and elucidating the functional properties of γδ-T cell subsets are fundamental to explore the therapeutic potential of γδ-T cells in both oncology and autoimmune diseases. **Methods:** γδ-T cells were enriched from PBMCs using multiple donors. Enriched γδ-T cells were transduced with virus constructs with different promoters and expanded under different culture conditions. The phenotypes of the cells were analyzed using multi-color full spectrum flow cytometry. The innate and CAR-mediated γδ-T cell killing activity were measured using flow cytometry and real-time imaging with Incucyte. In vivo target cell killing activity was demonstrated using NSG mice inoculated with luciferase expressing cancer cell lines. **Results:** We found that the abundance and composition of γδ-T varied significantly among different donors. We observed that the cytotoxicity properties of γδ-T cells, both innate- and CAR-dependent, were strongly influenced by donor variability and method of expansion. We developed efficient γδ-T cell expansion protocols that achieved up to >1,000,000-fold cell expansion over a 3-week period of culture without apparent signs of exhaustion or impaired cytotoxicity. Our expansion method generated potent Vδ2 T cells and a unique subset of Vδ1 T cells that highly express CD107, Granzyme B, IFN-γ, and IL-2. We screened viral constructs and identified viral envelops and promoters that support robust viral transduction and CAR expression in γδ-T cells. We found that CAR-γδ-T with mixture of both CAR-Vδ2 and -Vδ1 present maintained complete tumor control during a 30-day serial stimulation assay, whereas CAR-Vδ2 T cells alone failed to supress tumor growth beyond the first 7-days across all tested donors. Sequential rechallenge with target tumor cells ultimately led to selective expansion of CAR-

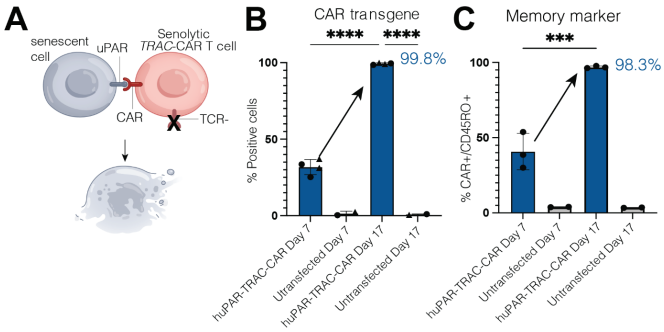
Vδ1 T cells. Vδ1 subtype enrichment could also be achieved in the absence of immune challenge with a small molecule compound. Finally, we demonstrated that CAR- γδ T cells could effectively control tumor growth in a NSG mouse model with disseminated tumor cells. **Conclusions:** We have established a unique and scalable allogeneic γδ-T platform with demonstrated CAR mediated in vitro and in vivo efficacies. We are able to generate Vδ1, Vδ2 and mixture of CAR- γδ T cells for potential off-the-shelf adoptive cell therapy in different cancer and autoimmune disease indications.

1814. Non-Viral Senolytic CRISPR CAR T Cell Products Autonomously Refine into Pure Cell Therapies

Lauren E. Sarko[1,2], Jeremiah Riendeau[3,4], Claire Shepley[1], David Givand[1], Roshini Traynor[1], Lei Zhao[5], Mackenzie Mnuk[1], Tyler Ulland[2], Mahau Dey[5], Melissa Skala[3,4], Krishanu Saha[1,3]

[1]Wisconsin Institute for Discovery, University of Wisconsin-Madison, Madison, WI,[2]Department of Pathology and Laboratory Medicine, University of Wisconsin-Madison, Madison, WI,[3]Department of Biomedical Engineering, University of Wisconsin-Madison, Madison, WI,[4]Morgridge Institute for Research, Madison, WI,[5]Department of Neurological Surgery, University of Wisconsin-Madison, Madison, WI

Cellular senescence is a biological process that reduces proliferation and helps to prevent the propagation of damaged cells. Controlling senescence within the body can be critical, as recent discoveries have identified its role in the pathophysiology of various disorders. Lysing senescent cells within the body using engineered cells can provide a new therapeutic modality to treat disease or prevent tissue dysfunction. Here, we exploit nonviral CRISPR genome editing reagents to efficiently generate T cells that express chimeric antigen receptors (CARs) against a senescence-associated cell surface protein, urokinase plasminogen activator receptor (uPAR). CAR T cells targeting uPAR were efficiently produced by knocking out the endogenous T cell receptor, alpha chain, gene (*TRAC*), and inserting a CAR transgene driven by the *TRAC* promoter. CAR T cells targeting murine and human uPAR proteins exhibit specific and significant cytotoxicity *in vitro* against induced and patient-derived senescent cells with limited off-target cytotoxicity against healthy dividing cells. During manufacturing, human *TRAC*-CAR cell products are autonomously refined into highly enriched CAR+ memory T cells, with purities reaching >98% CAR+/*TRAC*-/CD45RO+ phenotypes within weeks (*Figure*). Facile manufacturing of gene-edited CAR T cell products could enable new senolytic therapy for aging and various senescence-associated pathologies.



huPAR-*TRAC*-CAR T Cells target senescent uPAR+ T cells in culture and display a central memory phenotype after manufacturing. (**A**) Schematic of huPAR-*TRAC*-CAR T cells eliminating uPAR positive senescent cells. (**B**) Summary of flow cytometry for CAR transgene integration of huPAR-*TRAC*-CAR T cells on days 7 and 17 post-nucleofection. huPAR-*TRAC*-CAR T cells showed significantly increased mCherry expression from day 7 to 17 relative to Untransfected T cells. (**C**) Summary of flow cytometry of CAR-2A-mCherry/CD45RO double positivity from day 7 to 17. huPAR-*TRAC*-CAR (blue) n=3, untransfected control (grey) n=2. Ordinary one-way ANOVA; *p≤0.05; **p≤0.01; ***p≤0.001; ****p≤0.0001. ANOVA, analysis of variance.

1815. Characterization of CNTY-101, an Allogeneic Anti-CD19 iPSC-Derived NK Product, for the Treatment of B Cell-Driven Autoimmune Diseases

Mark Mendonca, Kaitlin Idank, Ciara Budd, Andriana Lebid, Dan Perry, Heidi Jessup, Buddha Gurung, Barry Morse, Jill Carton, Liam Campion, Michael F. Naso

Century Therapeutics, Philadelphia, PA

Achieving long-term remission in B cell-driven autoimmune diseases (AID), including Systemic Lupus Erythematosus (SLE), remains a substantial unmet need. Recently, numerous autologous anti-CD19 CAR T therapies have shown promise in treating patients affected by Systemic Lupus Erythematosus (SLE) by depleting autoreactive B cells. Allogeneic induced pluripotent stem cell (iPSC)-derived cells present a unique opportunity to offer a clonal, off-the-shelf cell therapy product. We describe a cell therapy product CNTY-101, with multiple precision gene edits, featuring antigen-specific killing of CD19+ B cells, homeostatic cytokine support for enhanced persistence, edits to prevent rejection by the patients' immune system, and a safety switch. In non-clinical studies, we demonstrate CNTY-101's ability to induce CD19-specific cytolysis of both CD19+ tumor cells and isolated B cells from multiple healthy and SLE patient donors. When compared with peripheral blood mononuclear cell (PBMC) derived CAR-T cells, CNTY-101 exhibits similar potency while observing less detectable inflammatory cytokine secretion after B cell depletion. The inclusion of the homeostatic cytokine transgene allows for the persistence of CNTY-101 several days after the depletion of CD19+ malignant and non-malignant B-cells. EGFRt is an elimination tag that can be engaged using anti-EGFR antibodies to deplete CNTY-101. The inclusion of Allo-EvasionTM edits (B2M and CIITA knockouts and HLA-E knock-in) allows for evasion from recipient PBMCs, enabling repeat dosing with and without lymphodepletion. CNTY-101, an off-the-shelf cell therapy product with multiple precision gene edits targeted to B Cell-driven diseases, including AID, demonstrates rapid B cell depletion with a favorable cytokine secretion profile, in vitro persistence and the opportunity for repeat dosing without host rejection.

1816. Universal Cloaking of Allogeneic Cell Therapies Against Natural Killer Cells via CD300a Agonism

Shuqi Zhang[1], Faith Thomas[1], Justin Fang[1], Jonathan Kurtz[1], Dustin Whitney[2], Gabriella Santone[1], Rodolfo Nazitto[1], David Monteiro[2], Daria Shlyueva[3], Francis Cervone[3], Chad Cowan[4], Grant Welstead[1]

[1]Discovery Biology, Clade Therapeutics, Cambridge, MA,[2]Computational Biology, Clade Therapeutics, Cambridge, MA,[3]Cell Engineering, Clade Therapeutics, Cambridge, MA,[4]Clade Therapeutics, Cambridge, MA

A major barrier to the persistence and efficacy of allogeneic, off-the-shelf T cell therapies is the host natural killer (NK) cell response. Natural ligand-based approaches, such as HLA-E, only inhibit subsets of NK cells whose frequencies vary host-to-host, preventing a universal solution to NK alloreactivity. Inspired by the earliest studies in inhibitory signaling, we engineered a class of synthetic ligands, called Trans Antigen Signaling Receptors (TASR), capable of agonizing inhibitory receptors previously inaccessible. One member, targeting CD300a, exhibited best-in-class performance and enhanced T cell persistence against all hosts tested (45/45), spanning diverse demographics and NK cell phenotypes. CAR-T cells co-expressing a CD300a TASR at the B2M locus and CD19-CAR at the TRAC locus via non-viral integration exhibited enhanced persistence and efficacy in a clinically relevant model system. Thus, CD300a TASRs represent a universal solution to NK alloreactivity, broadening the population that could be effectively treated by next-generation allogeneic cell therapies.

1817. Highly Stable Liquid IL-7 and IL-15 Closed System Solution Formulation for Sterility Assurance and Reproducible Biological Activity in Cell Therapy Application

Ezequiel Zylberberg[1], Juan Manuel Rodriguez[2], Julio Cesar Vega[2], Mahajoub B. Bello Roufai[3]

[1]Research and Process Development, Akron Biotech, Boca Raton, FL,[2]Research and Development, Laboratorio Pablo Cassará S.R.L,, Autónoma de Buenos Aires, Argentina,[3]Research and Process Development, Akron Biotech, FL, Sarasota, FL

Cytokines are important factors for T cell development and homeostasis. In addition to the TCR and costimulatory receptors, cytokines provide stimulatory signals for full T cell activation, and have pleiotropic effects on T cell proliferation, differentiation and function. Cytokines IL-2, IL-7 and IL-15 have been found to stimulate the growth of T cells and are used at specific concentration optimized depending on the production platforms. We have developed and distributed rHu IL-7 and rHu IL-15 in their lyophilized form and documented under Drug master File # MF026084. We have also launched our Closed system Solutions ™ (CSS) line of cGMP products that functionally closes critical unit operations in cell and gene therapy manufacturing workflow. Evolving cell therapy processes are requiring lower Million International Units (MIU), preferably in the single use format to accommodate speed, cell stimulation consistency (biological activity) and sterility assurance, moving away from the traditional lyophilized formats. Consequently, the future of the industry requires the development of formulations at low MIU, in which the biological activity of these cytokines is maintained for an extended period. However, cytokines are known to be susceptible to chemical and physical instability, especially at low concentration. They are also susceptible to aggregation, surface adsorption, and oxidation. Therefore, the formulation of the cytokines at lower protein concentrations in small bioprocessing bags, compatible with their chemical, physical and biological stability in an emerging industry need. Leveraging our decade long expertise in bioproducts including cytokines, we previously developed and launch a CSS format of rHu IL-2 that available on the market. Here we report the formulation development of our CSS format for rHu IL-7 and rHu IL-15 at low concentration and comparative results from three lead formulations designed based on their buffering and their compatibility with stabilizing agents, excipients and bioprocess bags to improve stability. We also report results comparing our formulated CSS format of rHu IL7 and rHu IL-15 to the traditional lyophilized format. The formulation development approach adopted for rHu IL-7 and rHu IL-15 CSS format could facilitate development of a wide range of cytokines in a format that meets new cell therapy processing modalities.

1818. Development and Evaluation of Immuno-Cell Growth Medium (Akron ICGM™): A Novel Xeno-Free Cell Expansion Medium Demonstrated to Support Superior Cell Activation, Viability, Transduction and Expansion

Jun Hung CHO[1], Cindy Farino Reyes[2], Darwing Rivas[3], Dennis O. Concepcion[1], Federico Franchi[2], Ryan R. Heetai[1], Ezequiel Zylberberg[4], Mahajoub Belcym Bello Roufai[1]

[1]Research and Process Development, Akron Biotech, FL, Sarasota, FL,[2]Cell Therapy, Cytiva, Marlborough, MA,[3]Research and Process Development, Akron Biotech, FL, Boca Raton, FL,[4]Research and Process Development, Akron Biotech, Boca Raton, FL

Over the past decade, significant progress has been made in T cell-based immunotherapy. Naturally occurring or genetically engineered T cells have been used to target cancer antigens in hematological malignancies and solid tumors. The approach has been proven to be promising and has been validated with recent clinical trial successes and FDA approvals of several Chimeric Antigen Receptor (CAR) T-cell therapy products. The treatment requires that T cells isolated from a patient blood be modified, culture-expanded ex vivo with a tumor cytotoxic phenotype and then re-infused back to the patient. Therefore, the development of growth medium that can effectively support ex vivo activation, transduction and expansion of isolated T-cells has been a point of focus for the industry with a few T-cell media already commercially available. Leveraging our decade long expertise in bioproducts including cytokines and human serum, we studied several media supplements to establish a basal T-cell media able to promote acceptable PBMC growth. From our initial development, five (5) Xeno-free lead formulations (A, B, C, D E) were nominated and further compared with commercially available T-cell media for PBMC expansion. Akron's Formulation C showed superior performance and was sent to several Akron partners for evaluation on their cell therapy platforms using T-cells isolated from healthy donors. Here,

we summarize data from our initial media development through the establishment of our five lead formulations and the comparison with commercially available T-cell media. We also report data obtained on T-cells from healthy donors evaluated of by Cytiva, one of the partners selected to test our novel Immuno-Cell Growth Medium (Akron ICGM™).

1819. New Technologies for CAR-T Characterization and Potency Testing

Christopher Dashiell, Julia Gilden, Pete Stecha, Aileen Paguio, Jim Hartnett, Brock Binkowski, Dan Lazar, Frank Fan, Mei Cong

Promega Corporation, Madison, WI

Despite significant clinical advances in CAR-T cell therapies for cancer, the ability to precisely and reproducibly analyze the potency and identity of these drug products remains a challenge. As new CAR-T products and production methodologies are developed, it is becoming increasingly important to be able to rigorously evaluate the relative potencies of each batch. We have developed two MOA-based bioluminescent assays that overcome challenges in quantitative assessment of T cell therapies. Both assays rely on split NanoBiT luciferase technology. In the HiBiT Target Cell Killing Assay, incubation of CAR-T cells with target cells stably expressing HiBiT results in lysis of the target cells and release of HiBiT proteins. These HiBiT proteins bind with high affinity to LgBiT in the detection reagent and form functional NanoBiT luciferase, resulting in production of light in proportion to target cell lysis. Lumit Immunoassays are a simple and homogeneous method for measuring cytokine production from engineered T cells. Monoclonal antibodies are covalently labeled with LgBiT and a low-affinity tag, SmBiT, which have negligible association in solution. When the antibodies are brought together on a common analyte, such as a cytokine, SmBiT and LgBiT can complement to form NanoBiT luciferase and produce light in proportion to the concentration of analyte in the sample. Lumit Immunoassays can be run directly on cells, removing media transfer, dilution, and wash steps that can be common sources of error. When autologous CAR-T products are being produced in parallel, it is essential to verify the identity of each product and ensure that it is administered to the correct patient. We will demonstrate an easy workflow for STR analysis that can be used to document the chain of identity for CAR-T products.

1820. Serum Proteomic Analysis Identifies Markers Associated with Anti-CD19 CAR T Therapeutic Response in Autoimmune Diseases

Justin Chou[1], Ricardo Grieshaber-Bouyer[2,3], Daniela Bohr[2,3], Christina Bergmann[2,3], Jule Taubmann[3], Fabian Müller[3,4], Aline Bozec[2,3], Tobias Rothe[2,3], Andreas Mackensen[3,4], James Chung[1], Georg Schett[2,3]

[1]Kyverna Therapeutics, Emeryville, CA,[2]Department of Internal Medicine 3 - Rheumatology and Immunology, Friedrich-Alexander Universität Erlangen-Nürnberg and Universitätsklinikum Erlangen, Erlangen, Germany,[3]Deutsches Zentrum Immuntherapie, Friedrich-Alexander Universität Erlangen-Nürnberg and Universitätsklinikum Erlangen, Erlangen, Germany,[4]Department of Internal Medicine 5-Hematology and Oncology, Friedrich-Alexander Universität Erlangen-Nürnberg and Universitätsklinikum Erlangen, Erlangen, Germany

Background: Anti-CD19 CAR T-cell therapy has demonstrated promising therapeutic efficacy and tolerability in patients with systemic lupus erythematosus (SLE), dermatomyositis (DM), and systemic sclerosis (SSc)[1-4]. The broad, deep, and short duration of B-cell depletion possible with anti-CD19 CAR T-cell therapy may provide sustained treatment-free remission for patients with autoimmune disease; however, the immunological impact of CAR T-cell therapy remains unclear. *Objectives*: In this study, we evaluated immunologic mechanisms and explored novel biomarkers by analyzing serum proteomic profiles at baseline and remission. *Methods*: Serum samples were collected prior to conditioning chemotherapy (baseline) and 3 months post-CAR T-cell infusion for 3 SLE, 3 diffuse SSc, and 2 DM patients treated at the Friedrich-Alexander University Erlangen-Nürnberg. Additional serum samples from 10 normal, 7 SLE, and 7 SSc donors were obtained from ProteoGenex. More than 7000 proteins were profiled with SomaScan® technology, which uses probe-tagged protein-binding aptamers for high-throughput proteomic profiling with an NGS readout. Differentially expressed proteins were identified by Wilcoxon test and used for Reactome pathway analysis. *Results*: We first evaluated data quality by checking the proteins known to be modulated by CAR T-cell therapy. Levels of IgM, IgA, and IgE were significantly reduced at 3 months post-CD19-CAR T-cell infusion, while IgG showed no significant change. When comparing normal donors with patient-derived samples at baseline, we observed elevated IFN signaling molecules (CXCL10 and MX1) in SLE samples and endothelial dysfunction markers (VEGF and ANG2) in SSc samples. Our proteomic analysis showed that HSF-1-mediated heat shock response (HSPA1A, DNAJA4, etc.) and type I IFN signaling (IFIT3, ISG15, etc.) were the two major pathways downregulated after CD19-CAR T-cell therapy (Figure 1). Moreover, an autoantigen poly(U)-binding-splicing factor 60 kDa (PUF60) was also reduced at post-infusion. These markers are selectively expressed by innate immune cells and co-expressed with proteins related to neutrophil degranulation and IL12 signaling. *Conclusions*: Our analysis provides new insights into the potential mechanisms of CAR T-cell treatment in patients with autoimmune diseases. These findings may facilitate future identification of markers predictive of CAR T-cell therapy response. *References*: 1. Feng J, et al. *Blood*. 2022;140 (suppl 1):10335-

10336. 2. Bergmann C, et al. *Ann Rheum Dis*. 2023;82:1117-1120. 3. Mackensen A, et al. *Nat Med*. 2022;28:2124-2132. 4. Haghikia A, et al. *Lancet Neurol*. 2023;22:1104-1105.

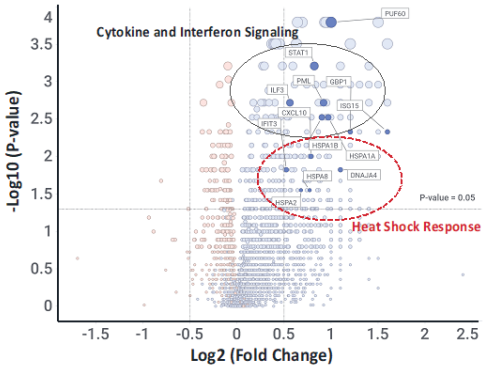


Figure 1. Proteins Differentially Expressed at Pre– and Post–CAR T-Cell Therapy
Heat shock protein mediated responses and type I IFN signatures were downregulated in patients who received anti-CD19 CAR T-cell therapy.

1821. eMHC-CAR as a Platform for Class I & Class II-Reactive Antigen-Specific T Cell Engagement, Depletion, and Tolerization

Cameron L. Gardner, Kerry Dobbs, Xiaobing Cui, Lindsay Whalen, Kristina Gurung, Ryan Grant, Sofia Makowska, Edward Dong, Hiromitsu Harimoto, Mattia G. Gollub, Eli N. Weinstein, Julie E. Norville, Elizabeth Wood

R&D, JURA Bio, Inc, Boston, MA

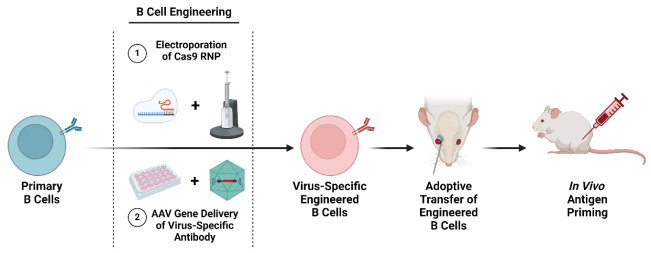
T cell mediated autoimmune diseases are driven by aberrant T cell recognition of innocuous peptides presented on major histocompatibility complexes I and II in healthy tissues. While the search space for peptide-MHC (pMHC) and TCR interactions is vast, there are a number of reported TCRs with crystal structure resolved cognate pMHC interactions. scFv based cell therapies targeting pan T cell markers have the potential to abrogate disease progression in autoimmune disease and T cell cancers, however targeting T cells in a non-specific manner carries significant risks such as immunosuppression, viral reemergence, cytokine release syndrome, neurotoxicity, and incomplete immune reconstitution in the case of bone marrow transplantation. Here, we present engineered major histocompatibility complex chimeric antigen receptors (eMHC-CARs), which functionalize the pMHC component of the pMHC-TCR immunological synapse to eliminate or tolerize TCR containing T cells which recognize a specified peptide driving pathogenesis. By functionalizing the pMHC trimer in crystal structure resolved pMHC-TCR interactions, we are able to target only T cells which interact with the specified peptide contributing to disease, leaving T cells without specificity to the pMHC complex untouched. To our knowledge, in addition to class I eMHC-CARS, we are the first to report human class II eMHC-CARs in human cells, and demonstrate that *in vitro*, and with preliminary *in vivo* mouse results, eMHC-CARs are specific, sensitive, and multifunctionalizable.

1822. Potent *In Vivo* Expansion of B Cells Engineered to Express Protective Antibodies

Grady Gastelum[1,2], Katrina Poljakov[1], Matthew Gray[3], Jennifer E. Adair[1], Justin Taylor[2]

[1]Translational Science and Therapeutics Division, Fred Hutchinson Cancer Research Center, Seattle, WA,[2]Division of Infectious Diseases & International Health, University of Virginia, Charlottesville, VA,[3]Vaccine and Infectious Disease Division, Fred Hutchinson Cancer Research Center, Seattle, WA

The COVID-19 pandemic and an estimated 15-19 billion global respiratory virus infections annually showcase the significant risk respiratory viruses pose to public health. While vaccines have helped reduce this risk, inducing potent and durable protective antibodies against the major families of respiratory viruses remains a challenge. While antibody infusions can provide protection against respiratory viruses, protection is typically short-lived and can lead to viral escape. To overcome these challenges, our lab is developing a CRISPR-based method to engineer B cells to produce multiple potent and broad virus-specific antibodies to provide lifelong protection against multiple respiratory viruses. We have previously shown that primary B cells can be engineered to express a Respiratory Syncytial Virus (RSV)-specific antibody in place of the antibody naturally expressed by cells. Following adoptive transfer of a high number of engineered and differentiated B cells into wild-type mice, protective levels of antibody were achieved seven days post transfer and returned to baseline levels 18 days later. Building off this and other studies, we now focus our efforts on investigating and optimizing the long-term protective ability of antibody-engineered B cells. Here we demonstrate that adoptive transfer of a low number of engineered B cells followed by *in vivo* antigen priming augments the antigen-specific immune response to achieve protective titers within 7 days. Ongoing work in our lab is exploring the durability of these responses and how to optimize them, with the goal of maintaining high antibody titers with minimal need for antigen boosting.



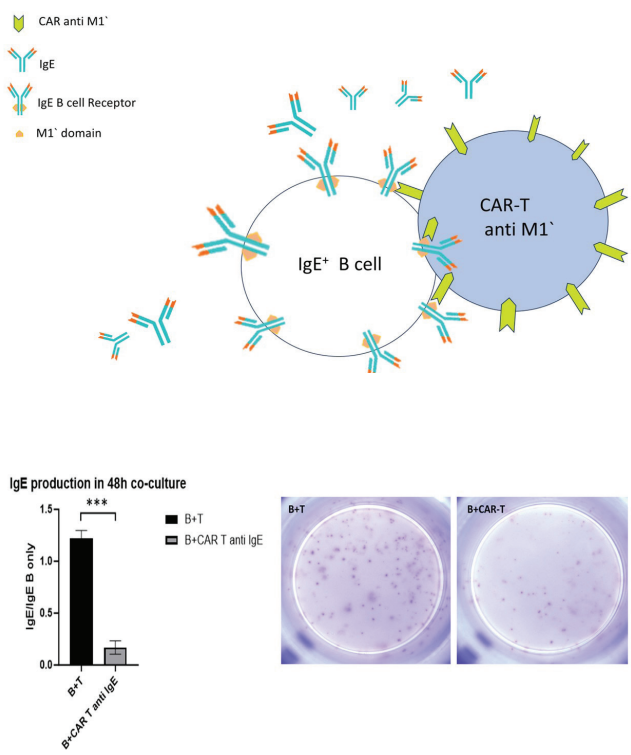
Experimental Overview: Primary mouse B cell engineering is accomplished through electroporation of Cas9 ribonucleoprotein targeting the heavy chain locus and AAV-mediated gene delivery of the virus-specific antibody. The engineered B cells are then adoptively transferred into host mice followed by *in vivo* antigen priming injections.

1823. Chimeric Antigen Receptor T Cells Targeting IgE B Cell Receptor Successfully Eliminate IgE-B Cells and Specifically Abolish IgE Production *In Vitro*

Tali Stauber[1], Miri Fried Horowitz[1], Maayan Zaba[1], Iris Dotan[1], Fred Finkelman[2], Alessio D. Nahmad[3], David Ochayon[1], Eldar Carmel[4], Adi Barzel[1]

[1]Tel Aviv University, Tel Aviv, Israel,[2]Cincinnati Children's Hospital, Cincinnati, OH,[3]Belinson Hospital, Petach-Tikva, Israel,[4]Sheba Medical Center, Ramat Gan, Israel

The prevalence of IgE mediated allergies has risen dramatically in the westernized world. Current treatment for allergies fails to offer a robust cure. In an attempt to eliminate all IgE producing cells, other groups have designed chimeric antigen receptor (CAR) T cells targeting IgE. However, these CARs target IgE epitopes found both on secreted IgE and on IgE B cell receptors (BCRs), which are membrane bound IgE antibodies. Thus, these previous anti-IgE CAR designs were sensitive to both activation and saturation by free IgE and in some cases also by FcεR bound IgE. In order to specifically target IgE expressing B cells, rather than free IgE, Genentech has developed the monoclonal antibody (mAb) Quilizumab. Quilizumab targets an epitope on the M1' domain, found only in the longer of two splice isoforms of IgE BCRs. In adults with inadequately controlled allergic asthma, Quilizumab reduced serum total and allergen-specific IgE by 30-40 %, but had no impact on asthma exacerbations, lung function, or patient-reported symptom measures. We conjecture that Quilizumab based CAR T cells, will outperform the Quilizumab mAb as well as previous CAR designs, and may serve as a much needed remedy for IgE mediated allergy. Quilizumab based 2nd generation CARs were expressed on human primary T lymphocytes via retroviral transduction. We tested the potency of the therapy on engineered NALM-6 cell line expressing membranal IgE (mIgE, modeling a BCR). mIgE-targeting CAR-T cells successfully eliminated IgE$^+$ expressing NALM6 cells but not wild type NALM6 or NALM6 that express the short isoform of mIgE (lacking the M1` segment). This targeted elimination was accompanied by increase of gamma interferon production by the effector cells. Importantly, the addition of high amounts of free-IgE to the culture did not affect the activity of the CAR-T against its target. To test the potency of the CAR-Ts against primary IgE$^+$ B cells, which are extremely sparse in blood, we used B cells from human tonsils and induced their class switching to IgE. Upon 2 days of co culture of isolated B cells with CAR-T cells, or non-transduced T cells as control, our CAR-T cells abolished IgE production, as measured by ELISA, and eliminated IgE secreting cells, as evident by ELISPOT. This elimination was specific to IgE, as IgG production was unaffected. In the near future, we plan to examine the potency of our therapy in vivo, in a mouse allergy model. Since mice do not express the M1` domain we designed transgenic mice in which the murine extra membranal proximal domain (EMPD) is replaced by the human one. CAR-T cells targeting membrane bound IgE are thus envisioned as a curative therapy for IgE mediated allergies, providing sustained surveillance against IgE$^+$ B cell resurgence and relieving the patient from all atopic diseases.



Friday Posters: In Vivo Therapy Approaches

1825. Circular RNA-Based CAR Cell Therapy for Potent and Long-Lasting Anti-Tumor Activity

Ashlesha Odak, Kshitij Parag-Sharma, Kelvin Muriuki, Aidan Simpson, Monica L. Jaffee, Rio S. Boothello, Weiyu Zhao, Brett Rickeard, Shivani Gaikwad, Jackson H. Lange, Britt A. DiMarzio, Sanjana Manja, Emily A. Clinton, Eric Lebish, Kavya Borde, Lauren Gambill, Sarah C. Bening, Alexander C. Lemaire, Wuhbet D. Abraham, Ryan T. Sowell, Jaspreet S. Khurana, Prashant R. Nambiar, Jacob R. Becraft, Tasuku Kitada

Strand Therapeutics Inc., Boston, MA

Stably integrated ex vivo engineered Chimeric Antigen Receptor (CAR) T cell therapy approaches have been effective for the treatment of hematological malignancies. However, broader patient access is limited by extremely complex, time-consuming, and expensive manufacturing. Recently, safety concerns have also arisen from random vector integration leading to secondary malignancies that necessitated black box warnings by the FDA. Additionally, these therapies may face antigen loss or escape-related relapses that require re-treatment with the same or different CAR target but existing modalities and manufacturing processes pose challenges owing to the long time period from target validation to the availability of an injectable drug product.

In vivo engineered, mRNA-based cell therapies can overcome these limitations of current cell therapies. However, for such RNA-based cell therapies, limited duration of conventional mRNA encoded CAR expression may preclude their ability to drive complete and durable clinical responses. To address the transient nature of mRNA expression and improve efficacy, we developed circular RNA (circRNA) sequences that drive higher levels and longer duration of CAR expression. We demonstrate that these novel circRNAs drive long-lasting and superior levels of CAR expression in freshly isolated non-activated primary human T cells as well as freshly isolated non-activated human peripheral blood mononuclear cells (PBMCs). These circRNA-CAR cells drive potent cytotoxic responses against target tumor cells in short and long-term co-cultures as well as upon multiple rounds of target tumor cell exposure. We further tested these circRNA-CAR cells in a relevant tumor mouse model and have compared efficacy over linear modified RNA and lentivirally transduced CARs over time. Our RNA-based approach thus enables potent activity and offers the added advantage of swiftly addressing antigen loss or escape-related relapses while significantly reducing manufacturing complexity and vein-to-vein time for patients.

1827. Characterization of VivoVecTM Pharmacokinetics Following Intravenous or Intranodal Administration Using a Vector RNA Genome Method

Soo Min Koo

Umoja BioPharma, Seattle, WA

Umoja is developing the VivoVecTM platform, a lentiviral vector designed to generate CAR Tcells in vivo. We have previously shown that in vivo administration of VivoVecTM particles (which are surface engineered to bind, activate, and transduce T cells) results in specific generation of CAR T cells with potent anti-tumor activity in preclinical models. Because VivoVecTM will be administered directly to patients, an understanding of in vivo biodistribution and pharmacokinetics of the VivoVecTM particles is critical; We developed a reverse transcription (RT)-PCR based method to detect viral RNA genomes present in whole blood samples and used the assay to evaluate VivoVecTM pharmacokinetics (PK) following intravenous (IV), intraperitoneal (IP), or intralymph node injection in mouse and canine models. VivoVecTM RNA genomes in blood were measured at multiple timepoints (5 min to 1 week) in 2 preclinical animal models via three routes of administration (ROA): IV, IP, and intralymph node injection in beagles, and IV injection in C57BL/6 mice. mRNA was isolated from whole blood, reverse transcribed into cDNA, and subsequently amplified by qPCR using product sequence specific primers. Negative control without reverse transcriptase confirmed absence of background. Spike-recovery studies were performed with known vector copy numbers into PBS and animal blood as positive controls to analyze linearity, precision, reproducibility and sensitivity of the assay. The vector RNA genome assay demonstrated reproducible extraction of high purity VivoVecTM particle mRNA from canine and mouse whole blood samples, including over freeze-and-thaw cycles of blood samples, down to a sensitivity of 115 transducing units (TU)/ml blood in spike-recovery studies. In a mouse PK study, when 4.2×107 transducing unit (TU)/animal VivoVecTM (a dose known to generate functional CAR T cells in tumor-bearing humanized mice) was injected IV, vector genomes were detected in peripheral circulation up to 2 hours post administration. Peak VivoVecTM RNA genome levels were detected at 5 minutes post administration, with a 700-fold reduction in vector genomes observed between 5 minutes and 2 hours post administration. At later timepoints, all samples were below the detection limit of the qPCR method (10 copies/well). In a canine biodistribution studies assessing RNA genomes at multiple timepoints (5 min to 1 week post administration), similar VivoVecTM PK profile was shown as mice when administered IV and VivoVecTM RNA genomes were detected in blood samples 24 hours after IP injection. However, VivoVecTM RNA genomes were not detected in blood following intra-lymph node injection. In conclusion, we have developed an RNA genome assay that successfully detected our lentiviral drug production in whole blood with high sensitivity following multiple ROAs. At pharmacologically active doses VivoVecTM particles demonstrated a short half-life in blood following IV administration and remained detectable in the circulating blood stream for only approximately 2 hours.

1828. Compositional Optimization of Lipid Nanoparticle for Intracellular Delivery of Proteins

Xiaoya (Julia) Lu, Yining Zhu, Sachin Kammula, Hai-Quan Mao

Johns Hopkins University, Baltimore, MD

Statement of Purpose: Protein-based therapeutics targeting intracellular entities, such as the CRISPR-Cas9 system, have been employed in gene and immune therapies for diverse diseases. Despite considerable efforts, an efficient delivery system for the *in vivo* intracellular delivery of protein therapeutics is still lacking. Lipid nanoparticles (LNPs) have been engineered as carriers for mRNA encoding antigens with validated biosafety profile.[1] However, their application in protein delivery has been relatively unexplored. Previously, our lab has developed a multi-step screening method to optimize the type of helper lipid and component ratios in an LNP formulation for gene delivery.[2] In this study, we aim to optimize the formulation for gene-editing proteins including Cre recombinase and Cas9 using this platform. In detail, with *in vitro* screening with HEK293 Ai9 cell line, and cluster-mode *in vivo* screening in Ai9 mice, we aim to discover formulations potentially targeting the spleen for T cells and B cells gene editing. **Methods:** As shown in Figure 1**a**, we generated a 486-formulation LNP library with DLin-MC3-DMA as ionizable lipid, cholesterol, DMG-PEG2000, and one of six helper phospholipids that were previously used in FDA-approved or experimental LNP formulations. By varying the following parameters: (1) combined molar percentage of DLin-MC3-DMA and helper lipids; (2) weight ratio of cholesterol to DMG-PEG2000; (3) weight ratio of DLin-MC3-DMA to helper lipid; and (4) the weight ratio of total weights of lipid to protein, formulations were generated with Cre recombinase to screen *in vitro* on HEK293 Ai9 cell line. The top 50 formulations encapsulating Cre recombinase were then divided into 10 clusters and administered intravenously (*i.v.*) into Ai9 mice for i*n vivo* cluster-mode screening. Analyzed by flow cytometry, top three clusters with the highest gene editing efficiency in spleen immune cells will be selected and tested individually on Ai9 mice. **Results:** To select LNP formulations that can successfully deliver proteins with intracellular targets, we first evaluated gene editing efficiency of the whole library treated encapsulating Cre

recombinase and measured the percentage of tdTom expression in HEK293 Ai9 cells (Fig. 1**b**). The composition variation has generated significantly different gene editing efficiencies under each helper lipid. Of 486 LNP formulations, the 50 top-performing LNPs are shown in Figure 1**c**. To evaluate these formulations' *in vivo* systemic gene editing efficiency, the LNPs with the best *in vitro* performances were tested by *i.v.* injections on Ai9 mice for cluster-mode screening. Three clusters have shown promising tdTom expression levels in T cells and B cells (Fig. 1**d-e**). The individual formulations within these clusters will be further evaluated. **Conclusion:** This LNP screening platform allows for the identification of the best performing LNPs for intracellular delivery of protein potentially for gene editing. In the cluster-mode screening, we see a high level of gene editing events in immune cells in the spleen in multiple clusters, which will serve as solid evidence for our next individual formulation testing.

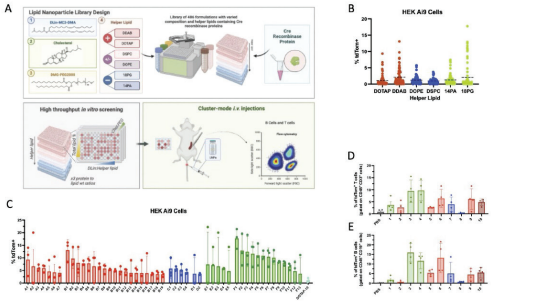


Figure 1. a) Schematic of multi-step screening for intracellular delivery of Cre recombinase **b)** Gene editing efficiency of LNPs in HEK293 Ai9 cells via high-throughput screening platform ($n = 3$). Assessed with tdTom expression. **c)** The top 50 formulations were selected. FACS was used to quantify the percentage of tdTom$^+$ cells in spleen of **d)** T cells (CD45$^+$CD3$^+$ cells) **e)** B cells (CD45$^+$CD19$^+$ cells)

1831. Selective and Efficient *In Vivo* HSC Transduction in Humanized Mice Mediated by Capsid Modified HDAd Vectors

Aphrodite Georgakopoulou, Hongjie Wang, Chang Li, Andre Lieber

University of Washington, Seattle, WA

We developed an *in vivo* HSC gene therapy approach that consists of HSC mobilization and intravenous injection of HDAd vectors that target either CD46 or desmoglein 2 (DSG2), receptors that are expressed at high levels on HSCs. While we have demonstrated safety and efficacy of the *in vivo* approach in CD46-transgenic mice and rhesus macaques, studies in mice with a humanized hematopoietic system could facilitate its potential clinical translation. NBSGW mice engraft cryo-preserved human CD34$^+$ cells in the bone marrow without preconditioning, however human chimerism in peripheral blood is usually low, and mobilization of human cells by G-CSF/AMD3100 for *in vivo* HSC transduction is inefficient. We show that low-dose total body irradiation (100Rad) or busulfan treatment (12.5mg/kg) increases the percentage of human CD45$^+$ cells in PBMCs two- and five-fold, respectively. In low-dose busulfan conditioned humanized mice, at 6 weeks after CD34$^+$ cell transplantation, G-CSF/AMD3100 mobilization (over 7 days) resulted in >30,000 human CD34$^+$ cells in the periphery at 40 min after AMD3100 injection (15% of human CD45$^+$ cells were CD34$^+$ positive). At the peak of mobilization, humanized mice were intravenously injected with a CD46-targeting HDAd5/35++ vector or a DSG2-targeting HDAd6/3+-GFP vector. Four weeks later, mice received one round of O^{6}BG/BCNU *in vivo* selection treatment which resulted in an increase in GFP marking within peripheral blood human CD45$^+$ cells. At week 7 after *in vivo* HSC transduction, the percentages of GFP$^+$/CD45$^+$ cells in the periphery were 7±3.8% and 16.1±8.83% in HDAd5/35++-GFP and HDAd6/3+-GFP *in vivo* transduced mice, respectively. A similar pattern of GFP marking rates were found in different human lineages (CD19, CD33, CD3, CD235a, CD34) in the bone morrow of these mice. These data show that in humanized mice, human HSCs can be efficiently mobilized with GCSF/AMD3100 and *in vivo* transduced with HDAd vectors. We also demonstrated efficient HSC mobilization within hours by other agents, including the small molecule cxcr2 inhibitor tGROβ and the VLA4 inhibitor WU-106 (given together with AMD3100). Furthermore, we used the humanized mouse model to test a new HDAd vector platform that targets two HSC receptors, an HSC attachment receptor (CD46) and an HSC internalization receptor ($\alpha_6/\beta_{1/4}$integrins (CD49f). CD49f expression is largely restricted to stem cells, specifically to primitive HSCs. Retargeting to this receptor was achieved by replacing the RGD (Arg-Gly-Asp) motif within the Ad5 penton with the functional core motif of laminin chain A IKVAV (Ile-Lys-Val-Ala-Val). After *in vivo* HSC transduction in humanized mice, we found significantly higher percentages of GFP$^+$ cells within CD34$^+$ subsets that are enriched for primitive HSCs with the laminin-modified vector compared to the parental vector. For example, 7.6±1.69% and 17.3±3.4% GFP-positive CD34$^+$/CD38$^-$/CD45RA$^-$ cells were measured in the bone marrow of mice, 3 days after *in vivo* transduction with HDAd5/35++-GFP and HDAd5/35++_laminin, respectively. The transduction efficiency in more committed progenitors was not significantly different. This suggests that CD46/CD49f-targeted vectors more selectively transduce primitive HSCs *in vivo*. Furthermore, we found that the double-retargeted vector triggered less proinflammatory cytokine release after intravenous injection into mice. Taken together, this indicates that this new HDAd5/35++-laminin vector platform is safer and more selective for primitive HSCs after *in vivo* transduction. In summary, we demonstrated that humanized mice are a suitable model for validating and optimizing our *in vivo* HSC gene therapy approach.

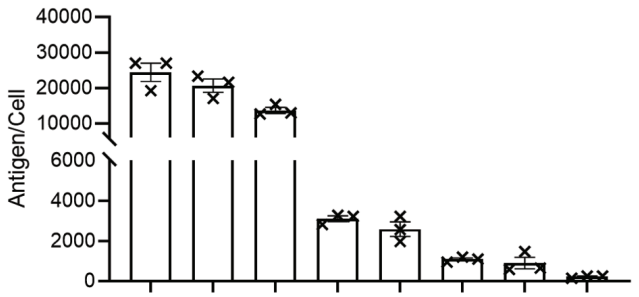
1832. HSC Antigen Density and Changes with Mobilization: Important Considerations for *In Vivo* HSC Targeting and Depletion

Justin Thomas, Dnyanada Pande, Mark R. Enstrom, Hans-Peter Kiem, Stefan Radtke

Fred Hutchinson Cancer Center, Seattle, WA

Hematopoietic stem cell (HSC) gene therapy is a promising treatment option for a variety of genetic blood diseases/disorders. However, current approaches are performed *ex vivo*, require genotoxic myeloablation, and depend on sophisticated infrastructure similar to bone marrow transplantation. Thus, *ex vivo* HSC gene therapy is expensive, geographically restricted, and can cause conditioning-related side effects. Direct application of HSC-targeted gene therapy agents in vivo would overcome these bottlenecks. Simultaneously, new conditioning/selection strategies including epitope engineering are developed to selectively remove endogenous or unmodified HSCs using non-genotoxic antibody-based conditioning regimens, respectively. Both approaches depend on HSC-specific antigens for either targeted delivery or selective killing. However, HSC antigens are

often not restricted to HSCs only. These so-called "antigen sinks" can reduce the targeting efficiency, lead to unwanted ablation of immune cells, or cause off-target effects. Here, we investigated the expression and cell-surface abundance of commonly targeted HSC antigens using bulk/single-cell RNAseq and quantitative flow-cytometry. We further studied the impact of mobilization on the transcription and cell surface presentation of antigens to inform the best route for the in vivo administration of HSC-targeted agents. Single-cell and bulk RNAseq revealed high expression of CD34, CD45, and CD164, low expression of CD133 and CD117, and very low to undetectable levels of CD33, CD90, and EPCR. Upon mobilization, expression of HSC antigens decreased but patterns remained unchanged. Antigen quantification by flow-cytometry demonstrated a very high CD164, CD34, and CD45 presentation on HSC (Figure 1). Significantly lower abundance of CD90, CD133, CD117, CD33, and ECPR was seen (Figure 1). Of note, surface expression of CD117, CD33 and CD164 was reduced on HSCs after mobilization, whereas all other markers remained stable or increased in abundance. Finally, assessment of total antigen within the bone marrow and peripheral blood demonstrated high expression of CD45, CD33, and CD164 on non-HSCs. Our data shows that the selection of the antigen for in vivo HSC gene therapy, ablation, or selection strongly depends on the abundance, mode of administration, and off-target presentation. Most highly expressed antigens were either impacted by mobilization or not HSC-specific, while more HSC-specific antigens were only presented at a low density. Important for the in vivo application of gene therapy or selection agents, presentation of the antigen outside the hematopoietic system has to be considered to reach high on-target specific, avoid antigen sinks, and reduce unwanted off-target effects.



#Antigens	CD164	CD34	CD45	CD90	CD133	CD117	CD33	EPCR
Steady state	Ø24K	Ø20K	Ø13K	Ø3.1K	Ø2.5K	Ø1.2K	Ø0.9K	Ø0.2K
Mobilized	Ø11K	Ø26K	Ø17K	Ø3.5K	Ø4.6K	Ø0.8K	Ø0.6K	Ø0.4K

1833. Intravital Visualization of Angiogenesis Using Endothelial Progenitor Cells in Diabetic Peripheral Ischemia Mouse Models

Hyungjin Kwon, Kubra Akyildiz, Hyunseok Kim
IVIM Technology, Songpa-gu, Seoul, Korea, Republic of

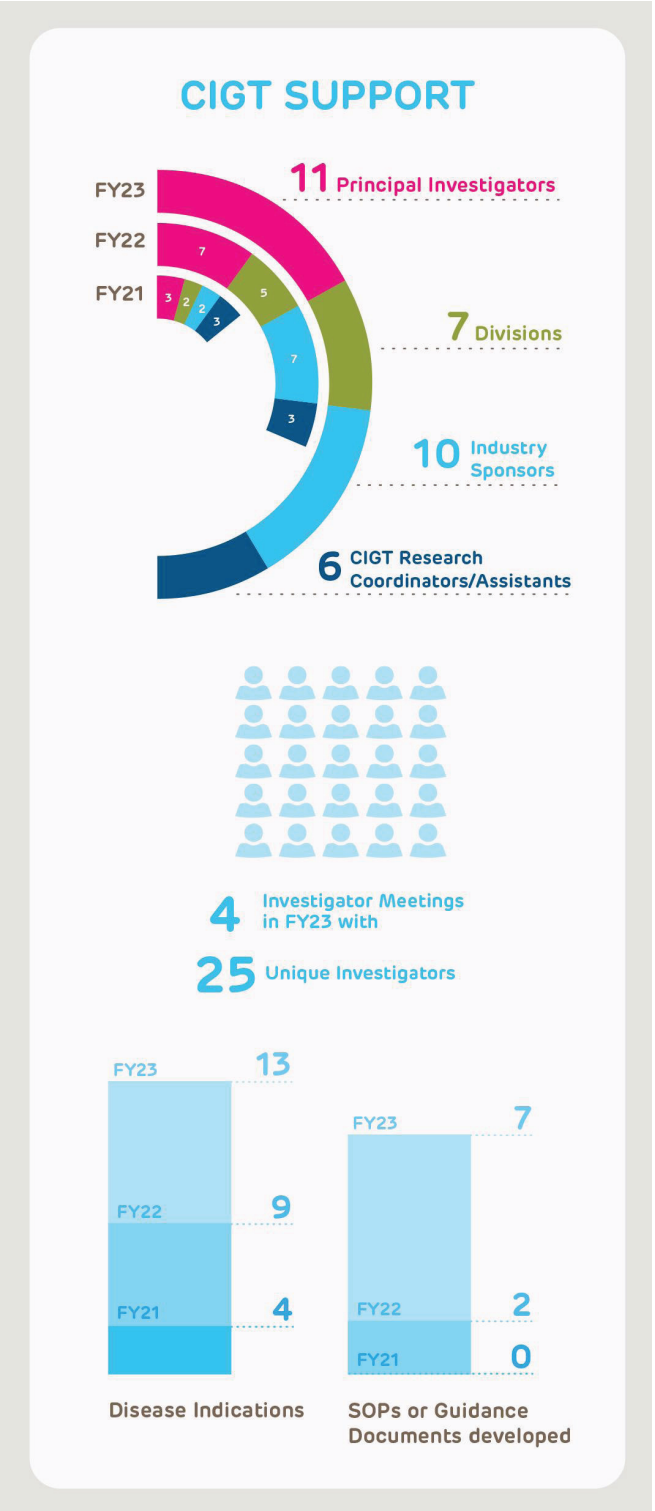
Understanding the physiological mechanisms of angiogenesis is crucial in researching treatments for various metabolic disorders and cancers. Particularly, given the detrimental effects of diabetes-induced microvascular damage leading to necrosis in the extremities and retinal complications, comprehending angiogenesis becomes pivotal. Through vascular regeneration, there is potential for preventing and treating such diseases, thereby significantly impacting the quality of life. In this study, we aimed to visualize angiogenesis using *in vivo* fluorescence microscopy. We employed a commonly used diabetic hind limb ischemia mouse model. After inducing diabetes, we performed venous ligation in the hind limb and injected endothelial progenitor cells (EPCs) to observe cell engraftment and the extent of vascular formation over time. To establish the mouse model, we induced diabetes by intraperitoneal injection of streptozotocin to Balb/c-nu/nu, monitoring blood glucose continuously. Subsequently, we surgically induced hind limb ischemia by ligating and cutting two veins in the hind limb with black silk. In the experimental group, endogenous EGFP-EPCs were injected around the incision site, while the control group did not receive this injection. Following this, we observed the formation of neovessels using FSD647 fluorescent dye-conjugated CD31 antibodies, visualizing the process with a different fluorescence. On the third day after EPC injection, we observed the emergence of GFP-positive new vessels, with some vessels showing colocalization with the CD31 marker. By the seventh day, we could visualize GFP-positive vessels bypassing the incision site. On days 14 and 21, GFP-positive vessels connecting the incision site were observed. In this study, we utilized *in vivo* microscopy to track the progression of angiogenesis in the hind limb ischemia mouse model. The *in vivo* microscope proves indispensable for vascular research, offering the unique advantage of high-resolution, real-time observation of blood vessel generation and structure. Furthermore, its ability to facilitate repetitive imaging within a single subject reduces potential artifacts arising from individual variations, underscoring its significance in the study of blood vessel.

1834. A Centralized Multi-Disciplinary Approach for Safe and Efficient Delivery of *In Vivo* Gene Therapy

Julianne LaRosa[1], Sarah McCague[1], Juliana Small[1], Jennifer Harding[2], Amy Tara Waldman[3], Benjamin Samelson-Jones[1], Susan Matesanz[1], Lindsey A. George[1]
[1]Clinical In Vivo Gene Therapy, Children's Hospital of Philadelphia, Philadelphia, PA,[2]Clinical In Vivo Gene Therapy, Children's Hospital of Philadelphia, Philadelphia, PA,[3]Clinical In Vivo Gene Therapy, Children's Hospital of Phliadelphia, Philadelphia, PA

Recent clinical successes with adeno-associated virus gene therapy highlight the potential for gene-based therapies to transform the care of patients with monogenic disorders. Clinical gene therapy efforts have historically been siloed within clinical divisions based on disease-specific expertise. This creates communication challenges and hurdles to the standardization of gene therapy research and practice within and across institutions. The increasing number of investigational and FDA approved gene therapy products underscores the need for gene therapy centers in hospital systems across the country that are equipped with the clinical, research, and administrative infrastructure to administer gene therapy drugs safely and efficiently. The Clinical In Vivo Gene Therapy Group (CIGT) was established in 2021 at the Children's Hospital of Philadelphia (CHOP) to provide centralized support for gene therapy efforts throughout the institution. The research group includes physicians, clinical investigators, regulatory specialists, clinical research coordinators, operation specialists, and a multidisciplinary consult team. CIGT is focused on the delivery of gene therapy for both investigational and commercial products across clinical divisions. In order to address emergent toxicities surrounding

gene therapy, CIGT formed a clinical consult service composed of physicians from hematology, cardiology, nephrology, hepatology, neurology, immunology, and metabolism. On-call physicians follow the patient, lead multidisciplinary case discussions, and provide consensus recommendations. An automated institutional registry within the electronic medical record platform was also developed to implement recommended lab safety monitoring as well as track long-term safety and efficacy outcomes. CIGT provides interdisciplinary opportunities for its growing network of clinicians and scientists to support continued education and to foster collaboration. Working closely with hospital leadership and administration, CIGT provides guidance and creates best practices related to the successful onboarding of gene therapy products, covering all aspects of the process such as manufacturer purchasing agreements, informed consent, EMR templates, patient education, and best clinical practices. CIGT's infrastructure establishes a framework to safely and efficiently conduct in vivo gene therapy trials as well as implement state-of-the-art clinical care to optimize patient outcomes. Thus far, CIGT services have treated more than 120 patients from 15 countries with investigational and commercial in vivo gene therapy products. CIGT supports 11 principal investigators across 7 clinical divisions (hematology, ophthalmology, neurology, endocrinology, metabolism, audiology, and cardiology) with 16 ongoing clinical trials across all phases of the product lifecycle. The program's success can be attributed to its cross functional team and prioritization of safety, education, and collaboration. Through its various initiatives and responsive infrastructure, CIGT enables the responsible growth of in vivo gene therapy at CHOP to ensure continued, safe access to treatments for patients with rare genetic disorders.



1835. Scoring-Based High-Throughput Screening Processes for Promoting the Accuracy of *In Vivo* Directed Evolution

Hongyoung Choi[1], Soonil Kwon[1], Sung Jin Lee[1], Subin Cho[2], Jinyoung Kim[2], Dohoon Kim[3], Youngbin Park[3], Changmin Kang[1]

[1]GluGene Therapeutics, Seoul, Korea, Republic of,[2]Yonsei University, Seoul, Korea, Republic of,[3]GluGene Therapeutics, Daejeon, Korea, Republic of

Adeno-associated virus (AAV) has been utilized as a delivery vehicle for in vivo gene therapy due to its efficacy and safety. Nevertheless, wild-type AAVs pose challenges such as off-targeting and immune responses. Directed evolution emerges as the most potent method for engineering novel AAV vectors capable of addressing these issues. Through the application of directed evolution, coupled with Next-Generation Sequencing (NGS) and in silico analysis, high-throughput screening is facilitated to identify novel AAV vectors. Using barcode matching consensus, we established a long-read sequencing system that precisely reads over the entire capsid region. This allows us to trace the mutations selected AAV vectors have by searching the matched barcode sequences. We introduced a 7-mer peptide insertion and an error-prone capsid library based on wild-type AAV1. Through in vivo selection in several mouse disease models, we constructed an in silico score database of selected AAV variants. The scores reflect in vivo properties such as target cell specificity and off-target effects. It is essential that these scores accurately represent the properties for the high-throughput screening process. To demonstrate this, we conducted in vivo validation for high-score AAV variants and confirmed that the properties follow the trend of the scores. This consistency enhances the feasibility of the high-throughput screening system. We anticipate that this study will provide a more sophisticated AAV vector engineering platform.

1836. Universal ddPCR Assay for Quantifying Cellular Kinetics and Biodistribution of CAR-T Cell Therapies in Preclinical and Clinical Trials

Hyun-Jung Kim, Hui Liu, Eric Lachacz, Anton AI Rosenbaum, Sonia Terrillon

1Integrated Bioanalysis, Clinical Pharmacology & Safety Sciences, R&D, AstraZeneca, South San Francisco, CA

Chimeric antigen receptor (CAR)-T cell therapy is a remarkable breakthrough in adoptive cell therapies with promising achievements in the treatment of blood-related cancers. This rapidly growing class of biotherapeutics requires sophisticated bioanalytical assays to support preclinical and clinical studies. Droplet Digital PCR (ddPCR) is an emerging innovative technology in bioanalytical laboratories enabling absolute and sensitive quantification of therapeutic transgenes employed in CAR-T therapies. However, there are limited bioanalytical guidelines for the validation of performance for pre-clinical and clinical sample analysis using ddPCR. Here, we describe developing and validating a universal ddPCR assay and critical considerations for ddPCR-specific assay deployment for use across multiple autologous CAR-T therapy assets. Briefly, primers and a probe targeting an essential element of the lentiviral vector used for CAR-T cell generation were designed. We then employed the universal CAR-T ddPCR assay for absolute quantification of CAR transgene copies in the blood and tissues of xenograft mice after administration of CAR-T cells. These preclinical data informed the cellular kinetics, biodistribution, and safety profiles to support IND applications of multiple products. The universal assay was then qualified and validated in a fit-for-purpose manner to support clinical studies. The qualification and validation demonstrated the assay to have acceptable accuracy, precision, linearity, dilutional linearity, sensitivity, specificity, matrix effect, and extraction efficiency. Briefly, at least two-thirds of replicates for each sample must have ≥10,000 accepted droplets. For the validation run, all plates should have three different levels of positive control (PC) and one non-template control (NTC) in triplicates. The mean of at least two-thirds of replicates for each of the high and middle PCs must have %RE ±30% of the expected target copies/well with %CV ≤ 30% (%RE ±55% and %CV ≤55% for low PC). For linearity, R^2 of the observed vs. expected copies/well determined by the assay of six different QC levels in triplicate must be ≥ 0.980 across the range of tested QCs. For specificity, the mean value of the NTC and matrix-only controls must be < 2.5 copies/well (50% of anticipated LLOQ). Since there is limited regulatory or industry guidance on extraction efficiency acceptance criteria, DNA extraction efficiency from five different levels of CAR-T cells spiked into the blood matrix should be evaluated. Next, we deployed the validated universal ddPCR assay in clinical studies. Finally, we describe our view on the best practices and critical considerations for ddPCR assay validation and deployment. The universal ddPCR assay approach and the deployment and validation strategies described herein can be followed to enable the transfer and use of high-quality ddPCR assays across multiple cell therapy products in preclinical and clinical studies.

1837. Methodologies of High-Throughput Screening Platforms to Enhance the Successful Rates of *In Vivo* Directed Evolution

Soonil Kwon[1], Sung Jin Lee[1], Hongyoung Choi[1], Subin Cho[2], JinYoung Kim[2], Dohoon Kim[1], Youngbin Park[3], ChangMin Kang[1]

[1]GluGene Therapeutics, Seoul, Korea, Republic of,[2]Yonsei University, Seoul, Korea, Republic of,[3]GluGene Therapeutics, Daejeon, Korea, Republic of

Adeno-associated viruses (AAVs) have recently emerged as potent gene carriers for numerous in vivo gene therapy trials. However, delivering AAVs, typically naturally occurring AAVs, specifically to targets while mitigating their exposure to neutralizing antibodies still poses significant challenges. Engineering external capsids has been considered a critical strategy to address these issues. In this context, we present a convergent methodology that utilizes directed evolution combined with in silico high-throughput iteration processes to create novel AAV capsids, enhancing their infectivity for specific targets. AAV capsid libraries were established through the capsid engineering method, employing 7-mer peptide insertion and error-prone PCR with random point mutations. We introduced random barcode sequences to enhance library iteration efficacy and high-throughput screening feasibility. Subsequently, we constructed a database composed of selected capsid sequences based on in silico scoring. Importantly, variants that received high scores through our in silico iteration process exhibited augmented tropism for targets in animal models, validating the elevated accuracy of our established iteration and scoring system. We will discuss in detail the methodologies and algorithms devised

to enhance the accuracy of in vivo directed evolution, combined with our in silico vector engineering platform, for developing novel AAV vectors with improved targeting capabilities.

1838. The Impact of Primary Myoblasts on Macrophage Polarization *In Vitro*

Ananya Goyal[1], Trevor R. Cardinal[2]

[1]California Polytechnic State University, San Luis Obispo, San Luis Obispo, CA,[2]Cal Poly, San Luis Obispo, CA

Peripheral Artery Disease (PAD) is characterized by the buildup of atherosclerotic plaque in the lower extremities that leads to downstream ischemia and affects roughly 200 million people worldwide. Revascularization procedures are available, however, 22% of PAD patients are left amputated. Alternatively, a potential cell therapy avenue involves stimulating arteriogenesis. In arteriogenesis, vessels around an occluded artery expand to form a bypass to restore blood flow. Bone marrow mononuclear cells (BM-MNCs) have been evaluated as candidates for PAD, however, in large trials there was little evidence of increased arteriogenesis. One limitation is that transplanted BM-MNCs are separated from their niche and are unable to communicate effectively with the myo-vascular niche, rendering them less effective than a tissue resident progenitor cell. Myoblasts are proposed as a potential cell therapy candidate for arteriogenesis due to their innate proximity to the musculature surrounding collaterals. Previously, we found that myoblasts can increase collateral capillary arteriogenesis in lean mice of a strain that possess fewer native collaterals and collateral arteriogenesis in mice with diet induced obesity. Macrophage recruitment is impaired during arteriogenesis in DIO (diet-induced obese) mice, implying that the cell type critical for arteriogenesis. Studying the relationship between macrophages and myoblasts is critical for understanding how to stimulating arteriogenesis. A relationship between macrophages and myoblasts has already been characterized in skeletal muscle regeneration. Post-injury, there is a phenotypic switch from the pro-inflammatory M1 macrophages to the pro-regenerative M2 phenotype. This switch assists regeneration initiation and coordinates myogenic events. Knowing that macrophages and myoblasts work in tandem for skeletal regeneration provides evidence that their established ability to communicate can be utilized in other regeneration events. Furthermore, arteriogenesis is orchestrated by macrophages where M1 macrophages make the phenotypic switch towards the M2 phenotype. This switch promotes arteriogenesis and is necessary for revascularization. The purpose of this study is to observe whether primary myoblasts are the drivers of this phenotypic switch from M1 to M2 macrophages *in vitro*. In this study, we found that upon *in vitro* coculture of RAW 264.7 macrophages and primary myoblasts, M0 and M1 macrophages make this switch towards M2 after 48 hours, indicated by a higher axial ratio, a marker of M2. These data imply that myoblast signaling induces regenerative macrophages in vitro, and therefore may drive this polarization in vivo. Overall, the purpose of this research was to determine the impact that coculture with primary myoblasts would have on RAW 264.7 macrophage polarization. It was found that coculture drove M0 and M1 macrophages towards an M2-like phenotype. Together these discoveries push us closer to developing a cell therapy to treat PAD patients and can help patients on their last limb.

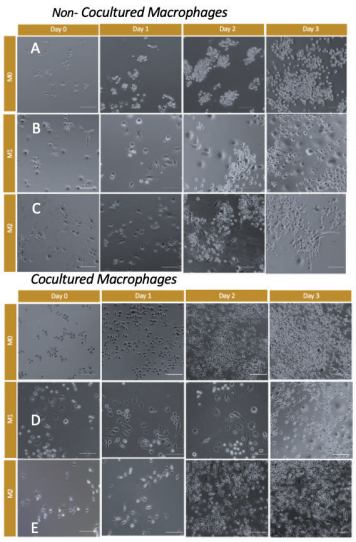


Figure 1. Differences in Macrophage Morphology in Cocultured with Primary Myoblasts vs Non-cocultured across 72 hours. A) Non-cocultured M0 macrophages across 72 hours (days 1-3). B) Non-cocultured M1 macrophages across 72 hours. C) Non-cocultured M2 macrophages across 72 hours. D) M0 macrophages cocultured with primary myoblasts before coculture (day 0) and across 72 hours. E) Cocultured M1 macrophages cocultured with primary myoblasts before coculture and across 72 hours. F) Cocultured M2 macrophages cocultured with primary myoblasts before coculture and across 72 hours. Images obtained at 10x magnification with an EVOS phase contrast microscope. Scale bar = 100um.

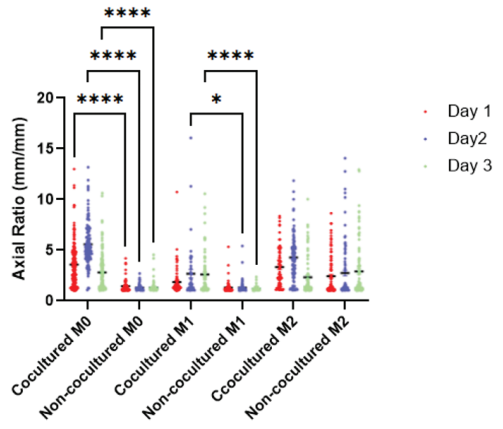


Figure 2. Differences in Axial Ratio of Macrophages Co-cultured or Not co-cultured with Myoblasts. Major Axis over Minor Axis of pre-polarized presumptive M0 (n = 105), M1 (n = 81), M2 (n=103) macrophages after 72 hours of co-culture compared to pre-polarized presumptive non-cocultured M0, M1, and M2 macrophages. Higher axial ratio is indicative of an elongated cell which provides evidence of M2 morphology. (*, p< 0.05 between co-cultured and not co-cultured of same time point for same polarization group.)

1839. *In Vivo* Delivery of an Engineered Lentiviral CAR19 Vector for the Treatment of Autoimmune Diseases

Daniel Grubaugh, Ronnie M. Russell, Debasish Boral, Aoi Wakabayashi, Corrine Little, Jeffery D. Foss, Julia L. A. Nguyen, James I. Andorko, Bruce C. Schnepp, Timothy D. Culp, Philip R. Johnson

Interius BioTherapeutics, Philadelphia, PA

Chimeric antigen receptor (CAR) T cell therapy has revolutionized the treatment of B cell malignancies and multiple myeloma. More recently, CAR T therapy has been highlighted as a potential intervention for autoimmune diseases. Conventional autologous CAR T therapy includes a multifaceted, complicated process including patient pretreatment using lymphodepleting chemotherapy, complex manufacturing and logistics for the generation and delivery of the

cell product which prolong vein-to-vein time. Alternative approaches meant to overcome these limitations, including allogenic CARs or faster manufacturing processes, are currently in clinical development. *In vivo* generation of CAR T cells is a fast-growing area of research, with the promise of reducing the complexity of autologous CAR generation, eliminating the need for lymphodepletion, and expanding patient access. We describe a lentiviral vector engineered to target CD7+ T cells and NK cells following a single intravenous administration to generate autologous CAR+ cells within the body, without lymphodepletion. This vector, INT2106, confers specific targeting of T cells and NK cells by pseudotyping the lentivirus with both a CD7 Binder and a detargeted VSV-G fusogen called "Gen 2.1 Fusogen". INT2106 delivers a fully human CAR19 transgene, resulting in the generation of functional CAR T and NK cells with specific activity against CD19+ B cells, the same cells targeted by autologous CAR T therapies being tested in clinical studies to combat autoimmune diseases. *In vitro* evaluation of INT2106 demonstrates specific transduction of CD7+ cells. Biologic activity of the fully human CAR19 was evident through specific killing of CD19-expressing B cell lymphoma lines across a range of Effector to Target cell ratios. This biological activity was confirmed to be dependent on CD19-expression on the tumor cells by data showing reduced killing of B cells with CD19 expression knocked out. Incubation of INT2106 with various B cell lymphoma lines or primary B cells isolated from healthy human donors does not result in the transduction of B cells, an important safety consideration regardless of whether the CAR cells are generated *in vivo* or *ex vivo*. An *in vivo* demonstration of INT2106 was performed using human CD34-engrafted NSG mice. Following treatment with INT2106 via a single intravenous tail vein injection, circulating B cells in vector-treated mice were depleted, with loss of CD19+ B cells across multiple tissues including the blood, spleen, and bone marrow. Coincident with B cell depletion, CAR19+ cells were detectable in the blood of treated mice, with peak concentrations measured approximately three weeks after administration. INT2106 treatment was well-tolerated with no vector associated safety signals observed. Taken together, these data suggest that intravenous administration of INT2106 can effectively target CD7+ cells, leading to functional CAR19+ cell generation and CD19+ B cell depletion, the intended biological effect. INT2106 is in development as an off-the-shelf treatment for autoimmune diseases without the need for patient preconditioning or cell manufacturing.

Friday Posters: Vector Product Engineering, Development, and Manufacturing

1840. Overcoming Traditional Limitations: GenScript's Advanced mRNA Applied Vector and Strain for Pertaining polyA Integrity and Efficient Plasmid Production

Xiaofei Long, Wenlu Zuo, Andrew Tsao, Qingwei Meng
GenScript Biotech Corporation, Nanjing, China

The burgeoning field of mRNA-based therapies and technologies, from personalized medicine to vaccine development, has catalyzed a growing demand for large-scale in vitro transcription (IVT) of mRNA. The prevalent methods for producing IVT mRNA typically utilize DNA templates derived from circular plasmids, offering efficient cloning of target DNA sequences and the advantages of straightforward bacterial propagation for rapid production. Among the various vectors, pUC57 and pVAX1 are particularly favoured by mRNA researchers. However, these traditional vectors are not without their challenges. They often face issues like less-than-ideal cloning efficiency, difficulties maintaining the stability and integrity of poly(A) sequences, and lower yields in plasmid production. These shortcomings can adversely affect the quality and quantity of IVT mRNA and prolong the preparation time for scalable IVT templates - a crucial aspect in therapeutic mRNA production and vaccine development. In response to these challenges, our team has developed a set of proprietary vectors, GS-mK and GS-CMV vectors, which are evolved versions of the conventional pUC57 and pVAX1 vectors, respectively. These vectors with built-in poly(A) tail sequences are specifically engineered for the efficient production of linearized IVT templates and many modifications include the removal of elements that negatively impact plasmid replication, DNA recombination, and poly(A) stability that enhance the PolyA integrity. Besides, Choosing the right bacterial strain for replicating plasmids with a poly(A) tail is crucial to maintaining the integrity of the poly(A) sequence. Bacteria strains exhibit varied levels of exonuclease activity, which can significantly affect the stability of the poly(A) tail in the plasmid DNA. However, even with some commercial strains to handle repetitive sequences, it may not fully resolve the instability issues associated with extended poly(A) sequences found in mRNA constructs. To tackle this challenge, GenScript has introduced a unique strain engineered to enhance the stability of poly(A) tails. The GenScript Poly(A) Strain v2 and v3 are composed of genetically edited genes or sequences, including *Rec*A, which have a direct or indirect impact on the recombination of cloned DNA. Extensive testing following cloning and large-scale plasmid preparation demonstrates its suitability for isolating plasmid clones that contain repeat elements like poly(A) tails.

1841. Breaking Barriers in Gene Therapy with Bacteriophage T4 Artificial Viral Vectors

Jingen Zhu, Venigalla B. Rao
Biology, The Catholic University of America, Washington, DC

The pursuit of effective gene therapy strategies has been significantly hampered by the limitations of existing viral vectors, particularly in the context of genetic disorders necessitating the delivery of large genes and/or genome editing complexes. Traditional viral vectors, such as adeno-associated viruses (AAVs) and lentiviruses, are limited by the cargo size, making them unsuitable for conditions like Duchenne muscular dystrophy (DMD), which is caused by mutations in the 2.2 MB dystrophin gene with an 11.2 kb coding region. We have developed a new bacteriophage-based approach to overcome these limitations by engineering bacteriophage T4 as an artificial viral vector (T4-AVV) for large gene delivery and genome remodeling. The T4 phage possesses a 120 × 86 nm capsid shell, capable of accommodating up to 171-Kbp therapeutic DNAs and thousands of protein copies. We engineered T4-AVVs by externally and internally incorporating various combinations of biomolecules, including DNA, RNA, proteins, and CRISPR ribonucleoproteins. Payload components are introduced

into the T4 capsid nanoparticles by sequential assembly *in vitro* using purified materials, and the assembled nanoparticles are coated with lipids to generate the AVVs. Remarkably, these T4-AVVs possess remarkable capabilities for delivering genetic material into human cells. They successfully packaged and delivered not only large genes, like the full-length dystrophin gene, but also performed complex genomic operations such as targeted genome editing, gene silencing, and site-specific recombination. These vectors demonstrated high efficiency in various applications, including the simultaneous delivery of multiple genes and proteins in their functional states. Furthermore, the T4-AVVs showed potential for broad applicability in gene therapy, offering a versatile and powerful tool for genome remodeling. Their ability to carry large genetic payloads and perform multiple genomic tasks in a single delivery system is particularly promising for treating disorders like DMD. The vectors' capacity to bypass the size limitations of traditional vectors opens new avenues for treating genetic disorders that involve large genes. In conclusion, the T4-AVVs offer a powerful tool for genome remodeling and broad applicability in gene therapy. In particular, AVVs in the future have the potential to transform therapies requiring delivery of large and multiple genes, genome editing machinery, targeted *in vivo* delivery, and personalized genetic medicine.

1842. Single-Use Fermenters for Plasmid and Minicircle DNA Production

Florian Weber[1], Chris Buschmann[1], Ram Shankar[1], Christina Ramke[2], Rudolph Klementz[2], Marco Leupold[2], Jens Rupprecht[2], Alyssa Vetter[3], Kathrin Teschner[4], Sandra Klausing[4], Marco Schmeer[1], Martin Schleef[1]

[1]PlasmidFactory GmbH, Bielefeld, Germany,[2]Sartorius Stedim Biotech GmbH, Göttingen, Germany,[3]Sartorius Xell GmbH, Schloß Holte-Stukenbrock, Germany,[4]Sartorius Xell GmbH, Schloss Holte-Stukenbrock, Germany

There is a surge in demand for GMP-grade plasmid DNA as starting materials for manufacturing crucial viral vectors, especially for late-phase clinical trials and for commercialization. The employment of single-use systems reduces development times through higher batch to batch turnover, allowing quicker supply of clinical material. Single-Use fermenters have been well documented for animal cell culture applications whereas for microbial cultivations, two major limitations were noted: poor oxygen transfer capabilities and insufficient heat removal. The Biostat STR® Microbial single-use fermentation system has been designed specially to overcome these challenges. Following the recommendations for process engineering characterization by the DECHEMA expert group on single-use technology, a volumetric oxygen mass transfer coefficient (k_La) of up to 675 h^{-1}, a maximum specific power input of 3 kW/m^3 and a maximum cooling rate of 29 $K{\cdot}h^{-1}$ were noted. At the R&D facility of PlasmidFactory GmbH, the Biostat STR® Microbial with 40 L working volume was tested for the fermentation of optimal plasmid production strains of *Escherichia coli*. Our tests comprised of batch and fed-batch, small and large plasmids including those for the production of AAV vectors as well as a particularly adapted fermentation process for the production of *minicircle* DNA. In addition to cascading stirrer speed against the DO level, we set a second level of control through pure oxygen sparging (max. 7 $L{\cdot}min^{-1}$) during maximum stirrer speed (500 rpm). Temperature control at a stable 37 °C was successful throughout. Efficient single-use exhaust cooling ensured that the bag pressure never exceeded 10 mbar. A simple batch process yielded a maximum optical density after 16 h fermentation time. In terms of biomass yield per batch, DNA product yield per biomass as well as DNA product quality (plasmid DNA homogeneity), the Biostat STR® single-use fermenter offered similar results as compared to conventional stainless-steel fermenters. The purified plasmid DNA was successfully used for AAV production within HEK293 cells (AAV6 carrying a GFP reporter gene construct) at shake flask as well as 2 L Bioreactor scale. Measured genomic titer and capsid titer were similar to those achieved in parallel AAV production using DNA from stainless steel fermenters. In the *minicircle* fermentation, CGE analysis of the DNA product showed effective control of induction and the expected level of recombination to yield *minicircle* DNA in the supercoiled conformation. The DNA product after lysis was quantified to be 914 mg from a single batch which is expected to yield over 100 mg of pure *minicircle* DNA. One of the major product quality attributes is the absence of cross-contamination which forms the strongest case for single-use technology. This fermentation system proves that microbial cultivations in the single-use space are no longer a compromise on yield but rather represent a true possibility to shift from conventional sterilizable fermenters. For GMP, this becomes necessary to accelerate production while easing quality control challenges.

1843. Aldevron's Nanoplasmid™: A Next Generation Vector Manufacturing Platform for Gene and Cell Therapy

Jeremy Luke[1], Tyler Kozisek[1], Jason Devlin[1], Patrick Alfredo Paez[1], Denis Arutyunov[1], Aaron Carnes[2], James Williams[1], Seshidhar Reddy Police[3], Venkata Indurthi[1]

[1]Aldevron, Fargo, ND,[2]Aldevron, Lincoln, NE,[3]Aldevron, Boston, MA

The same plasmid manufacturing technology has been used for decades in vaccination, cell and gene therapy, and as a raw material in viral vector and mRNA production. While canonical *Escherichia coli* plasmids contain antibiotic resistance markers and have backbones that are greater than 2000 bp, smaller backbones were shown to have reduced cell-transfection-associated toxicity and transgene silencing. Aldevron's Nanoplasmid™ vector has a small backbone (<500 bp) and no antibiotic markers. It has proven to be a transformative replacement in various applications, offering greater safety and efficiency than traditional plasmids. Nanoplasmid vectors use the antibiotic-free *E. coli* maintenance system based on sucrose selection. Notably, these vectors also contain a specialized bacterial R6K replication origin in place of the traditional pUC origin, making the Nanoplasmid vectors replication-incompatible outside of a set of engineered strains. These features dramatically reduce the risk of horizontal gene transfer in case of inadvertent exposure to the plasmid. In addition, Nanoplasmid vectors can be produced in high yields (1-2 gram/L) and quality in bacterial fermentation using proprietary strains and growth conditions. Nanoplasmid transposon vectors and Nanoplasmid Homology-Directed Repair (HDR) donor template vectors for CRISPR/Cas9 mediated gene editing demonstrated superior cell viability and gene integration frequencies during non-viral CAR-T cell manufacturing.
• Poseida Therapeutics showed that Nanoplasmid vectors have better efficacy and equal safety in patients compared to standard plasmids

when used for CAR-T production with the piggyBac® transposition (*Clinical Trial NCT03288493, P BCMA-101*). • Genentech reported improved CRISPR HDR knock-in by comparing a linear and a traditional pUC plasmid against a Nanoplasmid vector as the donor template. Their studies concluded that Nanoplasmid vectors resulted in twice the production of edited CAR-T cells compared to the traditional pUC plasmid and three-fold compared to the linear donor (Oh et al., *Journal of Experimental Medicine*, 2022). • Editas Medicines used Nanoplasmid vectors for the Selection by Essential-gene Exon Knock-in (SLEEK) HDR approach, producing knock-in rates of 90% (Allen et al., *Nature Biotechnology,* 2023). • Stanford University researchers used Nanoplasmid vectors in a CRISPR method termed "Homology-Independent Targeted Insertion" (HITI) and achieved approximately 80% edited cells after the 14-day protocol (Hyatt Balke-Want et al., *Molecular Cancer*, 2023). Independent evidence suggests that using Aldevron's Nanoplasmid vector platform in CRISPR *ex vivo* gene editing applications leads to 80-90% editing rates and reduced cellular toxicity compared to traditional vectors.

1844. Manufacturing Process Development and Analytical Characterization of CBN-1000, a New Viral Vector Derived from Human Bocavirus

Gillian Payne[1], Joshua Merritt[2], George Richards[2], Po-Yi Ho[1], Yousuf Aqeel[1], Alfred G. Tamayo[1], Ryan Farrell[2], Frederick Metters[2], Sung Jo[1], Shu-Hao Liou[2], Brooke DeSimone[1], Pooneh Hajian[1]

[1]Analytical Sciences, Carbon Biosciences, Waltham, MA,[2]Process Development and Manufacturing, Carbon Biosciences, Waltham, MA

CBN-1000 is a new parvoviral vector derived from human bocavirus 1 (HBoV1) virus. The CBN-1000 capsid is remarkable in that it has specific tropism for differentiated human bronchial airway epithelium and is able to package an AAV2 ITR-flanked genome payload of >5.5 kb, which is significantly larger than the 4.7 kb cargo capacity of conventional AAV vectors. Because of the expanded payload size and lung tropism, CBN-1000 vectors are ideal for the delivery of genes for the treatment of genetic lung diseases which are too large for AAV vectors. CGT-001 is comprised of a CBN-1000 vector with the full-length gene for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) for the treatment of patients with cystic fibrosis. A plasmid-based mammalian cell manufacturing process has been developed for the efficient packaging of a CFTR expression cassette into HBoV1 capsids. An affinity chromatography-free purification process has also been developed which results in high purity vector product. The process development, scale-up and manufacturing of this new hybrid vector and in-depth characterization of CGT-001 will be described.

1845. Navigating Pitfalls in the Adenovirus Development: From Drug Discovery to Clinics and Beyond

Emilie Gateau[1], Dmitrii Sorokin[1], Helmut Kewes[2], Petri Priha[3], Vasily Medvedev[1], Hanna P. Lesch[1]

[1]Exothera, Jumet, Belgium,[2]Cytiva, Cologne, Germany,[3]Valo Therapeutics Oy, Helsinki, Finland

Following COVID-19 pandemic response, there has been a steady increase in the number of clinical trials with recombinant Adenovirus (rAdV) for human gene therapy, oncolytic cancer therapy or as a human vaccine. There are several common pitfalls developers might encounter in their journey, including poor target identification of the proper lead candidates and serotypes as well as consideration of safety and efficacy which can all impact a program's success. We have built an Adenovirus platform with a rational CMC strategy to support the discovery process and to mitigate the risks during early stages of development. Analytical development serves as the foundational basis for the development of new therapies. We have been using qualified assays (viral particle concentration by ddPCR, infectious titer by TCID50, commercial titer kits, and next generation sequencing (NGS) for vector identity and genetic stability) to characterize, assess, and ensure the quality, safety, and efficacy of these complex products. We have performed ddPCR primer design and standardized sample treatment to improve the consistency of virus particle quantitative analysis across various adenovirus serotypes. Same approach is applicable for also for screening and selection of rare and chimeric serotypes. Preclinical decisions, like host cell line selection for virus seed stock generation and process development, are crucial for future clinical manufacturing. In addition, the use of standard cell lines like adherent HEK293 or A549 cells with safety concerns associated for the use of Fetal Bovine Serum (FBS). To address this concern, we have adapted an adherent A549 cell line to serum-free conditions by testing 5 different media over 5 passages and identifying 2 compositions of serum-free media, supporting cell growth. In the case of non-replicating adenoviruses, HEK293 cells may also increase the risks of contamination with Replication competent adenoviruses (RCA). We have preferred Cytiva's CAP™ cells, primary human amniocytes immortalized by modified adenoviral genetic E1/pIX functions, specifically designed to minimize RCA during production. These cells are fully documented and validated to meet regulatory requirements and they present good growth characteristics as well as improved quality (less than 1 RCA particle in 5E+10 rAdV particles in CAP cells, compared to 5-500 RCA particles in HEK293F cells). Early technological platform selection significantly influences clinical manufacturing challenges, such as scalability and batch consistency. We have been utilizing Design of Experiment (DoE) and mathematical modelling of the scale-dependent parameters to build a consistent manufacturing processes and scalability for stirred-tank, rocking angle and fixed-bed bioreactors, while working with suspension and adherent cells. As an example, for one of the oncolytic rAdV, produced in the adherent A549 cell line we identified three key process parameters using DoE approach at the small scale (T-Flasks), leading to 3.8 times higher productivity (infectious units per cm^2) compared to the reference process. An established factor-response mathematical model (RSq=0,96 and PValue=< 0,0001) was found reliable and was used to scale-up the process to the fixed-bed bioreactor and equivalent productivity increase (3,3 times) from reference process,

was maintained after application of the best DoE conditions to scale-X Hydro bioreactor at 2,4 m2 scale. Knowing about pitfalls on the road ahead and spending time and resources for critical decisions is the key to success in producing an adenoviral product. We have developed a wide variety of processes allowing safe production of AdV for all types of applications.

1846. Further Development of the TRiP System™: A Transgene Repression Technology Enhancing Lentiviral and AAV Vector Production

Ben M. Alberts[1], Cristina Nogueira[1], Magdalena Martin-Urdiroz[1], Alissa S. Bray[1], Ian A. Tovar Matthews[2], Desislava V. Simova[2], Magda Reis[3], Catarina R. Vieira[2], Adam Kuca[1], Elizabeth A. Ellis[2], Hannah J. Stewart[3], Jordan Wright[1], Kyriacos A. Mitrophanous[1], Nicholas G. Clarkson[2], Dan C. Farley[1]

[1]Innovation Group, Oxford Biomedica, Oxford, United Kingdom,[2]Process Development, Oxford Biomedica, Oxford, United Kingdom,[3]Product Feasibility Group, Oxford Biomedica, Oxford, United Kingdom

The impact of transgene expression during production of viral vectors can lead to changes in the feed material going into previously well-characterised processing steps, requiring extended process development. In addition, the total process yields can be lower than expected for typical 'benchmark' levels if the transgene affects vector particle assembly, release or activity, and the presence of the therapeutic protein within the final product may be undesirable. We previously developed the Transgene Repression in vector Production (TRiP) System to overcome these problems, since only the viral proteins will be expressed during production. TRiP employs the bacterial protein tryptophan RNA-binding attenuation protein (TRAP) from *Bacillus Subtilis*, which binds to its 55 nucleotide RNA binding sequence (tbs) inserted into the 5' UTR of the transgene mRNA to mediate a translation block. Here we report improvements to the TRiP System™ wherein a standardised, short TRiP 5' UTR-tbs sequence has been engineered to mediate both efficient transgene repression in production cells, and to maintain high expression in target cells. Upstream of the tbs, the TRiP 5' UTR comprises a 12 nucleotide leader derived from the human EF1α promoter with its native run of 5' terminal pyrimidines, which allow for predictable transcription initiation when paired with the typical constitutive promoters employed in gene therapy cassettes, as well as tissue-specific promoters. The tbs has been re-designed at its 3' end to overlap with the primary AUG codon of the transgene to better occlude it from scanning ribosomes when bound by TRAP, whilst also maintaining an efficient Kozak sequence for ribosomal RNA binding during translation initiation in target cells. We show improvements in production of LVs when the TRiP 5' UTR-tbs configuration is onboarded onto novel '2KO-LV' genomes (part of the TetraVecta™ System), which are mutated for aberrant splicing activity from the major splice donor in the packaging signal. In standard 3rd generation LVs, such aberrant splicing produces transgene-encoding mRNAs that the TRiP System™ represses much less efficiently, making the 2KO-LV platform the ideal partner to TRiP. Here we provide an example of the transition of a third-party LV-CAR product from small scale feasibility studies to a commercially relevant production scale model, utilising these and other novel LV production technologies. Over a 20-fold final yield increase was realised by utilising the 2KO-LV/TRiP technology components of the TetraVecta™ System, in combination with a novel LV production enhancer Ingenol-3-angelate (I3A) that contributed ~3-fold of this increase. Further, we show by mass spectrometry that the TRAP protein is detectable only as a minor residual (<1% of total protein) within HIV-1 based LV product that has been concentrated and purified by a commercially relevant process. Moreover, we have not been able to detect pre-existing antibodies to the TRAP protein in a screen of >160 healthy human sera, indicating that residual TRAP is unlikely to limit gene transfer. Finally, we applied the TRiP System™ to a wide range of transgenes within ssAAV vectors, including those containing a CMV-driven, pro-apoptotic Caspase-8 or Bax gene, where TRiP was able to fully rescue titres by 150-fold and 677-fold, respectively.

1847. Start with Stable: Stepping onto TetraVecta™ Packaging Cells to Make a Clearer Pathway to LentiStable™ Producer Cell Lines

Emma S. Whiteley[1], Emma Burton[2], Magdalena Martin-Urdiroz[1], Joana S. Boura[1], Ben M. Alberts[1], Jordan Wright[1], Kyriacos A. Mitrophanous[1], Hannah J. Stewart[2], Dan C. Farley[1]

[1]Innovation Group, Oxford Biomedica, Oxford, United Kingdom,[2]Product Feasibility Group, Oxford Biomedica, Oxford, United Kingdom

The use of HIV-1 based Lentiviral vectors (LVs) to deliver transgenes encoding Chimeric Antigen Receptors (CARs) is the industry standard in the generation of CAR-T cells for cancer immunotherapy. The commercial success of any given CAR-T therapy is multifaceted but is broadly in tension between the desire to traverse the clinical development pathway quickly, and the need to ensure a high-quality product is manufactured as economically as possible. The primary method of generating LV-CARs is via transient transfection of HEK293T cells (typically suspension, serum-free) at 50-200L scale, although up to 2000L is now feasible. The use of stable producer cell lines (PCLs) to manufacture LVs is desirable due to reduced costs, ability to scale to large volumes, improved batch consistency and a more streamlined production process, compared to the standard transient transfection process. However, transfection-based production is sufficient to provide enough LV product to service clinical trials and consequently is typically progressed for commercial supply in a number of jurisdictions. This creates an inertia that halts the possibility of realising the benefits of PCLs due to the need to perform comparability studies between LV product made by both processes. The generation of LV-CAR PCLs capable of producing high titre LV has had limited success until recently, due to the constitutive expression of the CAR molecule negatively impacting production cell survival. Oxford Biomedica (OXB) has developed the LV TetraVecta™ System, which enables effective repression of the transgene in production cells, thus opening the pathway to LentiStable™ LV-CAR PCLs. Since the generation of PCLs typically requires 6-9 months to complete, OXB also offers a transition platform that allows a flexible but simplified LV production process based on off-the-shelf TetraVecta™ packaging cell lines (PaCLs) that contain all necessary LV packaging components for

the production of LV particles, as well as TRiP System™ components that repress the transgene. Here we provide data exemplifying the use of TetraVecta™ PaCLs in the production of LV-CARs and demonstrate comparable or greater yields than the fully transient process. The process scaled well into a 7L Stirred Tank Reactor (STR), and following downstream processing, the concentrated and purified LV was capable of effectively transducing human primary T-cells. We envision that the use of TetraVecta™ PaCLs as the first step of LV-CAR product development will [1] reduce costs and logistics associated with sourcing GMP plasmids for all LV packaging components, [2] reduce process development timelines and improve batch-to-batch consistency owing to the invariance of LV particle production, and [3] close the comparability gap requirements between LV product made using PaCLs and PCLs. Should a PCL be developed in parallel to clinical trials a more straightforward transition to PCLs would be enabled, and the benefits of a fully transfection-free commercial product would be realised.

1848. Enabling Closed Chromatography Processes Using a Gamma Sterilized and Scalable Chromatography Product Platform from Small to Large Scale

Alexander Helling, Swapnil Chaudhari
Sartorius Stedim Biotech GmbH, Goettingen, Germany

With an increasing number of cell and gene therapy trials on the horizon, the demand for cost-effective and high-quality manufacturing of gene delivery tools such as viral vectors, exosomes and pDNA is growing. Although there is still little agreement on the optimal sequence of unit operations required for manufacturing high yields of vectors, chromatography, particularly anion exchange chromatography, is commonly used for primary capture from cell culture supernatant or polishing albeit with typically low recoveries or yields. Membrane chromatography has already proven to be an outstanding technology in terms of chromatographic performance, recovery or yield and productivity in several processes. This study illustrates the scalability of a cellulose-based membrane chromatography portfolio from development to manufacturing scale using both model and real-application feed streams. It includes strong (Q) anion, weak anion, and heparin functionalized membranes. The high permeability of the chromatographic membrane allows loading several hundred column (membrane) volumes without a significant pressure increase at a flow rate of 10 column volumes per minute thereby significantly reducing process times and minimizing the exposure time of the target to demanding process conditions. An outlook is provided regarding the commercial availability of this novel and innovative membrane chromatography portfolio designed for single use, closed and aseptic processing, which aids in implementing cost-effective and high-quality manufacturing of gene delivery tools such as viral vectors, exosomes and pDNA.

1850. Examining the Development Challenges of AAV and LVV Manufacturing Processes: A Comparative Perspective

Margherita Neri, Luca Allievi, Martina Brunati, Samuele Corbetta, Manuela Cota, Luca Crippa, Silvia Ungari, Emanuele Simonetti, Giuliana Vallanti
AGC Biologics, Milano, Italy

AGC biologics is a leading global CDMO, providing world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA, viral vectors and genetically engineered cells. Adeno Associate Vectors (AAV) and Lentiviral vectors (LVV), produced with transient transfection in HEK293 and HEK293T cells are extensively used for genetic modification in gene therapy for *ex-vivo* and *in-vivo* treatments. AGC Biologics developed BravoAAV and ProntoLVV platforms for the manufacturing of AAV and LVV at different scales in adhesion and suspension. Both platforms are flexible to be adapted to different GOI (Gene Of Interest). Of note, BravoAAV positively applies to different AAV serotypes. The two classes of vectors we are dealing with (AAV and LVV) share some characteristics but are different from many points of view. These resulted in the development of dedicated manufacturing platforms even if starting from common process steps using same equipment and common set of reagents but that faced different challenges. We are going to compare the two manufacturing processes, step by step, underlining differences and common features. Starting point of the processes is the thawing of packaging cells. HEK 293T and HEK 293 cells are used for LVV and AAV production respectively. It has to be taken into account that those cells have different behavior in terms of growth rate and aggregation profile. Indeed, duplication time of HEK 293 cell is higher than HEK293T requiring more time to reach the correct cell density for transfection. Transient transfection is of course performed with dedicated plasmids, but with the same transfection reagent (PEIPro). Vector production after transfection requires more time for AAV compared to LVV, resulting in delayed harvests respect to LVV process. The strong proteic capsid of AAV ensures their higher resistance to shear stress, allowing to perform harvest later than for LVV and also HEK293 cell lysis. On the contrary, the presence of the envelope in LVV is the cause of the high sensitivity of the vector to temperature, pH and shear stress. For this reason, clarification of LVV produced in suspension is challenging, as the whole cell mass have to be separated from the supernatant. Historically, the purification of LVV has primarily relied on AEX Chromatography, whereas for AAV, a wide range of affinity resins is available on the market. Despite their high cost, these affinity resins ensure both high purity and good vector recovery. During the execution of concentration and diafiltration steps, AAV is more resistant to shear stress and high recoveries of infective vector are more easily achieved. In contrast, LVVs require more gentle conditions and are also susceptible to aggregation phenomena due to the stickiness of their envelopes, negatively affecting the last critical step: sterilizing filtration. Sterilizing filtration is performed with 0.22 μm filters for both platforms. Since LVV diameter is approximately 0.10 μm, the presence of vector aggregates can cause filter clogging and a decrease in recovery. The yield of this step for LVV is around 40-50%. Due to their compact size and reduced inclination to aggregate, AAVs demonstrate a more straightforward behavior during sterilizing filtration. These characteristics prevent membrane clogging and lead to

higher recovery rates in the process, up to 90%. Further details can be dissected considering application of vector (in-vivo vs ex-vivo), dose and transgene nature. Also regarding the analytical panel there are some assays that are shared by the products and others that are specific and dedicated. The knowledge of the biology of AAV and LVV and the implications in the manufacturing process and assay development is a key factor for the flexibility, capacity and capability of a CDMO.

1851. Transformative Advances in Lentiviral Vector Manufacturing: Unlocking Efficiency and Cost-Effectiveness with Tet-Off PCL Innovation

Soodeh Baghaee Ravari
Genezen, Technical Review, IN

Lentiviral vectors (LVV) serve as efficient tools for delivering therapeutic transgenes into both dividing and nondividing cells, facilitating sustained gene expression. However, challenges in current cGMP LVV production processes, utilizing transient transfection methods, include batch-to-batch variability and heightened costs associated with LVV manufacturing. These challenges contribute to the overall high production costs of Cell and Gene Therapy (CGT) products. In response to these challenges, Genezen has implemented a proven Tet-off Producer Cell Line (PCL) system to overcome the limitations of traditional production methods. Through meticulous evaluation, we have successfully adapted a PCL that consistently achieves raw IU titers exceeding 6e6/mL in four 200L productions across two distinct VSVG enveloped Transgenes of Interest (TOIs). This innovative approach demonstrated superior titers with better residual profile in a comparative study with one of the TOIs tested. The Tet-off PCL system offers numerous advantages, including improved downstream processing (DSP) with enhanced overall recoveries, a superior residual profile, and the ability to generate multiple sublots that can be combined into a super lot with functional titers surpassing 5e8 IU/mL.By eliminating the requirement for cGMP-grade plasmids in lentiviral vector production, Genezen's approach holds the promise of significantly reduced costs, improved commercial scalability, and consistent batch yields when compared to current industry-standard manufacturing processes. This innovation represents a substantial step forward in the field of gene and cell therapy, with the potential to enhance patient access to medicines based on lentiviral vectors.

1852. Large-Scale Adherent Cell Manufacturing Platform for Stem Cell and Seed Train Applications

Matt Kremer
Univercells Technologies by Donaldson, Nivelles, Belgium

Adherent cell-based manufacturing platforms have long represented the gold standard for both stem cell and viral vector manufacturing applications, due to rapid implementation possibilities, straightforward process optimization and relatively high productivities. In recent years, they have however also been increasingly discarded as a viable option by organizations looking to achieve scale-up to commercial manufacturing. Limitations of traditional adherent manufacturing platforms largely justified this choice as they have historically been associated with poor scalability and high cost per dose. Efforts to use automated alternatives, such as packed-bed bioreactors, have provided significant improvements but remain limited by critical factors, such as poor scalability, high operating footprint, and, crucially, the inability to efficiently harvest cells from adherent bioreactors. This ability to harvest has been seen as a key limitation, not only for manufacturing of cells as a therapeutic agent but also in generating large-scale seed train for adherent cell-based viral vectors manufacturing. In this presentation, the speaker will unveil a novel approach unlocking cost-efficient large-scale manufacturing of adherent cells and cell-based products. An introduction to the scale-X™ bioreactor will be provided, demonstrating how structured fixed-bed technology enables low-cost, low-footprint automated cell harvest through a blend of enzymatic and mechanical actions. More specifically, the speaker will: - Present the results of an extensive study demonstrating efficient harvest of HEK293 and Vero cells for seed train applications using scale-X cell collect, with >90% viability of the harvested cells and proof-of-concept for fixed-bed to fixed-bed seed train - Showcase an intensified, automated seed train workflow with reduced process steps and drastic reduction in required clean room area - Detail how implementing this workflow enables significant cost reduction with 21%, 40% and 32% decrease of total facility-, materials- and labor- associated costs, respectively - Present several cases studies demonstrating the application of the scale-X cell collect to harvesting stem cell lines with high efficiency and high viability

1853. Developing High Titer Stable Producer Cell Line Platform for Lentiviral Vector Manufacture

Bofu Xue
Shenzhen Eureka Biotechnology Co., Limited, Shenzhen, China

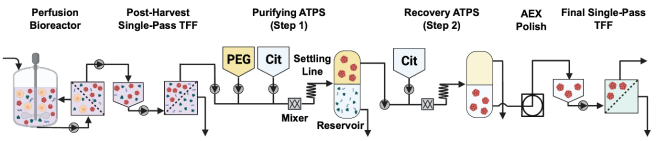
Lentivirus is one of the widely used viral vectors in cell & gene therapy, and the mainstream production system is the four-plasmid transient transfection system which faces the challenges of high plasmids costs and batch variability at a commercial production scale. To address these challenges, Eureka Biotechnology has developed a suspension-mode, inducible lentiviral vector stable production system (EuLV System). Our stable production system comprises two innovative technology platforms: the packaging cell line (PCL) and the stable producer cell line (SPCL). The packaging cell line integrates lentivirus packaging components (Rev, Gag/Pol, and VSV-G) into the genome of suspension HEK293T cells. The PCL platform uses one plasmid transient transfection for LVV production, significantly reducing plasmid production costs and process complexity. The stable producer cell line platform offers a plasmid-free method for LVV production. It integrates the Gene of Interest (GOI) into the packaging cell line genome and utilizes double molecular switchers for precise gene expression control. This platform eliminates plasmid manufacture and transfection steps, produces 10 to 100 folds higher titers (2E8 TU/ml), enhances scalability, and greatly improves process robustness. It enables cost-effective, scalable, high-titer, and high-quality lentiviral vector production and is pivotal for the future of commercial-scale LVV manufacturing. The EuLV System simplifies the CMC process, and benefits to the cost down. It represents a promising solution for solving the bottlenecks of the current LVV manufacturing.

1854. Continuous Manufacturing for Viral Vectors Using an Aqueous Two-Phase Extraction Platform

Natalie M. Nold[1], Sheridan Waldack[1], Grace James[1], Emily Agustin-Mazariegos[2], Abigail Ruthenberg[1], Taravat Sarvari[1], Eric Pearson[1], Lynn Manchester[1], Trisha Colling[1], Amanda Bekkala[1], Bradley Priem[3], Justin Sargunas[3], Michael Betenbaugh[3], Caryn L. Heldt[1]

[1]Chemical Engineering, Michigan Technological University, Houghton, MI,[2]Grand Rapids Community College, Grand Rapids, MI,[3]Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD

Standard bioprocessing methods for viral vectors, which occur in batch mode, are outstandingly expensive. More economical manufacturing methods could alleviate the cost of these ground-breaking therapies. Continuous processing promises increased flexibility and productivity compared to traditional batch chromatography-based processes. Aqueous two-phase systems (ATPS) combine product capture and purification by partitioning the viral product and contaminating host-cell proteins and DNA to different phases. Our process consists of two stages: the first ATPS stage separates the bulk of the viral product from the bulk of the impurities, while the second stage recovers the viral product from the viscous polymer phase to ease further processing. ATPS relies on raw materials rather than expensive proprietary consumables such as chromatography resin, which translates into over 50% lower production costs and 90% lower capital costs compared to traditional processes above a 11 kg/year production scale. Optimization studies in batch mode have shown 66-100% overall product recovery for adeno-associated virus (AAV), herpes simplex virus (HSV), influenza B virus, and porcine parvovirus (PPV), with lentivirus results pending. A multi-hour continuous run of our two-step ATPS for PPV showed statistically similar PPV recovery results compared to batch studies. Batch studies showed a consistent output of contaminating protein (~11 g/mL) and DNA (~8 ng/mL) regardless of the input titer of these impurities. The output DNA titer for our continuous process matched that of batch studies, however, the output protein impurity titer was about five-fold higher at 61 mcg/mL. A polishing flow-through anion exchange membrane was added post-ATPS to provide more robust impurity removal. The filter succeeded in removing an additional 87% of DNA with 90% PPV recovery, but did not succeed in removing protein impurities. A flow-through cation exchange membrane will be tested in the future to help remove protein impurities. Continuous operation of ATPS requires in-process monitoring. One of our biggest challenges is control and monitoring of the mixing and settling. Turbidity monitoring using absorbance measurements at 800 nm ensures sufficient mixing and settling during ATPS. The turbidity readings were 20% higher for batch mixing as compared to the stirred mixing used in continuous ATPS. Turbidity was able to demonstrate that an inline static mixer was not sufficient to mix the phases and have desired separation of viral product and contaminants, as well as distinguish settled ATPS. A control strategy using turbidity measurements could be used to adjust mixing speed and to adjust settling time in order to reach sufficient mixing and settling rates. This proof-of-concept study demonstrates that a two-step ATPS could be incorporated into a fully continuous purification process that will make viral vector manufacturing more flexible and cost-effective.



Process diagram of an end-to-end viral vector purification system using ATPS.

1855. Overcoming Challenges to the Commercialization of Lentiviral-Based Therapies: A Scalable and Robust Production Process Using a Suspension Transient Transfection Platform

Jiasong Jiang[1], Leila M. Aneke[1], F. Michael Haller[1], Vijetha Bhat[1], Chien-Ting Li[1], Victor Paredes[1], Peng Wang[1], Miguel Betancourt[2], Thanh Dang[2], Phi Le[2], Aaron Bedee[2], Ares Ayandokon[2], Peng Lu[2], Shuo Lu[2], Mariya Viskovska[2], Senthil Ramaswamy[1], Wenling Dong[2], Bingnan Gu[1]

[1]Research & Development, Lonza Houston Inc., Houston, TX,[2]Process Development, Lonza Houston Inc., Houston, TX

Due to their ability to deliver larger cargo, transduce both dividing and non-dividing cells and maintain long-term and high expression levels, lentiviral vectors (LVV) are increasingly becoming a tool of choice in cell and gene therapy applications. This increases the pressure, particularly on CDMO's, to meet the markets' demand for safe, 3rd generation viral vectors with adaptable, scalable and high-yielding manufacturing processes. While continuing to develop new tools for LVV manufacture, such as a stable producer cell line, we are presenting here the results obtained for our proprietary HEK293T 2G7 suspension cell line for LVV production by transient transfection in serum-free, chemically-defined and animal component-free media and its subsequent downstream purification. The process was developed using LVV-CD19 as model construct in addition to the commonly used LVV-GFP due to altered downstream performance observed by us when switching the gene of interest from GFP to a therapeutically relevant gene. The obtained process was confirmed repeatedly in a 3 L STR for both LVV-GFP and LVV-CD19, transferred to our process development department and tested there at 50 L scale using LVV-CD19, with an overall process recovery, including clarification, of 24 %. The bulk drug substance (BDS) was analyzed for infective, genomic and capsid titer, as well as potency by T-Cell transduction and residual host cell DNA and host cell protein content.

1856. A Novel, Synthetic DNA Alternative for Lentiviral Vectors Manufacturing

Andreia M. Silva, Fabian Trick, David Wilson, Ivana Pastierikova, Luca Distefano, Marco Guarrera, Martin Cusack, Alexander Pekarsky, Jorge Omar Yanez Cuna, Joel de Beer

Anjarium Biosciences AG, Schlieren, Switzerland

Lentivirus vectors (LVV) represent vital products and tools within the cell and gene therapy domains. LVV are conventionally produced in

cellular expression systems upon transfection with plasmids of bacterial origin that encode the gene-of-interest (GOI), packaging proteins, and envelope proteins. Scaling up the production of these plasmids is costly, and time-consuming. We have developed a novel, optimized synthetic linear double-stranded DNA with customizable hairpin-ended structures for LVV production. Our cell-free, enzymatic production process is readily scalable and results in high-fidelity, high-yield, and high-purity DNA in a short timeframe. Additionally, our synthetic DNA constructs are devoid of bacterial backbone, thereby reducing the risk of introducing non-relevant and antibiotic resistance elements into the LVV production process - a significant safety and regulatory concern. Our DNA platform encodes the elements of a 3rd generation lentivirus system. LVV were produced by transient cell transfection at optimal conditions determined by Design of Experiments. Functionality and titer of the produced LVV was evaluated by cell transduction assay and p24 ELISA. Here, we show that synthetic DNA constructs codifying for the GOI, Rev, Gag-Pol, and VDV-G generated through our enzymatic, cell-free process are produced at high purity. GOIs encoded in our DNA platform include reporters of different size, anti-CD19 Chimeric Antigen Receptor (CAR), under the control of different promoters. All LVV produced were functional, effectively transducing HEK293T cells. CD19 CAR was also effectively expressed at the surface of Jurkat cells upon transduction with the produced LVV, further demonstrating the feasibility of our DNA in applications for CAR-T cell therapies development. In conclusion, our synthetic DNA platform can be used as an alternative to bacterial plasmids in LVV production.

1857. Efficient Separation of Plasmid Isoforms Using Anion Exchange Chromatography (AEX) and by Capillary Electrophoresis with Laser Induced Fluorescence Detection (CE-LIF)

Syam Pillai[1], Kiran Krishnan[1], Upul Halambage[1], John Bowen[2], Tatiana Nanda[1]

[1]Analytical Development, SK Pharmteco - US, King of Prussia, PA,[2]Plasmid Operations, SK Pharmteco - US, King of Prussia, PA

Precise qualitative and quantitative determination of individual plasmid DNA (pDNA) isoforms in the final product is the major quality determinant, as high purity supercoiled (SC) pDNA is a prerequisite for all gene therapy (GT) platforms. AEX -high performance liquid chromatography (HPLC) exploits the different physicochemical properties of the open circular (OC) linear (LN) and supercoiled (SC) pDNA in ascending salt gradients to separate the isoforms where SC isoforms with a higher charge density elute later than OC with a lower charge density. However, quantitative resolution of the OC and LN isoforms are challenging due to the physical retardation of OC isoforms in the chromatographic stationary phase and overlapping of LN isoforms with OC and SC isoforms. This challenge is further aggravated by the pDNA size, that contributed to the elasticity and other physicochemical factors related to hydrodynamics affecting the channel/pore size of the columns. Circular plasmids are sensitive to homologous recombination resulting in the formation of a dimeric molecule, in addition, to the multimers formed by the aggregation of plasmid during high salt purification processes. The AEX methods have limitation to precisely determine these dimers and multimers. In the present work, we introduce an AEX method for the effective separation of pDNA isoforms for the plasmid sizes ranging from 2.9 through 23.6 Kb size and development of a CE-LIF method for quantitation of the plasmid isoforms, specifically for the separation of dimeric/multimeric pDNA, and the comparison of two methods for a range of plasmid sizes for effective topology analysis. This will enable a unique opportunity to close the gap in a complete plasmid characterization process and ensure the highest quality plasmid for further GT manufacturing.

1858. Novel Ligand Outperforms State-of-the-Art Ligand in Lentivirus Purification by Anion Exchange Chromatography

Nicolas Meier, Andreia M. Silva, Fabian Trick, Joel de Beer

Anjarium Biosciences AG, Schlieren, Switzerland

Lentivirus vectors (LVV) are widely used in cell engineering for cell therapy applications, and are leveraged in the manufacturing of an increasing number of products such as CAR-T cells. LVV used for cell engineering are produced using cell expression systems at varying scales and thus their isolation from conditioned media containing host-cell and cell culture-derived contaminants is a crucial step during production. LVV purification is usually performed by anion exchange chromatography (AEX), but the state-of-the-art run conditions required often lead to significant loss of functional virus due to LVV's high sensitivity to pH, salt concentration, and shear stress. Here we present a novel functionalized resin that enhances the purification of LVVs, enabling the optimized high yield recovery of functional LVV under mild conditions, outperforming the benchmark ligand used for LVV purification. Different resin ligands were screened for optimal binding and elution of enveloped biological particles, with optimal elution conditions at salt concentrations ranging between 150-275 mM and pH 7.0-8.0. Collected fractions were analyzed for viral recovery by HEK293T cell transduction assay and p24 ELISA. Depletion of contaminants was assessed by total protein quantification assay, HEK293 Host Cell protein ELISA and QuantiFluor dsDNA assays. We show that LVV can be successfully purified in both capturing and polishing mode from a diverse set of clarified conditioned media. Due to the mild elution conditions, viral titer recoveries of up to 4-fold higher were achieved with nearly 2-fold improved contaminants clearance (HCP and dsDNA) compared to the benchmark DEAE ligand. Our novel ligand is a promising alternative in LVV downstream processing, leading to significant increases in process productivity by recovering purer LVV at high yield.

1859. Purification Platform for Viral Vectors Using Aqueous Two Phase Systems

Natalie M. Nold, Taravat Sarvari, Sheridan Waldack, Grace James, Abigail Ruthenberg, Liza Korolkov, Lynn Manchester, Trisha Colling, Amanda Bekkala, Eric Pearson, Caryn L. Heldt

Chemical Engineering, Michigan Technological University, Houghton, MI

The costly manufacturing methods used for viral vectors are a key reason why gene therapies can be prohibitively expensive, often costing over $1 million per patient treated. More economical manufacturing methods could lower costs and improve patient access. Continuous manufacturing could reduce the cost of viral vector manufacturing

by increasing process time while reducing both operating costs and capital costs. Unfortunately, traditional production methods, including ultracentrifugation and bind-and-elute chromatography, are not easily compatible with continuous operation. Our lab has developed a continuous purification method using aqueous two-phase systems (ATPS), which combines product capture and purification by partitioning the viral product and contaminating host-cell impurities to different aqueous phases. ATPS consists of polyethylene glycol polymer and citrate salt that separate at high concentrations into two aqueous phases. Since ATPS relies on biocompatible and environmentally-friendly raw materials rather than expensive proprietary consumables, such as chromatography resin, ATPS requires 50% lower production costs compared to traditional processes at large manufacturing scales. Our ATPS consists of two steps, as shown in Figure 1. The first step purifies the virus from host cell contaminants and the second step extracts the product from the viscous polymer phase to ease processing downstream. ATPS holds promise as a viral vector purification platform with 66-100% overall product recovery for adeno-associated virus (AAV), herpes simplex virus, porcine parvovirus (PPV), and influenza B virus; results are pending for lentivirus. ATPS delivers a consistent low output of impurities; for PPV ATPS, ATPS outputs ~11 mcg/mL of contaminating protein and ~8 ng/mL of contaminating DNA regardless of starting titer. ATPS ability to enrich the full capsid percentage of AAV will be tested using biolayer interferometry. Our investigation of ATPS for multiple viral vectors has uncovered the key driving forces of electrostatic and hydrophobic forces that enable quick process development for a wide variety of viral products. This could unlock cost-effective manufacturing for viral gene therapy vectors and make these ground-break therapies more affordable for patients.

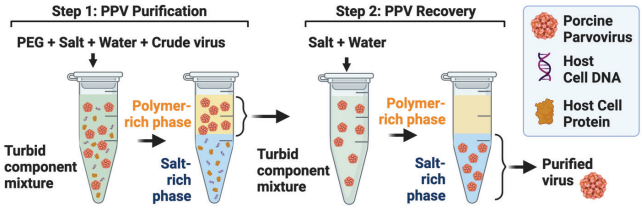


Figure 1: Schematic of the two-step ATPS process. PPV is purified from host cell impurities in the first ATPS step. Virus-laden PEG-rich phase is then mixed with fresh citrate at different chemical conditions to recover PPV into the salt phase in the second ATPS step. Figure created in Biorender.

Friday Posters: Cell Therapy Product Engineering, Development, and Manufacturing

1860. Mitigating Risk for Transfer of Living Materials into Closed Systems

Donald C. Singer[1], David J. Root[2]

[1]Ecolab Life Sciences, Phoenixville, PA,[2]Ecolab Life Sciences, Horsham, PA

One of the biggest challenges for developers and manufacturers of cell and gene therapies is to design a robust process to transfer living material into closed systems such as isolators, without introducing contamination into the grade A/ISO 5 area or damaging them from the bio-decontamination process. Different processes (automated and manual) and strategies are being taken into consideration by various cell therapy manufacturers, as well as some novel strategies to overcome this challenge. Use of isolators with automated bio-decontamination systems are becoming a first intent consideration for robust process design and should be integrated with procedures that ensure protection of living material viability.

1861. The Myth of the "Non-GMP" Space: The Criticality of Establishing Good Aseptic Practices in the Development Environment

Amanda Curtis

ValSource, Downingtown, PA

Microbial control concepts such as good aseptic practices and segregation control are well-known and accepted practices in GMP manufacturing of cell and gene therapies. However, a gap is still commonly seen in the development space, in which functions such as Research and Development or Process Development do not consistently consider and adhere to good aseptic practices when developing and designing new products and processes. Adherence to applicable microbial control concepts during the design and clinical phases can improve the quality of the work performed in the development space, lead to more robust processes, decrease the risk of contamination during process performance, and enhance the transfer of processes to the GMP environment. This presentation will discuss relevant microbial control concepts from the perspective of a GMP microbiologist with experience investigating and responding to contamination events in early phase clinical products with a contract manufacturing organization, in which patient treatment was ultimately delayed due to poor process design. The presentation will provide industry examples of contamination events in the development space and handoffs to the GMP space that could have been prevented or mitigated via application of microbial control concepts.

1862. Validation of a Spectral Flow Cytometry Platform for Use in a GxP Environment

Christopher Colvin[1], Emma Kraus[1], Austin Lawrence[1], Victoria Best[2], Sapna Varia[2]

[1]Analytical Sciences, AmplifyBio, West Jefferson, OH,[2]AmplifyBio, West Jefferson, OH

Background Spectral flow cytometry is a versatile and efficient platform for performing complex immunophenotyping and population biodistribution testing for discovery, preclinical and clinical immune-monitoring applications. Spectral flow cytometers utilize spectral unmixing technology, which allows for a large number of parameters (>25) to be interrogated simultaneously at relatively low cost. Though this increase in panel size allows for the detection of a broader range of markers, little is known about the platform's suitability for use in a GxP environment. The goal of this study was to qualify a 5-laser Cytek® Aurora using pre-defined reference samples to evaluate multiple performance parameters. *Methods* The Cytek® Aurora (5-laser, 64 channel with small particle detector configuration) and its SpectroFlow acquisition software were evaluated for a range of parameters that

included: template creation and user access control, template and file corruptibility, FCS integrity and data export, reference library performance, unmixing and compensation functions, small particle detection, precision, reproducibility, and accuracy. To assist with this process, commercially available 10-500 nm size beads, single color compensation beads, and characterized reference PBMCs were used. The Cytek® Aurora 25-color immunoprofiling assay (qualified by the vendor) was also used to eliminate effects of panel design and operator skill in the interpretation of instrument performance. All acquired data were analyzed using FlowJo Software. *Results* SpectroFlow acquisition software performed consistently and allowed for repeated use of access-controlled templates without corruption (n=15 independent runs). The spectral unmixing and traditional compensation algorithms were easy to use and showed comparable results when applied to a 7-parameter panel. When assessing spectral unmixing only, precision runs showed average %CVs of 9.4%, and average percent recoveries of 113% for populations of known frequency. The reference library, utilized by SpectroFlow for instrument setup, could also be used across different instrument settings without any effect on the accuracy of the data. The small particle detector, performed as indicated, consistently resolving bead standards ranging from 80-200 nm. Importantly, when testing the performance of the Cytek® Aurora system on a 25-color panel, there was no loss of resolution or accuracy noted, as the average %CV was 11.9%, and average recovery was 102%. In summary, spectral flow cytometry is a superior and cost-effective approach for applications that require high parameter testing, such as: product characterization, or immune monitoring/immunogenicity testing. The Cytek® Aurora also demonstrated flexibility for use in various discovery applications, due to its ability to toggle between compensation and unmixing, and its small particle detection function. Most importantly, its performance can also be qualified as suitable for use in a GxP environment.

1863. The AREM: A Systematic Risk-Based Approach to Contamination Control and Sterility Assurance for Aseptic Processing

Tiffany A. Baker[1], Darius Pillsbury[2]

[1]Valsource, Inc, Providence, RI,[2]Valsource Inc, New Paltz, NY

Establishing a robust Contamination Control Strategy (CCS) for an Advanced Therapy Medicinal Product (ATMP) also referred to as Cell and Gene Therapy Products is of utmost importance for success of consistent supply of drug product that is safe for patients and meets product quality requirements to support all stages of the product lifecycle. As most ATMPs are required to be sterile, aseptic processing is required at many steps of the manufacturing process. These manufacturing processes present unique and different challenges from those processes used for the manufacture of better understood biological and pharmaceutical product types. A lack of effective process controls can lead to contamination of drug product resulting in patients not receiving these important therapies and the overall clinical programs being significantly delayed and even study termination. As many of these novel manufacturing processes (e.g. for the manufacture of a cell therapy or gene-modified cell therapy) cannot be terminally sterilized, aseptic processing controls throughout the manufacture of the product over multiple unit operations and extended durations of time are required. Thus an assessment tool not only fit for fill and finish operations but also to the other process unit operations, many of which are highly manual and complex, is required for the purposes of managing the aseptic focused risks. In this presentation, a systematic approach to managing the unique risks of aseptic processing of ATMPs, the Aseptic Risk Evaluation Model (AREM) is presented. The AREM utilizes the collective product knowledge and process understanding and is compliant with the evolving global regulatory landscape and based on industry best practices. This risk-based approach has been successfully leveraged to improve processes that went on to be commercially approved. The objectives of this presentation are so that: • Attendees will gain tactical knowledge on how to perform a risk-based approach for all aseptic process steps using the Aseptic Risk Evaluation Method. • Attendees will learn how to leverage the output of the AREM to determine the strategy for process design improvement, APS design, aseptic operator qualification design, and a QA oversight model.• A Case Study overview will be provided to give attendees a real-time look at how the tool has been successfully used.

1864. Comparative Analysis of Ultra-Pure Recombinant Human Serum Albumin and Serum-Derived Human Albumin on Oxidative Stress, Expansion, Viability, and Phenotype of Human T Cells

Mark Stathos, Jake Hanks

InVitria, Aurora, CO

The rise of cell therapy has substantially increased the demand for inexpensive, effective, and consistent methods to grow primary human cells at large scale and in serum-free conditions. As in serum, human serum albumin (HSA) is often one of the most abundant proteins in culture systems for therapeutic cells. Despite this, the importance of HSA is often overlooked beyond its role in osmotic pressure regulation. However, HSA is also capable of binding to a diverse array of hormones, small molecules, and fatty acids. When serum-derived HSA is used in cell culture systems, these bound substances are also included whereas their levels are greatly reduced in culture systems using high purity delipidated recombinant HSA (rHSA). The effects of this difference on cell therapy products are unclear. We hypothesized that an ultrapure, delipidated rHSA, having more available binding sites, would better be able to reduce oxidative stress on cells resulting in beneficial changes to T cell phenotype such as a reduction in senescence and exhaustion. To that end, we characterized the phenotype of activated primary human T cells with flow panels designed to target several markers of exhaustion and senescence, cultured them in delipidated rHSA or serum-derived HSA for several days then recharacterized them using the same flow panels. Our results suggest that high purity albumin reduced expression of markers associated with undesirable phenotypes which shows promise for improving the efficacy of T cell therapies.

1865. Novel Experimental Approach to Improve Scale-Up of Microcarriers-Based Cell Culture in Bioreactors

Eyram Marcelle Koudji, Terri Jerbi, Alex Sargent

Process Development, Charles River Laboratories, Hanover, MD

The scaling of adherent cells cultivated on microcarriers in bioreactors can be challenging due to differences in the bioreactors' design and

shear force across multiple platforms. The difference in design makes scaling up the agitation particularly complex in striking a balance between maintaining all the microcarriers in suspension and using an optimal speed to reduce shear stress on the cells. Here, we compared different computational and experimental methods to determine the optimal agitation rate for expansion of adherent cells on microcarriers at small and large scales. We demonstrated a novel experimental approach to optimize agitation rate for microcarrier culture that was scalable in different bioreactors platforms. When compared to computational methods like tip-speed conversion and power input ratio, our approach substantially increased the agitation rate that was best for microcarriers suspension and cell expansion. Optimal agitation rate was consistent across the different working volumes of the bioreactors. This study shows that our novel experimental approach can improve scale-up of microcarriers-based cell culture compared to computational methods. Defining better methods to successfully scale adherent cell expansion in bioreactors is important to reduce cost, maximize cell yield, and produce a higher quality product.

1866. A New Tissue Culture Surface Enabling Serum-Free and Coating-Free Adherent Culture of Human Mesenchymal Stromal Cells

Josh DiGiacomo[1], Isha Dey[2], Rayees B. Ahamed[2], Kanika Singh[3], Arafat Fasuyi[1], Debra Johnson[1], Sune Sporring[4], Matthew Dallas[1]

[1]Thermo Fisher Scientific, Frederick, MD,[2]Thermo Fisher Scientific, Bengaluru, India,[3]Thermo Fisher Scientific, Frederick, India,[4]Thermo Fisher Scientific, Roskilde, Denmark

Due to their capabilities in differentiation, immune regulation, tissue repair, and cell signaling, human mesenchymal stromal cells (hMSCs) have found applications in therapies for a wide variety of diseases such as bone and cartilage disorders, diabetes, neurological disorders, and more. High quantities of hMSCs are required to prepare therapeutic doses and consequentially scale-up to larger cell cultures is required. Therefore, the process of culturing hMSCs needs to be efficient, reliable, and consistent in order to provide adequate quantities of cells for both research purposes and therapeutic development. The adherent culture of hMSCs typically requires the presence of serum and/or coating with extracellular matrix (ECM). These factors reduce the efficiency and reproducibility of the hMSC culture process by introducing variable culture conditions, requiring additional time, and adding extra costs. We recently developed the enhanced Nunclon™ Supra cell culture surface which eliminates these issues by allowing for the adherent culture of hMSCs under both serum-free and coating-free conditions. The treatment that generates the Supra surface can be applied to plastic cell culture vessels spanning a large range of sizes, from 96-well plates to T175 flasks. Using hMSCs derived from adipose tissue, bone marrow, and Warton's jelly, we demonstrated that serum-free, coating-free culture on the Nunclon™ Supra cell culture surface allows for comparable hMSC culture metrics such as adherence, doubling time, morphology, and dissociation compared with serum-free, ECM-coated culture on a standard cell culture surface. In addition, we also demonstrated that hMSCs cultured under serum-free, coating-free conditions on the Nunclon™ Supra surface maintain equivalent expression of multipotency markers and demonstrate comparable differentiation potential when compared to hMSCs cultured under serum-free, ECM-coated conditions. Taken together, these results indicate that the enhanced Nunclon™ Supra cell culture surface can play an important role in simplifying hMSC culture workflows and easing the transition to large volume hMSC culture.

1867. A Fast Extraction-Free Direct qPCR Method for Quantification of Residual Host Cell DNA in Viral Vector Products

Paul Li[1], Zexin Zhou[2], Ziqiang Li[2], Jiale Qu[2], Bin Liang[2], Jiayong Lao[2], Danlan Qiu[2]

[1]PackGene Biotech, Worcester, MA,[2]PackGene Biotech, Guangzhou, China

Residual host cell DNA (hcDNA) contamination presents a potential concern in purified viral vector products, including adeno-associated virus, adenovirus, and lentivirus. Regulatory agencies have established permissible limits to govern residual hcDNA levels in purified products. In the industrial cGMP setting, qPCR is widely acknowledged as the gold standard method for detecting residual host cell DNA. The inherent challenge with qPCR lies in its susceptibility to various sample matrices. Additionally, in viral vector products, a substantial portion of host cell DNA is either encapsulated within the viral capsid or attached to the capsid surface. Achieving effective hcDNA release necessitates a sample pretreatment method capable of degrading the capsid. The traditional approach involves employing a DNA extraction kit for the purification of hcDNA. However, this method is intricate and time-consuming, requiring skilled operators and potentially resulting in significant DNA loss. The recovery rate of DNA post-treatment may vary from 150% to 50%, introducing notable instability into quantitative hcDNA results and compromising detection accuracy. In response to these challenges, we have developed an extraction-free method utilizing a proprietary direct qPCR lysis buffer named AceLysis Buffer. The sample is diluted based on its hcDNA content, followed by a 1:1 mixture with AceLysis Buffer. Nucleic acids in the sample are released through 15 minutes of enzymatic digestion. The processed samples, following digestion and lysis, can be directly subjected to qPCR. This method, facile and expeditious in operation, obviates the need for high-temperature and prolonged DNA extraction, offering superior sample pretreatment performance compared to conventional methods. The recovery rate of DNA spiked into samples ranges from 85% to 110%, with a median of 95.53% (n=72), and the intermediate precision coefficient of variation (CV) is below 15%. In summary, the implementation of AceLysis Buffer for sample pretreatment expedites hcDNA detection and significantly enhances qPCR-based hcDNA detection, resulting in consistent and stable results.

1868. Performance Evaluation of an At-Line, Automated, and Rapid Mycoplasma Detection System for Cell and Gene Therapy Products

Ling Zhao, Zi Qin, Zhixing Yang

Huzhou Shenke Biotechnology Co., Ltd., Huzhou, China

Mycoplasma contaminations present frequent challenges in Cell and Gene Therapies. Unfortunately the compendial methods for their detection can be time-consuming, while general PCR methods require nucleic acid extraction and purification from samples. The AdvSHENTEK Mycoplasma DetectInnova System transformed RMM (rapid microbiological method) into a streamlined, fast, fully

automated, and easily portable laboratory system. It integrates nucleic acid extraction and real-time PCR assay, allowing a turnaround time of 2.5 hours from sample to result. Importantly it only requires less than 5 minutes of hands-on time, without specific PCR expertise to operate. The validation study shows the following feature: (1) Detection range: The AdvSHENTEK Mycoplasma DetectInnova System can load 4 different PCR assays in every run, and allow genomic DNA detection of over 180 species of Mycoplasma, Spiroplasma and Acholeplasma. The internal control in each sample ensure reliability of results by monitoring all steps from sample extraction through amplicon detection. (2) LOD (10cfu/mL) in different sample matrices: Limit of Detection (LoD) studies have been performed with a particular focus on species listed by the Pharmacopeias (not limited to below strains), in matrices of high protein, high cell density and media using 1 mL sample volume.Direct testing of 0.2 mL samples showed the positive results of mycoplasma detection by the system, so it is intended to facilitate in-process sample testing (raw materials, culture media, cell banks...) with very limited volume availability. (3) Specificity: The assay was unaffected by the presence of foreign genomic DNA of Clostridium (Clostridium acetobutylicum, Clostridium perfringens, Clostridium sporogenes), Lactobacillus (Lactobacterium thermophilum), streptococcus (Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus mutans), mammalian cell lines (CHO, Vero, 293T, HEK293, MDCK, NS0, Sf9, Hi5) and bacteria cell lines (E.coli, Pichia pastoris, Saccharomyces cerevisiae, Hansenula polymorpha) without any cross-reactivity. In conclusion, the AdvSHENTEK Mycoplasma DetectInnova System meets the pharmacopoeial requirements with a Limit of Detection of 10cfu/ml, and accommodates diverse sample types at various stages of Cell and Gene therapy manufacturing process, with the fully automated test system and hands-on time of less than 5 minutes. By utilizing pre-filled cassettes, the system significantly minimizes hands-on time, offering a streamlined solution for on-demand testing across various cell and gene therapy products. Table 1 MycoSHENTEK® Detection Level at 10 CFU/mL in Different Matrices

Sample Matrix	**Strain**			
Mycoplasma orale	Mycoplasma hyorhinis	Mycoplasma pneumoniae		
10 CFU/mL	**10 CFU/mL**	**10 CFU/mL**		
5% human albumin	24/24	24/24	24/24	
CHO-S	10^6 cells/mL	24/24	24/24	24/24
CHO-S	10^7 cells/mL	24/24	24/24	24/24
DMEM (20%FBS, 10%DMSO)	24/24	24/24	24/24	
ExpiCHO™ (10%DMSO)	24/24	24/24	24/24	

Note: 24/24=24 out of 24 test results positive



1869. Framework for Sustaining Excellence: Adapting Quality Systems in Advanced Therapies Amidst Dynamic and Fast-Paced Changes

Javier Cardenas
Azzur Group, LLC, Hatboro, PA, CA

Introduction: The rapid advancement of cell and gene therapies holds immense promise for revolutionizing medical treatment, yet the current landscape is marked by formidable challenges. Issues such as complex manufacturing processes, evolving technologies, and dynamic regulatory landscapes contribute to bottlenecks in product approvals. This abstract aims to address these challenges by exploring the adaptation of quality systems to streamline the approval process for cell and gene therapies. Methods: Our approach involved an extensive review of current regulatory guidelines and FDA / developer communications to gain insights into the existing regulatory frameworks governing cell and gene therapies. Additionally, we examined the progression of these therapies from academic discovery to the clinical and commercial manufacturing development lifecycle. This comprehensive analysis provided a foundation for designing a framework capable of adapting to the inherent variability in both the process and product as they evolve throughout the lifecycle. Results: The results of our research highlight the opportunity to design a dynamic framework that facilitates rapid adaptation to changes in the cell and gene therapy lifecycle. This framework allows for the swift integration of new technologies and methodologies, ensuring that quality systems and controls remain effective throughout the product development stages. Furthermore, our findings underscore the need for increased flexibility and interpretation within the regulatory landscape to accommodate the unique challenges posed by the evolving nature of these therapies. Conclusions: In conclusion, our review demonstrates that by streamlining quality systems and controls and implementing a flexible regulatory approach, significant time savings can be achieved in the approval process for cell and gene therapies. The adaptability of our framework ensures that regulatory requirements align seamlessly with the evolving nature of these therapies, ultimately expediting the translation of groundbreaking scientific discoveries into clinically and commercially viable treatments. This approach marks a crucial step towards unlocking the full potential of cell and gene therapies in a more efficient and timely manner.

1870. Strategies and Standards for Fit-for-Purpose Assay Design: A Focus on Cell Count and Cell Viability

Sumona Sarkar[1], Laura Pierce[2]
[1]Biosystems and Biomaterials Division, National Institute of Standards and Technology, Gaithersburg, MD,[2]National Institute of Standards and Technology, Gaithersburg, MD

The manufacturing and release of cellular therapy products (CTPs) requires high quality, robust, and validated analytical methods. Here we describe several international standardization efforts that support the fit-for-purpose development analytical methods to characterize cells and establish critical quality attributes. The ISO 23033 standard for the testing and characterization of CTPs provides a framework for establishing quality attribute that are product and MOA dependent

(fit-for-purpose). A series of ISO Cell Counting Standards (ISO 20391-1&2) establish methods for evaluating the quality of cell counting methods to support method selection and optimization. An ISO General Cell Viability standard (ISO/CD 8934) is currently under development to support the development of fit-for-purpose cell viability assays, where viability of cells is defined based on the intended use of the measurement. Use cases that demonstrate the utility of these standards are described for model Jurkat cell systems and model immunotherapy cell systems (Figure 1). These standards are critical for supporting innovation while reducing barriers to translation and reducing product development costs by easing the burden on any single entity to establish processes for analytical method development. The importance and utility of standards in cell and gene therapy is further supported by the recent deployment of the FDA CBER "Voluntary Consensus Standards Recognition Program for Regenerative Medicine Therapies" which has recognized several of the standards described here.

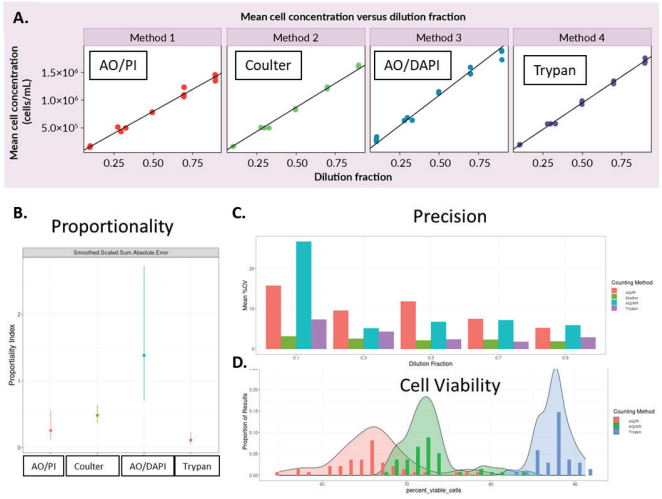


Figure 1: Example of using ISO 20391 to evaluate and compare the quality of different cell counting method using the open-source Cell Counting Method Evaluation Tool (COMET). Analysis indicates that AO/DAPI has a less proportional response than the other methods and AO/PI is less precise. The Coulter and Automated Trypan methods have similar performance, however, Coulter does not provide viability information. There is a bias in cell count and cell viability results. In this case, the Automated Trypan would be the selected method, for it's greater performance and ability to evaluate cell viability. However, the AO/PI can also be a reasonable candidate if for example the cell viability results are more meaningful for the application. These conclusions could be made without the use of a reference material or reference cell counting method. A) Mean cell concentration versus dilution fraction indicating average of the triplicate observations for each sample with error bars indicating the prediction intervals computed using the fitted flexible polynomial model. B) Proportionality Index (PI) value for each counting method, computed using the sum of the residuals. The vertical bars represent bootstrap confidence intervals computed at the requested confidence level. C) Mean %CV for each method at each dilution fraction. D) Histogram showing % viable cells for each method and the proportion of results at each measured viability

1871. Developing Image-Based Cell Assays to Effectively Monitor T-Cell Apoptosis and CAR Expression

Yongyang Huang[1], Ning Guo[2], Mackenzie Pierce[1], Leo L. Chan[1], Bo Lin[1], Yi Yang[2]

[1]Revvity Health Sciences, Inc., Lawrence, MA,[2]Novartis, Cambridge, MA

Chimeric antigen receptor (CAR)-T cell therapy is one of the novel cellular therapeutic options for cancer patients, such as the B-cell malignancies. To effectively develop and manufacture CAR-T products, it is imperative to identify and assess the critical quality attributes (CQAs) of CAR-T cells. Recently, the Food and Drug Administration (FDA) has published the chemistry manufacturing and controls (CMC) guidance for human gene therapy. Cell concentration, cell viability, and CAR expression are important quality attributes that should be reliably measured during the entire process. In this work, we developed an image-based method to quickly count viable T cell, measure viability, and assess CAR expression. Using this new method, we compare different gene delivery procedures to make CAR T cells, mostly through the electroporation process. Our results showed that high percentage of apoptotic cells were introduced during the electroporation process, regardless of the CAR vectors. Cell viabilities were checked daily with AO/PI dual fluorescent staining method and the viability data were correlated with apoptosis data. On Day 1 after transduction, measured cell viabilities of transduced SupT1 cell samples decreased to ~60% - ~70%. Compared to measured cell viabilities of un-transduced and electroporation SupT1 controls of over 90%, it was identified that the introduction of plasmids, other than the electroporation process itself, induced 20 - 30% additional cell death. Our apoptosis data further confirmed that most of the cell death after electroporation were potentially due to cell apoptosis. The transduced SupT1 cell samples were able to recover as their viabilities increased to >90% on Day 3. Percentages of CAR expression in transduced SupT1 cell samples were characterized by staining with APC-conjugated specific anti-CAR antibody. As for the percentages of CAR expression measurements, we observed similar CAR expression results in different transduced SupT1 cell samples on Day 1 and Day 2. We confirmed the percentages of CAR expression results using the flow cytometer and did not see significant differences between Cellaca® PLX Image Cytometer and flow cytometer results. With the advantages of ease of use, visual verification with captured cell images, and higher-throughput capability, Cellaca® PLX Image Cytometer may be potentially used as the convenient benchtop system for rapid assessment of the quantity and quality of CAR-T cells, which may ultimately improve the productivity of development and manufacture of CAR-T products.

Friday Posters: Pharmacology/ Toxicology Studies and Analytics/Assay Development

1872. Establishing Novel, Ultra-Sensitive, Widely Applicable qPCR and ddPCR Protocols to Support Gene and Cell Therapy

Bin Xu

Accurant Biotech, Cranbury, NJ

The FDA requires that a Quantitative Polymerase Chain Reaction method for gene therapy have a lower limit of quantitation (LLOQ) of less than 50 copies of viral gene in one microgram of DNA. However, the FDA has minimal guidance for other aspects of qPCR method validation. Thus, a fit-for-purpose adaptation of biological method validation guidelines from the FDA is commonly utilized for qPCR method development and validation by biopharmaceutical labs. Using this template, consensus parameters and criteria have been conceived in several white papers by leading scientists in qPCR applications. It is generally recognized that high concentrations > 500 ng / 100 µL of DNA template can dramatically decrease amplification efficiency due to insufficient denaturation of dsDNA. It is commonly recommended to use 10 to 100 ng of DNA template in 100 µL reaction. As FDA recommendations are listed per microgram of DNA, this means that an LLOQ of 5 copies / 100 µL reaction must be achieved. Such a task is exceptionally difficult to do reliably with current technologies. Moreover, if 20 ng of gDNA were used in the more common 20 µL qPCR reaction, it would require an even stricter reported LLOQ of 1 copy / reaction. To address this pressing issue, we employed a high concentration of **endonuclease-fragmented genomic DNA** 1 µg in 20 µL qPCR reactions (**Figure 1**). Through optimization, we were not only able to achieve good dilutional linearity (**Figure 2**) and acceptable amplification efficiencies (**Table 1**), but we found that such fragmented genomic DNA can make the assay more robust against changes in amplification efficiency, ensuring data reproducibility, while maintaining accuracy and precision very well under BMV guidelines. A reported LLOQ of 8 copies of target gene per 1 µg of DNA template can be achieved consistently (**Table 2**). In addition, we can use the reference gene Ct value to normalize target gene copy number in multiplex reactions. Thus, we assert that this optimized qPCR method represents a significant technical breakthrough and could mark a new chapter for qPCR applications. We have integrated this protocol into the validation of multiple qPCR methods, including a VSV-G gene qPCR assay for replicative component lentivirus as part of Car-T risk assessment, and a WPRE enhancer qPCR assay, which measures a sequence in the Car-T DNA construct to track Car-T cell populations. ddPCR has an advantage of offering absolute quantitation, a robust method that can often produce better LLOQ and LODs per reaction than qPCR. Our ddPCR strategy employed 300 ng endonuclease-fragmented template DNA in a 20 µL reaction. By adapting our novel set of protocols, we established a powerful ddPCR method development and validation system. Multiple ddPCR methods validated for different matrices, consistently reached an LLOQ between 1 to 8 copies per 20 µL reaction, equivalent to an LLOQ of 3.3 to 26.7 copies per 1 µg of DNA. **Table 3** shows data from a ddPCR run using blood DNA, demonstrating an LLOQ of 2 copies / 20 µL reaction, while **Figure 3** demonstrates that such a run exhibits near perfect dilutional linearity alongside a manufactured standard curve. In **Figure 4,** we provide a schematic that highlights our typical ddPCR method validation. In summary, our qPCR and ddPCR assay development and validation system presents an efficient methodology in quantitating target genes with ultrasensitivity, accuracy, and precision. Detailed technical information will be released in our presentation. Some recently published qPCR/ddPCR methods will be used as references to compare with our methods and results, to demonstrate how our novel methodologies advance the field.

Figure 1. Amplification Curves of 8 Calibrators in a Multiplex qPCR Assay

In this VSV-G qPCR assay for RCL activity, eight standard amplification curves (red) represent 8, 80, ..., 8 x 10^7 copies of VSV-G per 20 µL reaction per 1 µg genomic DNA. Conversely, the blue amplification curves represent a reference gene from the human genome.

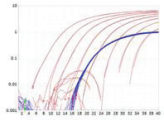


Figure 2. Linear Regression of 8 Calibrators in a Multiplex qPCR Assay

Our novel multiplex qPCR assay exhibits exceptionally high dilutional linearity ($r^2 > 0.999$) across the entire quantitation range (8 – 8 x 10^7 copies).

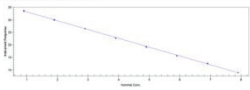


Figure 3. Linear Regression of 8 Calibrators in a Multiplex ddPCR Assay

Our novel multiplex qPCR assay exhibits exceptionally high dilutional linearity ($r^2 > 0.999$) across the entire quantitation range (2 – 100000 copies).

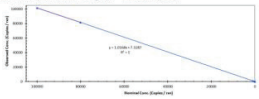
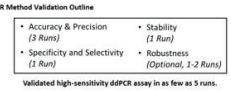


Figure 4. ddPCR Method Validation Outline



Validated high-sensitivity ddPCR assay in as few as 5 runs.

Table 1. Amplification Efficiencies of a Multiplex qPCR Assay at Various hgDNA Concentrations

TE Buffer	100 ng Undigested	1000 ng Undigested	1000 ng Digested
Amp. Efficiency	Amp. Efficiency	Amp. Efficiency	Amp. Efficiency
99.21%	92.54%	90.83%	91.49%
Ct (Std 1) = 9.229	Ct (Std 1) = 9.364	Ct (Std 1) = 9.190	Ct (Std 1) = 8.854

Table 2. The Multiplex qPCR Assay Exhibits Accuracy and Precision Well Within Acceptance Criteria between a Quantitation Range of 8 – 8x10^7 Copies / Reaction

For our novel qPCR assay, we observe accuracy and precision well within industry-established recommendations (%RE < ± 25% of nominal value, %CV < 25%) across the entire quantitation range (8 – 8x10^7 copies / reaction, corresponding to $\log_{10}$(copies/rxn) of 0.903 – 7.903).

Stds	Nominal Conc. $\log_{10}$(Copies/rxn)	Nominal Copies/rxn	TE Buffer		100 ng Undigested		1000 ng Undigested		1000 ng Digested	
			% RE	% CV	% RE	% CV	% RE	% CV	% RE	% CV
Std 1	7.903	8 x 10^7	0.1%	0.1%	0.4%	-1.9%	0.0%	-1.0%	0.2%	-1.2%
Std 2	6.903	8 x 10^6	0.0%	0.3%	0.0%	-0.2%	0.4%	0.3%	0.2%	0.0%
Std 3	5.903	8 x 10^5	0.4%	-0.2%	0.7%	0.7%	0.0%	1.4%	0.1%	0.5%
Std 4	4.903	8 x 10^4	0.3%	0.5%	0.5%	2.7%	0.5%	1.6%	0.4%	2.6%
Std 5	3.903	8 x 10^3	0.5%	0.9%	0.0%	3.4%	0.2%	0.7%	0.2%	2.3%
Std 6	2.803	8 x 10^2	0.2%	0.3%	0.0%	3.0%	0.1%	-4.5%	0.7%	-2.2%
Std 7	1.903	8 x 10^1	0.3%	-3.2%	0.1%	-8.9%	1.5%	-6.6%	0.9%	-6.6%
Std 8	0.903	8	11.7%	2.6%	9.5%	-10.1%	4.5%	13.9%	12.0%	4.2%

Table 3. Example of a Multiplex ddPCR Curve that Exhibits an Observed LLOQ of 2 Copies / 20 µL Reaction

In this mock standard curve that utilized our novel ddPCR assay, the standards spanning 100000 copies / 20 µL reaction (recommended ULOQ for the instrument by the manufacturer) down to 2 copies / 20 µL reaction exhibited an accuracy and precision that exceeded the current consensus acceptance criteria. Using this novel ddPCR assay, LLOQs ranging between 2 – 8 copies / 20 µL reaction are commonly observed for various matrices.

Stds	Nominal Conc. (Copies/ rxn)	Recorded Conc. (Copies / rxn)				% RE	% CV	PASS / FAIL Acceptance Criteria
		Replicate 1	Replicate 2	Replicate 3	Average			
Std 1	100000	102693.8	99579.6	102389.6	101554.3	1.6%	1.7%	PASS
Std 2	80000	82933.6	80726.9	80895.2	81518.6	1.9%	1.5%	PASS
Std 3	800	863.7	856.7	837.7	852.6	6.6%	1.6%	PASS
Std 4	64	54.5	64.5	67.0	62.0	-3.1%	10.7%	PASS
Std 5	32	26.3	32.7	26.9	28.6	-10.5%	12.3%	PASS
Std 6	16	14.2	15.1	16.1	15.1	-5.4%	6.3%	PASS
Std 7	8	12.1	8.7	5.9	8.9	11.3%	34.9%	PASS
Std 8	4	4.6	5.7	3.4	4.6	14.2%	25.2%	PASS
Std 9	2	2.2	4.2	1.2	2.5	26.7%	60.3%	PASS
Std 10	1	0.0	0.0	3.3	1.1	10.0%	173.2%	FAIL
Std 11	0	0.0	0.0	0.0	N/A	N/A	N/A	FAIL
Observed LLOQ for Curve: 2 copies / 20 µL Reaction								

GCC Consensus for ddPCR Method Validation Passing Criteria:

Expectations for Accuracy (%RE):
- %RE of < ±35% for samples with ≥ 50 copies / 20 µL ddPCR reaction.
- %RE of < ±50% for samples with < 50 copies / 20 µL ddPCR reaction.

Expectations for Precision (%CV):
- %CV of < 40% for samples with ≥ 50 copies / 20 µL ddPCR reaction.
- %CV of < 80% for samples with < 50 copies / 20 µL ddPCR reaction.

1873. mRNA/LNP Multiattribute Quantitation of Payload(s), Size and Heterogeneity with Size Exclusion Chromatography Coupled to Multiangle Light Scattering

Mateusz Imiolek[1], Lavelay Kizekai[2], Bala Addepalli[2], Szabolcs Fekete[1], Matthew Lauber[2]

[1]Waters Corporation, Geneva, Switzerland,[2]Waters Corporation, Milford, MA

Bringing cell and gene therapy drugs to market offers a chance for curing previously intractable diseases, but it comes with a formidable challenge of ensuring the safety and efficacy of increasingly complex therapeutics. These drugs are multicomponent ensembles (carrier + payloads), which means there are an array of critical quality attributes (CQAs) that must be monitored at each stage of their development - creating bottlenecks and delaying the process. High-throughput analytical methods that permit multiattribute measurements constitute a potential solution to this issue. Among the most efficient are chromatographic separations that can be coupled to advanced detectors, as with size exclusion chromatography paired with multiangle light scattering (SEC-MALS), which is of growing importance to emerging new modalities (AAVs, mRNA/LNPs etc.). Significant attention is being placed on creating fit-for-purpose SEC methods for lipid nanoparticles (LNPs) and their payload drug substances. Optimized widepore, low adsorption SEC columns (>450 Å) have been developed such that new techniques can be envisaged. Past investigators have applied a positive surface potential

gel permeation resin to perform LNP size exclusion chromatography. There is risk to this approach due to the potential adsorption and incomplete elution of free nucleic acid impurities. As such, we have alternatively investigated non-ionic, hydrophilic packing materials for SEC of LNPs. We have compared the recovery of the LNP materials across different columns and found optimal conditions that led to over >75% recovery with minimal tailing allowing for MALS mediated size, mass and heterogeneity measurements. Additional investigations are underway to further improve our understanding of analyte of recovery. Moreover, we developed conditions for a complete online disruption of the LNP carrier which enables a direct measurement of the nucleic acid payload(s) with a 2 ng as the limit of detection (LOD). We show that efficient size based separation is crucial for dual payload quantitation of gene editing therapy oriented LNPs containing both mRNA and gRNA. Various denaturing agents and LNP formulations (>5) were investigated to ensure universality of the disrupting conditions and the creation of a platform method. In summary, we demonstrate the versatile use of SEC-MALS to extract multiple CQAs (purity related e.g. RNA integrity or presence of process related impurities or strength related such as mRNA content) measurements for LNP RNA drug products and drug substances. With its deployability, SEC-MALS is coming to establish a cost and time-effective means to comprehensively characterizing a range of new nucleic acid therapies.

1874. Label-Free *In Vitro* Assay to Qualify Functional T-Cells as Starting Material for the Manufacturing of Advanced Therapies

Alberto Muniz[1], Vanessa Figliuolo Da Paz[1], Komali Pentakota[1], Rachel Beddard[2], Scott Jones[2], Anand Srinivasan[1]

[1]The Center for Innovations and Biodesign, BioBridge Global, San Antonio, TX,[2]Research and Development, BioBridge Global, San Antonio, TX

Background: Advanced therapies are hindered by the lack of standardization in the quality of the cells used as starting material for manufacturing. There are several manufacturing processes for CAR-T-cell therapies with many variabilities that affect the critical quality attributes of the resulting product. However, these processes seem to have the same measure of characterization, often limited to the expression of phenotypic markers. Qualification based on phenotypic markers do not truly account for the functional variabilities in the cells prior to manufacturing. Hence, the assessment of the function of donor-derived T-cells is crucial to the manufacturing and the potency of the product. Current *in vitro* methods for analyzing the function of T-cells suffer from long assay duration, fluorescence labeling, and a lack of data collection in real time. At BioBridge Global, we have developed an impedance-based label-free assay, for continuous monitoring of T-cells mediated killing of cancer cells as a measure of quality of the T-cells prior to manufacturing. **Methods:** T-cells were isolated from buffy coat of healthy donors and activated *in vitro* with anti-CD3 and anti-CD28. Epithelial lung cancer cell line A549 was used as a model for adherent tumor cells. Different concentrations of T-cells (effector) were challenged against A549 cells (target), and the rate of killing was quantified. The kinetics of activation of the T-cells was quantified between day-6 and day-9. The ability of T-cells to kill cancer cells was calculated by measuring the change in electrical impedance over time. This assay was also benchmarked with the current methodology of measuring cancer-cell killing using flow cytometry. **Results:** Our results demonstrated that the interaction of activated T-cells with cancer cells led to a decrease in impedance, indicating the killing of the cancer cells. T-cell mediated cytolysis of target cells was detected as early as 36 hours. The rate of T-cells killing cancer cells were directly correlated to the ratio of effector and target cells. The results obtained from our experiments performed with cells from three healthy donors demonstrated the variance in the functionality of T-cells as starting materials. We also observed differences in the rate of activation of T-cells derived from three healthy donors. **Conclusions:** In advanced therapies, we remain focused on the quality of the end product administered to the patients. It is equally important to invest significant attention to the functional attributes of the starting materials used to manufacture potent batches of cell and gene therapies. Our results demonstrate the potential of our assay as a quick and consistent method to qualify functional T-cells as starting material for the manufacturing of CAR-T cell therapies.

1875. Universal Pharmacokinetic Assessment of Anti-CD19 CAR-T Using Biocytometry

Martin Cienciala[1], Hynek Kasl[1], Pavel Fikar[1], Laura Berne[1], Monika Holubova[2,3], Daniel Lysak[2], Daniel Georgiev[1,4]

[1]Sampling Human, Inc., Berkeley, CA,[2]Department of Hematology and Oncology, University Hospital Pilsen, Pilsen, Czech Republic,[3]Biomedical Center of the Faculty of Medicine, Charles University, Pilsen, Czech Republic,[4]Faculty of Applied Sciences, University of West Bohemia, Pilsen, Czech Republic

Introduction: There is a critical clinical need for a technology capable of monitoring therapeutic activity in real time after infusion of cell therapies. High sensitivity cell enumeration of anti-CD19 CAR-T cells with a wide dynamic range was performed using biocytometry. This proof-of-concept study establishes the performance capability of the platform, employing the CD19 antigen for universal anti-CD19 CAR-T detection. The platform employs a homogeneous workflow, enabling routine testing of patients undergoing treatment and providing high resolution activity data of therapeutic cells in vivo. Methods:The performance of the platform was evaluated using a dilution series of anti-CD19 CAR-T cells in peripheral blood. The samples were prepared by combining PBMCs collected from a patient undergoing CAR-T therapy with peripheral blood isolated from a healthy individual. Each sample containing the equivalent of 1×105 leukocytes was further analyzed using the biocytometry workflow and BIOS(anti-CD19CAR) bioparticle system. Samples were processed without any additional washing or separation steps, with assay readout being performed on a microplate reader. Results: The technical validation of biocytometry demonstrated strong alignment with flow cytometry analysis. The sensitivity threshold for routine assessments was established at 1×10-4. Notably, biocytometry consistently showed a null limit of blank in samples devoid of any CAR-T cells, representing a significant improvement over flow cytometry, which can produce false positive events when employing methodology for universal CAR-T assessment. Conclusion: Biocytometry facilitates the universal quantification of anti-CD19 CAR-T cells and notably exhibits no background signal in samples obtained from healthy individuals. The unique homogeneous assay workflow provides a significant advantage for clinicians, featuring

a streamlined, high-throughput protocol that can be easily performed by laboratory technicians with minimal training requirements. Continued refinement of this platform promises to significantly improve the sensitivity of pharmacokinetic measurements, extending beyond the current limit of 1×10-4, holding substantial promise for future applications such as precision re-dosing.

1876. Impact of Pre-Existing Neutralizing Antibodies on Transduction, Safety, and Shedding of AAVhu68.FXN Following Intraparenchymal Injection into the Dentate Nucleus

Gourav R. Choudhury[1], Juliette Hordeaux[1], H. Isaac Chen[2], Joel M. Stein[3], Cecilia D. Dyer[1], Chelsea K. Wallace[1], Elizabeth L. Buza[1], Jessica A. Chichester[1], Liyang Han[1], Peter Bell[1], James M. Wilson[1]

[1]Gene Therapy Program, University of Pennsylvania, Philadelphia, PA,[2]Department of Neurosurgery, University of Pennsylvania, Philadelphia, PA,[3]Department of Radiology, University of Pennsylvania, Philadelphia, PA

Neutralizing antibodies (NAbs) generated in response to natural adeno-associated virus (AAV) exposure represent a major obstacle to the success of intravenous AAV-based gene therapies. NAbs can significantly impede AAV transduction and reduce drug safety; thus, high NAb titers are an exclusion criterion for many AAV-based clinical trials. The high prevalence of NAbs in humans renders many potential patients ineligible to participate. However, the brain is an immunologically privileged site, and some studies suggest that direct brain administration might be effective for correcting diseases that affect the central nervous system in an NAb-positive patient. Therefore, the development of safe and effective AAV gene therapies for neurological diseases such as Friedreich's ataxia involves understanding the potential impact of circulating NAbs on vector transduction and transgene expression in the brain. In the current study, we sought to evaluate the impact of circulating NAbs on AAV vector transduction and transgene expression following intraparenchymal injection to the brain. Rhesus macaques (*Macaca mulatta*) with variable antibody titers (0 to 1:160; NAb <5 (n=1); NAb 1: 20 (n=1), NAb 1: 160 (n=1)) received magnetic resonance imaging-guided bilateral injections of AAVhu68.hFXN at $1.6x10^9$ genome copies to the dentate nuclei (DN). We monitored the animals for 28 days via daily clinical observation, periodic blood work, and cerebrospinal fluid (CSF) collection. Brains were collected and processed for histopathological and transgene expression analysis upon necropsy (Day 28). All animals remained clinically unremarkable throughout the study and survived until the study endpoint. Evaluation of blood and CSF clinical pathology (including liver enzymes, coagulation parameters and biomarkers for cardiotoxicity) were unremarkable across the groups. Similarly, serum biomarkers for cardiac toxicity and coagulopathies were unremarkable in the treated animals. Intraparenchymal administration of the AAV vector did not induce a NAb response, even in those animals with high circulating NAb levels at dosing. Vector shedding was not detected in urine or feces at any time point. Evaluation of transgene expression via in situ hybridization indicated that similar hFXN expression levels in the DN were achieved in all treated animals independent of circulating NAb levels at the time of vector administration. In conclusion, the current study demonstrated that the presence of circulating NAbs did not impact vector transduction or transgene expression in monkeys upon intra-dentate nucleus administration of AAV vector, and that the treatment was safe and well tolerated. These preclinical data provide support for extending clinical trial inclusion criteria to permit the participation of NAb-positive patients in AAV-based clinical trials involving direct AAV administration to the brain.

1877. Is My Next-Generation Sequencing Deep Enough?

Tommaso Ocari[1], Takahiro Nemoto[2], Emilia Zin[1], Deniz Dalkara[1], Ulisse Ferrari[1]

[1]Institut de la Vision, Sorbonne Université, CNRS, Inserm, Paris, France,[2]Premium Research Institute for Human Metaverse Medicine (WPI-PRIMe), Osaka, Japan

Next-generation sequencing (NGS) has revolutionized genomics research since the early 2000s. Thanks to this technique, some fields of evolutionary biology such as directed evolution and deep mutational scanning have benefited from the availability of big data, improving the characterization of variants with desired properties. In these cases, NGS is used to screen biological libraries in order to measure the concentrations of different mutants by reading and counting them. Yet, because of several amplification steps in the experimental protocols, like PCR amplification or bacterial growth, NGS datasets suffer from large noise errors that are often neglected. To gain deeper insight in this phenomenon we have developed a statistical model of the entire NGS protocol, incorporating the exponential processes, inspired by Galton-Watson process theory. Our model predicts a simple proportionality law between the variance and the mean of the counts data, that we have validated in both real data and in-silico simulations. Finally, starting from this noise model, we provided a practical guideline on the optimal NGS depth for accurately measuring concentrations.

1878. De-Risking Clinical Trials Leveraging MRI-Guided Intraparenchymal Delivery by Validating Commercially Available Infusion Pumps for Gene and Cell Therapy Infusions

Stephanie Korszen, Princeton Saroha, Nate Willliams, Juan Carrasquillo, Chip Smith, Zachary Carr, Jeremy Stigall, Ernesto Salegio

ClearPoint Neuro, Inc., Solana Beach, CA

One of the most promising routes of administration (ROAs) for gene and cell therapies intended for the treatment of neurological disorders is direct intraparenchymal brain delivery. This ROA significantly reduces the volume of therapeutic needed and the risk of off-target toxicity, while also ensuring the therapy reaches its intended target. To facilitate the biodistribution of therapeutics within the desired target regions, a pressurized driving force such as that of convection-enhanced delivery (CED) is recommended. This requires an infusion pump with the ability to accurately and consistently deliver infusates on a microliter scale and at slow flow rates. An additional challenge is presented when performing infusions with MRI-guidance, as the infusion pump also needs to be MRI compatible. Most commercially available infusion pumps cleared for clinical use are not suitable for

MRI-guided intraparenchymal infusions, due to insufficient accuracy, inability to deliver at low flow rates, lack of MRI compatibility, or prohibitively high hold up / dead volumes required. In this study, we evaluated 3 commercially available infusion pumps using a buffer of similar density to that of suspension buffers currently used in many cell and gene therapy products. As part of our compatibility design to mimic a clinically-relevant pressurized system, we evaluated the accuracy and reliability of fluid delivery at the intended flow rates and volumes while delivering into an agar gel that mimics the backpressure of brain tissue. Our results indicated that there are multiple infusions pumps that can achieve sufficient accuracy at the desired flow rates and volumes used in clinical trials and preclinical studies of intraparenchymally-delivered therapeutics. The data and findings presented herein provide useful information when establishing drug delivery systems that will ultimately affect the design and conduct of associated clinical trials.

1879. MRI-Guided Intracerebroventricular and Intraparenchymal Dosing in a Swine Model

Maggie A. Mohr, Andy R. Carlson, Jenifer A. Sweet, Vinisha A. Pandya, Mark Johnson, Christen Simon
Northern Biomedical Research, Norton Shores, MI

Convection-enhanced delivery (CED) of gene and cell therapies directly into the brain offers a more precise therapeutic target when compared with systemic delivery. Moreover, bypassing the blood brain barrier decreases the risk of systemic toxicity. Real-time guidance using magnetic resonance imaging (MRI) allows for accurate targeting of regions of interest while considering individual differences in brain anatomy and vasculature, allowing immediate adjustments to be made to prevent delivery into unintended regions and subsequent off-target effects. Demonstrating safety and efficacy of new gene and cell therapies in large animal models is a fundamental step in pre-clinical translational research involving CED. Furthermore, choosing size-relevant models for clinical imaging and surgical techniques is paramount to the success and translation of pre-clinical studies. The large animal model of choice for many years has been the non-human primate (NHP), but due to disruptions in the NHP supply chain, it is necessary to evaluate alternative species. Pigs are gaining considerable attention as size-relevant models for clinical imaging and surgical techniques. The current study aimed to determine the efficacy of MRI-guided intracerebroventricular (ICV) and intraparenchymal CED in Gottingen minipigs using a custom-designed MR-compatible frame. Here we demonstrate successful MRI-guided ICV dosing using CED in the Gottingen minipig. Additionally, we demonstrate successful MRI-guided CED into several intraparenchymal sites, including the caudate, putamen, and substantia nigra of the Gottingen minipig. The success of this study demonstrates the practicality of the Gottingen minipig as an alternative large animal model in a pre-clinical setting for MRI-guided testing of therapeutics using CED.

1880. Achieving Uniform Vector Genome Transduction in the Brain Tissue after Intraparenchymal Delivery of Adeno-Associated Virus Gene Therapy Using Computational Fluid Dynamics and Quantitative Systems Pharmacology Modeling

Glen Ko, Zhiwei Zhang, Jiaolong Jiang, Victor (Seungchan) Yang, John Pappas
RES Group, Needham Heights, MA

Introduction: Gene therapy for neurodegenerative diseases like Alzheimer's, Parkinson's, and Huntington's often relies on intraparenchymal (IP) delivery, directly infusing the target tissue. While this ensures that the therapy reaches target areas, localized high vector genome transduction may pose risks of immunogenicity or toxicity. A mathematical model offers a method for investigating infusion conditions, enabling the delivery of an effective dose across a broad target area while minimizing exposure to localized high levels. **Methods**: Our model combines Computational Fluid Dynamics (CFD) to describe the hydrodynamics of the viral vector in tissue extracellular matrix, and Quantitative Systems Pharmacology (QSP) to describe the biological steps of the vector transduction (entry to the cell, cytoplasmic and nuclear processing, therapeutic protein expression). We have applied the CFD-QSP model to examine the infusion conditions of IP delivery to basal ganglia tissues for gene therapy treatment of Huntington's disease. **Results**: The CFD-QSP model successfully described the hydrodynamic flow of the infusate in the extracellular space and cellular uptake/processing of the therapeutic vector. Key findings include: (1) Spatial distribution of vector genome transduction is influenced by the competition between vector particle entry and intracellular trafficking governed by the vector particle characteristics, and the flow velocity in the extracellular space governed by infusion volume and rate and tissue size. (2) Achieving a uniform spatial distribution of the vector genome transduction becomes more difficult for larger tissues, posing translational challenges from preclinical data in smaller species to humans. (3) Enhancing uniform spatial distribution is feasible by optimizing infusion conditions, which include the number and strategic placement of infusions within the tissue. **Conclusion**: Calibrated to preclinical data, the novel CFD-QSP model for gene therapy delivery to brain tissues via IP administration provides a useful tool to study optimum infusion parameters for supporting human dose selection in clinical trials.

1881. Intraparenchymal Infusions to Deep Brain Structures Using Low Field MRI

Katelyn L. McKnight[1], Yixuan Liu[2], Russell R. Lonser[2], Orlando P. Simonetti[2], Stephanie Korszen[1], Kyle C. Wu[2]
[1]ClearPoint Neuro, Inc., Solana Beach, CA,[2]The Ohio State University Wexner Medical Center, Columbus, OH

The blood brain barrier (BBB) poses a significant challenge to delivering therapeutics for treatment of neurologic disorders. While many development efforts are focused on trying to develop treatments that can be delivered systemically (i.e., intravenous infusion), advances in image guided technologies have significantly improved the ability to deliver gene and cell therapies to targets within the

central nervous system (CNS) with extreme precision. The safest and most feasible approach for direct delivery employs a percutaneous approach paired with intraprocedural magnetic resonance imaging (MRI) technology; allowing for safe and accurate delivery that can be monitored in near real-time. Given the increasing volume of clinical trials in CNS gene and cell therapies that require direct delivery, the role of visualizing infusions within the brain is essential to scale-up of neurosurgical delivery. To this aim, the work herein presents the first study of intraparenchymal infusion guided by low field MRI. The 0.55T MAGNETOM Free.Max (Siemens Healthineers AG; Erlangen, Germany) was first validated for accuracy using a phantom, and was then used with an intraoperative MRI stereotactic frame (ClearPoint Neuro, Solana Beach, CA) to infuse contrast agent into multiple targets within the CNS of Yorkshire pigs. The results herein confirm the ability of the Free.Max to achieve submillimetric accuracy in targeting, and present imaging from infusions to the pig thalamus. These initial findings show great promise in the potential for eventually obviating the need to utilize high field MRI and greatly improve the affordability, accessibility, and surgical workflow options of direct brain delivery of gene and cell therapies.

1882. Characterization of Dorsal Root Ganglionopathy Following Intrathecal Delivery of High-Dose AAV9.CAG.EGFP Vector to Adult Rats

Yulia Dzhashiashvili, Brian M. Kelly, Greglynn D. Gibbs, Joan Wicks, Esteban A. Engel, Elizabeth Ramsburg

Spark Therapeutics, Inc., Philadelphia, PA

Intra-cerebrospinal fluid (CSF) AAV administration, as well as intravenous (IV) AAV administration, especially at high doses, have been reported to result in adverse histopathological findings in dorsal root ganglia (DRGs) of non-human primates (NHPs) and other animal species, including rats. Considering that DRG toxicity is an emerging safety concern for central nervous system (CNS)-directed AAV gene therapies, understanding the pathophysiology of the toxicity is critical to evaluating the risks associated with AAV vectors. In this study, we delivered AAV9 vector at a high dose ($3.1e^{12}$ vg) to lumbar CSF of adult Wistar Han rats to: 1) evaluate the susceptibility of the rat species to AAV-induced DRG pathology; 2) investigate their relevance as a preclinical model to monitor DRG toxicity, using non-invasive biomarker and neurofunctional assessments. At 45 days post-AAV administration, the microscopic histopathological changes in DRGs, such as increased cellularity, neuronal degeneration, and mononuclear cell infiltration, were predominantly minimal-to-mild in severity. The transduction efficacy, as evidenced by vector genome levels in DRGs, was positively correlated with levels of neurofilament light chain (NF-L), a biomarker of neuroaxonal injury, in plasma and serum of AAV-injected rats. A time-course analysis of NFL in serum revealed that values peaked between 15 to 30 days post-AAV administration. Moreover, nerve conduction velocity (NCV) of sciatic nerve, measured at 30 days post-dosing, was found to be significantly reduced compared to baseline NCV. To gain insights into mechanisms of DRG toxicity, a subset of animals was injected with AAV9 vector containing a modified CAG.EGFP cassette, which lacks both the Kozak sequence and the initial ATG codon, resulting in dramatically diminished EGFP protein expression. Notably, the severity of neuropathological findings in these animals was comparable to that observed in animals injected with AAV9 vector containing a full-length CAG.EGFP expression cassette. This suggests that the toxicity is not caused solely by transgene overexpression, as other reports have indicated. Overall, the data from this study support the possibility of using rats as a preclinical model for AAV-induced DRG toxicity assessment.

1883. Characterization of a Novel C9orf72 $(G4C2)_{80}$ Knockin Rat Model

Claire Meurice[1], Zhengyu Luo[1,2], Renee Bodinizzo[3], Yao Chen[4], Jaunetta Hill[5], Ye He[1,2], John Bladon[1,2], Juliana Laverde Paz[6], Zane Zeier[6], Mikhail Levit[4], Robert Jackson[1,2], James Dodge[5], Martin Goulet[1], Steven Rodriguez[5], Giulio Tomassy[1], Christian Mueller[1]

[1]Genomic Medicine Unit, Sanofi, Waltham, MA,[2]In Vivo Working Group, Sanofi, Cambridge, MA,[3]In Vivo Research Center, Sanofi, Cambridge, MA,[4]Precision Medicine and Computational Biology, Sanofi, Cambridge, MA,[5]Rare and Neurological Disease, Sanofi, Cambridge, MA,[6]University of Miami Miller School of Medicine, Miami, FL

GGGGCC (G4C2) hexanucleotide repeat expansion in the first intronic region of open reading frame 72 on chromosome 9 (C9orf72) is the most common genetic cause of Amyotrophic Lateral Sclerosis (ALS). G4C2 repeat expansion alters the expression of C9orf72 splice variants, leading to haploinsufficiency and gain of function toxicity: aberrant expression of pathological C9orf72 variants, RNA toxicity, and production of toxic dipeptide repeat proteins (DPRs). Understanding of mechanisms that lead to the development of C9orf72-ALS is complicated by the lack of effective rodent models that recapitulate disease progression. Developing such models is paramount for advancing our understanding of the disease and for the preclinical exploration of novel therapies. The C9orf72 $(G4C2)_{80}$ knockin rat model is a new model reported to exhibit motor and grip strength impairment as early as 8-mths-old, C9orf72 haploinsufficiency and an abnormal immune phenotype; the original publication did not explore all hallmarks of the disease (e.g., RNA foci, DPRs). To evaluate the usefulness of this model for preclinical research, we established and monitored a new colony of C9orf72 $(G4C2)_{80}$ knockin rats at Sanofi, aged them through 16-mths-old, and performed all *in vivo* procedures according to IACUC approved protocols at an accredited institution. Extensive behavioral characterization - locomotor, coordination, gait and grip strength assessments, using Open Field, Rotarod, Catwalk and Grip Force Test respectively - revealed no genotype dependent abnormalities, and this was corroborated by a lack of change in serum neurofilament-light and pathological assessments of brain, spinal cord and muscle following necropsy. While C9orf72 knockin rats exhibited C9orf72 haploinsufficiency, as previously published, and frequent sense and infrequent antisense foci, no dipeptide repeat proteins were detected up to 16-mths-old by IHC. Sanofi colony rats also did not have the published immune phenotype: altered thymus and cervical lymph node weight. Our results suggest that while C9orf72 $(G4C2)_{80}$ knockin rats exhibit haploinsufficiency and RNA foci, this model may be used for some pharmacokinetics/pharmacodynamic studies, but it does not provide a useful tool for efficacy studies.

Friday Posters: Molecular and Cellular Methods

1884. Assay Validation and Clinical Sample Stability of Recombinant AAV Vector in Five Human Shedding Matrices

Christopher Mcpharlin[1], Haiyan Ma[1], Allison Swarthout[1], Kristin N. Bell[1], Peter Ross[2]

[1]Northern Biomolecular Services, Kalamazoo, MI,[2]Bioanalytical, Kriya Therapeutics, Durham, NC

KRIYA-748 is an AAV-based vector that delivers a gated ion channel to the trigeminal nerve for trigeminal neuralgia, a disease characterized by frequent, debilitating facial pain attacks. Once expressed, the channel is activated by orally-administered varenicline ("Chantix®") leading to reduced excitation of transduced neurons. Assessments of vector shedding in clinical gene therapy trials are important to understand the likelihood of transmission of vector to untreated individuals and to provide information for environmental assessment. Regulatory authorities recommend the use of quantitative polymerase chain reaction (qPCR) assays to analyze vector shedding because of the high sensitivity and specificity of this method. Current guidance provides latitude in the approach used for vector shedding assessments based on product-specific variables such as route of administration, replication competent vs. incompetent vectors, and presence of potentially oncolytic transgenes. For vector shedding, assay validation provides a thorough understanding of the capabilities and limits of the method which in turn allow for higher confidence in the results. Since vector shedding studies are typically conducted in multiple matrices each with their own particular challenges for qPCR detection, it is important to understand how different matrices impact the reported result, the recoveries of the vector genomes from each matrix, and the stability of the test article vector genomes in these matrices. Here we report the method validation of a qPCR assay for analysis of recombinant AAV (rAAV) vector genomes across five human clinical matrices; plasma, tear swab, saliva, nasal swab, and urine, and recommend parameters to evaluate during method development and validation for similar replication incompetent AAV gene therapy products. The recoveries of the vector genomes from the five matrices and the stability of the vector genomes in the five matrices were then evaluated using the validated qPCR assay. **Method Validation, Results, and Conclusion:** Ten validation runs were performed with serially diluted DNA standards, negative controls, and QCs, plated in the presence of DNA extracted from each of the shedding matrices evaluated. Each completed run met the validation acceptance criteria for target gene amplification. The ULOQ, LLOQ, and LOD were 10^8, 50, and 10 double stranded copies per reaction, respectively. All five shedding matrices QC sets passed acceptance criteria demonstrating that target DNA detection in plasma, urine, saliva, tear swab, and nasal swab can be accurately and precisely interpolated from a standard curve that does not contain matrix genomic DNA. The extensive evaluation of the fit-for-purpose validation parameters confirms the applicability of the qPCR method for clinical vector shedding assessments. **Recoveries of Vector Genomes from Five Clinical Matrices:** 39% - 55% from plasma; 111% - 140% from urine; 58% - 70% from tear swab; 59% - 70% from saliva swab; 56% - 69% from nasal swab. **Stability of Vector Genomes in Five Clinical Matrices:** The rAAV vector genomes were found stable when tested under the following storage conditions: 4 hours @ RT (15 to 30°C), 24 hours @ 2 to 8°C, 3 cycles of freeze/thaw, 1 month @ -10 to -30°C, 1 month @ -60 to -90°C, and 6 months @ -60 to -90°C.

1885. Characterization of Residual DNA Impurities in rAAV-Based Therapies through Short and Long-Read Sequencing

Jawhara Karam, Tomoko Maekawa

Passage Bio, Philadelphia, PA

The genetic payload of recombinant adeno-associated viral (rAAV) vectors is a critical quality attribute for the vector's efficacy and safety. Impurities (e.g., residual DNA derived from host cells and plasmids utilized during the manufacturing process) in rAAV therapies must be kept at minimal levels to reduce the potential of adverse events in patients. Therefore, characterizing residual DNA within the rAAV vector remains of paramount concern for manufacturers. Gold-standard methods to quantify DNA copies, such as qPCR and ddPCR, are used to quantitate the concentration of residuals in a product. However, these methods rely on the presence of a short amplicon and may underestimate the total number of residuals by failing to detect residuals without the amplicon sequence. Therefore, we established short and long-read next-generation-sequencing methods for a more thorough characterization of residual DNAs within the sample. Illumina short-read sequencing is advantageous in the fact that reads across short regions of sample DNA have a high level of depth coverage. However, Illumina cannot provide a complete size distribution of residuals since sample DNA must be fragmented as part of the library preparation. Furthermore, size selection during library preparation reduces the presence of residual fragments below the targeted fragment size. To address these gaps, long-read sequencing utilizing the Oxford Nanopore platform was performed in conjunction with Illumina short-read sequencing. Downstream analysis for both sequencing methods involved quality control and mapping to reference sequences. Samples are first mapped to the cis plasmid containing the transgene, followed by mapping to the rep/cap plasmid, helper plasmid, human genome, E. coli, and lambda phage. Illumina data showed ≥ 99% of reads mapping to the rAAV genome with up to 1% residual DNA impurities. Comparable results were demonstrated with long-read sequencing. Furthermore, the long-read sequencing data provided insight into the size distribution of residual DNA from the host organism, cis plasmid backbone, and plasmids (helper and rep/cap). Together, this information can be used to provide a more complete profile of the impurities present in rAAV vectors.

1886. Quantification of Residual Plasmid DNA Impurities Utilizing Short and Long ddPCR Amplicons

Sarah Monaco, Tomoko Maekawa

Passage Bio, Philadelphia, PA

Passage Bio is a gene therapy company developing recombinant adeno-associated viral (rAAV) products using a triple transient transfection system with a cis plasmid containing the transgene, a Helper plasmid, and a RepCap plasmid. To assess product quality and safety, it is essential to monitor process-related impurities including residual

plasmid DNA concentrations. The current residual plasmid DNA assay for GMP release testing utilizes a short Droplet Digital™ PCR (ddPCR) amplicon targeting the Kanamycin resistance gene (KanaR), present in all three plasmids. The current method quantifies total residual plasmid concentration, but cannot determine contributions from each plasmid, which is critical for evaluating potential adverse events in patients (e.g., introduction of a potential oncogene derived from the helper plasmid). To circumvent this limitation we developed short ddPCR amplicon assays to specifically quantify residual Helper and RepCap plasmid DNA. Our results demonstrate that the cis plasmid (~80%) is the largest contributor to residual plasmid DNA impurities, followed by the RepCap plasmid (~15%) and the Helper plasmid (~5%); despite the 1:10:10 molar ratio used during transfection. We also developed long ddPCR amplicon (~700bp) methods to assess the size distribution of each residual plasmid DNA, which is important due to safety limitations setting a threshold on larger amplicons in products. We found that residual cis plasmid DNA (most likely originating from the cis plasmid backbone) was longer in size than that from the Helper and RepCap plasmid-specific sequences. Collectively, our results indicate that residual plasmid DNA impurities from the cis plasmid backbone may present a greater likelihood of assembly into rAAV vectors due to their proximity to ITRs. Our novel ddPCR methods are a useful tool for product characterization studies as well as future process development.

1887. Development of an *In Vitro* Potency Assay for an AAV Gene Therapy Delivering a Functional Version of *SHANK3*

Michael A. Murphy, Michael De Petrillo, Kalyn Bauldwin

Analytical Development and QC, Advanced Medicine Partners, Durham, NC

Patients with *SHANK3* haploinsufficiency present with a debilitating neurodevelopmental disorder including Phelan-McDermid syndrome (PMS) that is characterized by severe intellectual disabilities, impaired or absent speech and language, deficits in motor skills, behavioral challenges, and autism and/or autistic features. SHANK3 is enriched in glutamatergic synapses where it participates in regulation of postsynaptic signaling by acting as a molecular scaffold through its interactions with other proteins located in the postsynaptic density. JAG201 is an AAV9-based gene therapy intended to deliver a functional version of *SHANK3* to treat PMS and *SHANK3* haploinsufficiency caused by loss of function mutation. Due to DNA packaging constraints of AAV capsids and the large size (1731 amino acids) of the multidomain-containing SHANK3, Jaguar Gene Therapy developed JAG201 to express a miniature version of human SHANK3 (miniSHANK3). To support product development, an *in vitro* assay was developed to characterize the potency of JAG201. The assay uses SHANK3 knockout (SKO) cells and is based upon the SHANK3 mechanism of action in which the protein functions as a scaffold within the postsynaptic density. Following transduction of the SKO cells with JAG201, cell lysates are prepared and added to microplates coated with a peptide from a SHANK3 binding protein. After capture of miniSHANK3 to the microplate through its interaction with the coated protein, an antibody specific for a second SHANK3 binding protein is used in conjunction with a horseradish peroxidase-conjugated antibody and a suitable enzyme substrate to produce antibody-specific fluorescence. The level of fluorescence is proportional to the quantity of SHANK3 binding partner and functional miniSHANK3 present in the lysate. Upon completion of initial development, the assay was qualified for accuracy, precision, linearity, range, and stability indication. All qualification results indicate that the assay is suitable for measuring potency to support JAG201 lot release and stability monitoring. Clinical testing of JAG201 is slated to start this year.

1888. A Novel Technology for Accurate Quantification of Viral Genome and Determination of Lipid Nanoparticle Encapsulation Efficiency

Subhanjan Mondal, Kevin Hsiao, Nathan Feirer

Promega Corporation, Fitchburg, WI

Adeno-associated viral (AAV) vectors show great promise as a delivery system for gene therapy. However, development of robust methods for quantitation, infectivity and dose-determination has remained a challenge. Lipid nanoparticles (LNP) have also emerged as a successful non-viral delivery system as evident from the success of the COVID-19 vaccines. For LNP characterization methods for determining encapsulation efficiency remains a technical gap. In this work we show that through use of a membrane impermeable small molecule (TruTiter Reagent) that binds to nucleic acid both DNA or RNA) and covalently conjugates with it we can accurately and consistently determine AAV viral genome titers and LNP encapsulation efficiency. For quantification of AAV viral genome titer, samples are typically pretreated with DNase to remove vector and packaging plasmid DNAs, and often time followed by proteinase K treatment to remove DNase and release DNase -resistant packaged viral genomes. The challenges using DNase include the incomplete digestion resulting in residual DNA outside the capsids and the inaccessibility of DNase to nucleic acid from partially ruptured viral capsids due to the large size of DNase. To address these challenges using TruTiter Reagent we first show that it is effectively excluded from AAV particles at all tested concentrations (< 100uM). We then developed a workflow for determination of viral genome titers that involves addition of the TruTiter™ reagent at 10uM to a sample for 30 minutes. This covalently crosslinks any nucleic acid that is free or from ruptured viruses. Then a neutralization solution is added that stops the crosslinking process, followed by proteinase K treatment to release the protected DNA. The sample is then analyzed using qPCR or dPCR. To determine if genome titer measurement using TruTiter results in accurate and consistent measurements, we treated AAV of different serotypes (AAV2, AAV9 and AAV8) with different temperature and cell lysis agents typically used in AAV manufacturing process and determined genome titers using both DNase and TruTiter™ methods. For samples treated at 65°C, the DNase treatment showed a two times higher average genome unit measurement and a 2.7 times greater standard deviation, indicative of TruTiter™ method being more robust for genome titer measurements. For both detergents genome tittering showed a greater standard deviation compared to the TruTiter™ method. This study demonstrates the inherent variability in viral genome quantitation when using DNase, and the variability is reduced when using a small molecule-based approach. Encapsulation efficiency for LNP is an important parameter for optimizing the right type of lipid or understanding LNP storage condition. TruTiter™ Reagent is excluded from the LNP particle, thus protecting the nucleic acid inside

and is utilized to determine encapsulation efficiency. We observed that lipids used in LNP are often inhibitors of Reverse Transcriptase and DNA polymerase making nucleic acid purification a prerequisite for analyzing LNP encapsulation efficiency. First, we used GFP mRNA encapsulated in a LNP and treated at different temperatures (22°C, 42°C and 55°C) to induce rupture of the lipid nanoparticle. Encapsulation efficiency was determined using PicoGreen or the TruTiter technology. TruTiter shows a significant reduction in encapsulation efficiency with increase in temperature whereas PicoGreen did not show any appreciable loss of encapsulation even when the LNP structural integrity was lost. Next, we used in *vitro* transcribed RNA (Firefly luciferase) and incubated with transfection reagents that do or do not form LNP complexes. Each sample was treated with and without the TruTiter™ Reagent. After the neutralization and nucleic acid extraction, nucleic acid was analyzed for amplification of the target gene using RT-qPCR.

1889. Development of Novel Bioluminescent Assays for Sensitive and Specific Quantitation of Double-Stranded RNA in mRNA Therapeutics

Jeff Nelson, Rich Moravec, Jun Wang, Jim Hartnett, Mei Cong, Jamison Grailer

Promega Corporation, Madison, WI

mRNA-based therapeutics have shown clinical efficacy, evidenced by the success of the mRNA vaccines for SARS-CoV-2. mRNA drug substance is typically generated by in vitro transcription (IVT). One byproduct and contaminant of IVT is the formation of double-stranded RNA (dsRNA). dsRNA is highly immunogenic, can induce inflammation and cell death, and inhibit protein translation, including the protein encoded by the therapeutic mRNA. As such, dsRNA must be measured for all IVT products to ensure safety and efficacy. However, existing dot bot and ELISA technology to detect dsRNA is neither sensitive nor quantitative. Herein, we describe the development of novel assay technologies for sensitive and specific quantitation of dsRNA in IVT products and following encapsulation of IVT products in lipid nanoparticles (LNPs). First, NanoBiT® split luciferase was applied to detect dsRNA in IVT drug substance. dsRNA binding domains were genetically attached to either SmBiT or LgBiT. In the presence of dsRNA, the SmBiT and LgBiT fusions bind via the dsRNA binding domains, resulting in dimerization and the complementation of functional NanoBiT® luciferase. NanoBiT® then generates light which is proportional to the amount of dsRNA in the sample. The assay detects dsRNA greater than or equal to 30 base pairs and is independent of the size and sequence of the dsRNA contaminant. The assay does not cross-react with single-stranded RNA or DNA and is highly sensitive, with a limit of quantitation less than 100 pg/ml. As a complementary tool to the biochemical assay, we developed a cell-based bioassay to detect dsRNA encapsulated in LNPs. dsRNA/LNPs are taken up by endocytosis where Toll-like receptor 3 (TLR3) detects dsRNA. Activation of TLR3 initiates a signaling pathway that is detected by a TLR3 pathway-specific promoter driving luciferase expression. This pathway likely represents the most physiologically relevant readout of dsRNA bioactivity. Both assays are developed in "add-mix-read" format with no wash steps or media transfer, and exhibit robust, sensitive, and repeatable performance for specific detection of dsRNA in mixed solutions.

1890. Comprehensive Analysis of Off-Target and On-Target Effects Resulting from Liver-Directed CRISPR-Cas9-Mediated Gene Targeting with Adeno-Associated Viral Vectors

Kshitiz Singh[1], Raffaele Fronza[2], Hanneke Evens[1], Marinee Chuah[1], Thierry Vandendriessche[3]

[1]Department of Gene Therapy & Regenerative Medicine, Free University of Brussels (VUB), Brussels, Belgium,[2]ProtaGene CGT GmbH, Heidelberg, Germany,[3]Free University of Brussels (VUB), Brussels, Belgium

Comprehensive genome-wide studies are needed to assess the consequences of adeno-associated virus (AAV) vector-mediated CRISPR-Cas-based gene editing. In this study, we evaluated CRISPR-Cas-mediated on-target and off-target effects and examined the genomic integration events of AAV vectors employed to deliver the CRISPR-Cas components to the liver in neonatal mice. The guide RNA (gRNA) was specifically designed to target the coagulation factor IX gene (*F9*). Whole-genome sequencing (WGS) was conducted to examine the on-target and off-target insertions/deletions (indels). On-target editing events were quantified using the CRISPResso algorithm, revealing efficient CRISPR targeting (36.45 ± 18.29%) in the *F9* locus. In contrast, off-target effects were very rare or below the WGS detection limit since only one single putative insertion was detected out of 118 reads (n = 3), based on >100 computationally predicted off-target sites. Genome-wide AAV integration events were identified by WGS and Shearing Extension Primer Tag Selection Ligation-Mediated PCR (S-EPTS/LM-PCR), demonstrating that AAV integration occurred preferentially in the CRISPR-Cas9 induced double-strand DNA breaks in the F9 locus. In contrast, AAV integrations outside this locus were not in proximity to any of ~5000 putative computationally predicted off-target sites (median distance of 70 kb) and likely represent canonical CRISPR-Cas9-independent AAV integrations.

1891. Real-Time Phase Shift Imaging and Single-Cell Tracking of Human Placental Cells for Discovering Alterations Induced During Lentiviral Transduction

Sarah E. Wachtman[1], Martin Rodriguez[1], Nicholas P. Edenhoffer[1], Ritu M. Ramamurthy[1], Christopher Doering[2], H. Trent Spencer[2], Anthony Atala[1], Christopher D. Porada[1], M Graça D. Almeida-Porada[1]

[1]Fetal Research and Therapy Program, Wake Forest Institute for Regenerative Medicine, Winston Salem, NC,[2]Dept. of Pediatrics, Aflac Cancer and Blood Disorders Center, Emory University and Children's Healthcare of Atlanta, Atlanta, GA

Cells that have been genetically engineered using viral vectors to encode therapeutic proteins have tremendous clinical potential. While characterization of the final clinical product is usually performed several passages after transduction, little is known about cellular kinetics and dynamics during and immediately after transduction. Therefore, multiple testing approaches were used to determine the

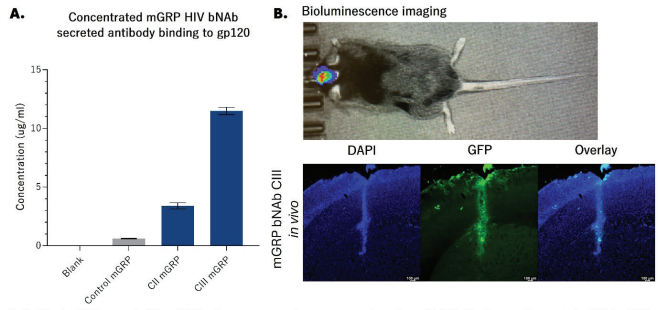
effects of lentiviral vector (LV) transduction on various cellular parameters. Real-time phase shift imaging and single-cell tracking of human placental cells (PLCs) transduced using a LV encoding a bioengineered FVIII transgene, mcoET3 (MOI = 5 and 10) was employed to investigate how LV transduction affects PLC kinetics and morphology during this period. PLC were cultured in a PHI HoloMonitor M4 and scanned for a total of 5 passages: one passage prior to transduction (36hrs), during transduction (24hrs), and three passages post-transduction (36hrs each). Non-transduced PLC (PLC-NT), PLC-MOI 5, and PLC-MOI 10 were monitored in parallel, and the experiment was repeated 3 times. Thirteen parameters of cell kinetics were monitored: proliferation, motility, motility speed, cell family tree, phase shift, optical volume, optical thickness, eccentricity, irregularity, surface area, migration, roughness, and roughness kurtosis. After the final monitoring session, RNA, DNA, and culture media were collected to quantify endogenous FVIII/vector-encoded FVIII expression (RT-qPCR), vector copy number (VCN; by qPCR), and FVIII activity (aPTT assay). FVIII mRNA expression, FVIII activity, and VCN confirmed efficient PLC transduction proportional to the MOI used. Baseline kinetics of the PLCs prior to transduction exhibited changes in motility (μm; matched pairs t-test, p=0.009), motility speed (μm/h; p=0.002), eccentricity (p=0.04), and irregularity (p=0.017). Within the first 4hrs post-transduction, both the PLC-MOI 5 and PLC-MOI 10 groups showed a statistically significant decrease in cell motility speed (μm/h) and optical volume (μm^3) when compared to the PLC-NT controls. Interestingly, by passage three post-transduction, all cell kinetic parameters normalized to baseline, and no significant differences were found between the groups. We hypothesize that because a decreased optical volume (OV) is most often due to the gain or loss of osmotically active solutes, it is possible that *clathrin-mediated endocytosis* of the LV leads to osmotic changes causing an OV decrease. Likewise, microtubules and microfilaments have dual functions, dynamically maintaining cell shape and enabling motility. MAP1-family proteins bind along the microtubule lattice and help tether incoming viral capsids to the microtubular network, thereby promoting cytoplasmic trafficking. Thus, it is possible that the presence of LV particles induces dysfunction of the microfilament network leading to decreased cell motility. However, by passage three, the abnormalities in cell function are restored to normal. These studies are critical to the continued development of LV-modified PLC, which are being developed for in utero applications. Further studies investigating how LV transduction affects each of these parameters and correlating them with specific biologic functions are underway.

1892. Development of HIV Neutralizing-Antibody Expressing Glial Progenitor Cells for Viral Clearance from the Brain

Lorissa Ingsay McDougall[1], Abdolrahim Abassi[2], Mohammad Sajadi[2], Alonso Heredia[2], Linda Chang[1], Yajie Liang[1], Chengyan Chu[1], Miroslaw Janowski[1], Piotr Walczak[1]

[1]Diagnostic Radiology and Nuclear Medicine, University of Maryland, Baltimore, Baltimore, MD,[2]Institute of Human Virology, University of Maryland, Baltimore, Baltimore, MD

Human immunodeficiency virus (HIV) infection occurs when CD4 T-cells recognize and bind the viral envelope glycoproteins on the chemokine receptor 5 (CCR5), enabling the virus to hijack these T-cells and spread throughout the lymph system and blood. Fortunately, due to improvements in the detection and treatment of HIV, the mortality rate has dropped to only 2%. However, a person with HIV (PWH) will be dependent on lifelong anti-retroviral medications, which often leads to complications involving liver and kidney toxicity, lipid changes in the gut, and neurological changes. Additionally, these treatments are inefficient at eradicating viral reservoirs from the brain, which often leads to systemic re-infection upon drug cessation. Therefore, our research aims to develop a protocol for the engineering of broad neutralizing antibody (bNAb) expressing primary glial progenitor cells (GRPs) by introducing a lentiviral construct encoding a bNAb against HIV. GRPs mature into support cells of the brain, which, when injected inter-arterially (IA), have been proposed to migrate across the endothelium into the brain and thus are our model of choice for the clearance of HIV-infected cells. Additionally, the lentiviral constructs were designed to include the heavy and light chains of the antibody, a linker and cleavage site, as well as a green fluorescent protein (GFP) for detection. We have shown successful transduction of the constructs (CII and CIII) in luciferin-producing immortalized mouse GRP cells (mGRP) through FACS cell sorting and release of the bNAbs *via* ELISA detection and flow cytometry of glycoprotein expressing B-lymphocyte cells. Additionally, stereotactic injection of these cells into the brains of IL34+ NOG mice identified survival of the mGRPs after three days *via* BLI imaging of luciferin metabolic activity and GFP-positive cells in the needle track of the brain slices. Therefore, we expect to observe antibody binding with GFP localization after injection of the mGRPs into humanized HIV-infected mice. Additionally, the development of genetically engineered primary mGRPs will allow for IA injection and a broader distribution of the cells in the brain, and a higher rate of HIV infected cell neutralization.



A. ELISA of mGRP control, CII and CIII cell supernatant shows greater detection of bNAb binding to glycoprotein 120 (gp120) when compared to the control in both CII (increase of 2.8ug/ml) and CIII (increase of 10.9ug/ml). **B.** BLI imaging of the mGRP cells three days post injection into the IL34+ NOG mice identified cell survival, and immunofluorescence of the GFP-positive mGRPs in the mouse brain.

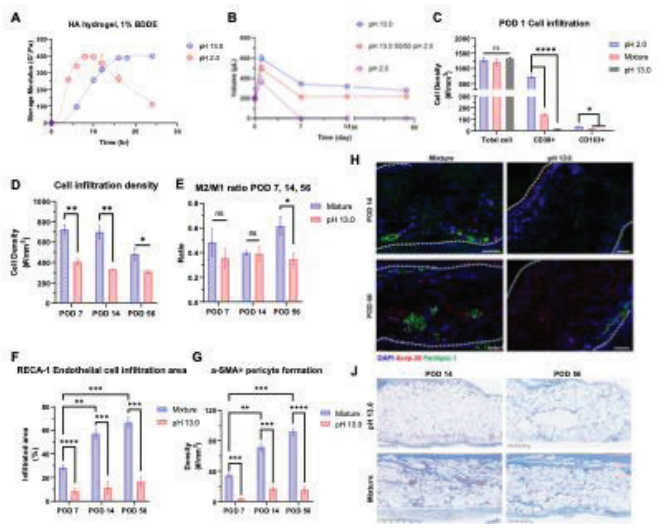
1893. Pro-Angiogenic Response to Hyaluronic Acid Hydrogel Matrix with a Tunable Degradation Profile

Kailei Ding, Jiayuan Kong, Hexiang Feng, Hai-Quan Mao

Johns Hopkins University, Baltimore, MD

Statement of Purpose: Hyaluronic acid (HA) hydrogel is widely used for cell theapy and tissue engineering due to its excellent biocompatibility and biodegradability profile. Here, we developed an HA hydrogel with tunable degradation kinetics by combining two HA hydrogel microparticles with different degradation rates, and tested the

hypothesis that the tunable, gradual degradation profile can enhance the recruitment and immunomodulation of host immune cells and improve the pro-angiogenic property of the hydrogel in vivo. We evaluated macrophage infiltration and polarization, hydrogel volume retention, pro-angiogenic effect, and subsequent soft tissue remodeling within the HA hydrogel. **Methods:** We first crosslinked HA using 1,4-butanediol diglycidyl ether (BDDE) at pH 2.0 and pH 13.0 to obtain two HA microgels with distinctively different degradation rates. To generate HA hydrogel with a tunable degradation rate, we mixed the two microgels at 1:1 ratio. The degradation rates of hydrogels were monitored by shear storage modulus (G') over time. The microgels prepared at pH 2.0, pH 13, and the mixed microgels (1:1 ratio) were tested to assess *in vivo* degradation and cell infiltration following subcutaneous injection in Sprague-Dawley rats (8-10 week old). $CD38^+$ and $CD163^+$ macrophages were examined by immunostaining. The pro-angiogenic and adipogenesis responses were assessed via immunostaining of endothelial cells/pericytes and adipocytes/collagen deposition, respectively. **Results:** HA hydrogels crosslinked with BDDE at pH 2.0 and pH 13.0 showed similar storage modulus (G'~250 Pa) but a distinct degradation profile (Fig. 1A). The shape retention and cell infiltration were examined after subcutaneous injection (Fig. 1B). The pH 2.0 HA hydrogel degraded within 7 days whereas the pH 13 hydrogel retained more than 50% of the peak volume by day 56. A substantial level of infiltrated macrophages was observed in the pH 2.0 microgel at 1 day post-injection due to rapid degradation (Fig. 1C). The microgel mixture consistently showed a higher macrophage count than the pH 13 microgel from day 7 to 56 post-injection (Fig. 1D-E). At both time points, endothelial cell (RECA-1+) and pericyte (-SMA+) infiltration was significantly greater in the microgel mixture than the pH 13 microgel (Fig. 1 F-G). Furthermore, the microgel mixture induced a higher degree of adipogenesis than the pH 13 microgel (Fig. 1H). Masson trichrome staining indicates more cellular content, collagen deposition, and neo-tissue formation in the microgel mixture at day 56 post injection compared to the pH 13 microgel (Fig. 1J). **Conclusion:** The engineered hydrolysable HA microgel mixture with a tunable degradation profile creates pores within the HA hydrogel gradually after implantation *in vivo*. The degraded HA microgel may serve as a biostimulatory cue facilitating infiltration and polarization of host macrophages to augment pro-angiogenic responses and potentially tissue remodeling. **Figure 1.** (A) Shear storage modulus change during the gelation process. (B) Volume retention of hydrogels *in vivo* over time. (C-E) Cell infiltration and polarization at 1 (C) and 7, 14, and 56 days (D-E) after subcutaneous injection in SD rats. (F-G) Endothelial cells infiltration (F) and pericytes formation (G) at 7, 14, 56 days after injection. (H-J) Adipose tissue formation (H) and collagen deposition (J) at 14 and 56 days after injection.



1894. A Rapid and Scalable 96-Well Platform to Screen CARs and TCRs

Ana Chadbourne, Alexander Boucher, Elise Kim, Peter Moua, Christopher B. Driscoll, Shen Lu, Joshua Ferrell, Andrew Basinski, Stacie Seidel, Cherylene Plewa

ElevateBio, Waltham, MA

Autologous adoptive cell transfer (ACT) therapy has revolutionized the landscape of cancer treatments and has shown great promise for patients with otherwise non-viable therapeutic alternatives. The early success of autologous Chimeric Antigen Receptor (CAR)-T therapies in heme indications in the clinic led to a rapid application to the solid tumor setting, expansion to allogeneic and iPSc derived cell modalities, as well as to other cells of the immune system such as NKs and macrophages. Regardless of the modality, identification of a target binder that will give rise to the CAR or T cell receptor (TCR) construct is a necessary first step in the development of engineered cell therapies. Screening platforms for nomination of CAR and TCR constructs typically rely on high throughput platforms for which the initial criteria involve selection of binder molecules that express at reasonable levels on the T cell surface. These platforms are seldom scalable, utilize non-MOI normalized lentiviral vector preparations and functional readouts are typically limited to IFNg production which may or may not correlate with cytotoxic activity. When using these platforms, top-candidate selection relies on time-consuming, larger-scale engineered cell production to produce enough cells to run *in vitro* functional assays required for candidate nomination and validation in *in vivo* tumor models. Here we report a rapid, mid-throughput, comprehensive, scalable CAR/TCR screening platform for the selection of candidate CARs and TCRs in primary T cells. The platform relies on a scalable mid-throughput lentiviral vector production platform, and a 96-well scalable T cell expansion that produces enough cells for characterization and functional assays including vector copy number (VCN), surface binder expression and function. The platform was utilized to screen candidate CARs by surface expression and VCN, tonic signaling, functional activation by engineered cell lines as well as cell lines expressing endogenous high, medium and low levels of target.

Within 3 rounds of screening, we were able to nominate top candidates ready for *in vivo* testing. Our proprietary CAR/TCR platform thus allows acceleration of nomination of binders for faster development of CAR and TCR therapies.

Friday Posters: Cell Therapy and Cell-Based Gene Therapy Trials

1895. Trial in Progress: A Phase 1/2 Study to Investigate the Safety and Efficacy of OBX-115 Engineered Tumor-Infiltrating Lymphocyte (TIL) Cell Therapy in Patients (Pts) with Advanced Solid Tumors

Adam J. Schoenfeld[1], Allison Betof Warner[2], Sajeve S. Thomas[3], Omid Hamid[4], Gino K. In[5], Alexander N. Shoushtari[1], Yazan Samhouri[6], Parameswaran Hari[7], Giridharan Ramsingh[7], Prakash Prabhakar[7], Lauren Mclaughlin[7], Jason A. Chesney[8]

[1]Memorial Sloan Kettering Cancer Center, New York, NY,[2]Stanford University School of Medicine, Stanford, CA,[3]Orlando Health Cancer Institute, Orlando, FL,[4]The Angeles Clinic and Research Institute, Los Angeles, CA,[5]Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA,[6]Allegheny Health Network Cancer Institute, Pittsburgh, PA,[7]Obsidian Therapeutics, Inc., Cambridge, MA,[8]UofL Health – Brown Cancer Center, Louisville, KY

Introduction: Immune checkpoint inhibitors (ICI) have improved outcomes for pts with solid tumor malignancies; however, most pts relapse and treatment options in this setting are limited. Unengineered TIL cell therapy has shown promising efficacy in pts with ICI-resistant melanoma (Rohaan *NEJM* 2022, Chesney *JITC* 2022) and non-small cell lung cancer ([NSCLC] Creelan *Nat Med* 2021, Schoenfeld SITC 2021), but requires co-administration of systemic high-dose IL2, which is associated with safety risks and limits pt eligibility. OBX-115 TIL are engineered to express membrane-bound IL15 (mbIL15) under dose-dependent regulation using acetazolamide (ACZ), an FDA-approved small-molecule diuretic, avoiding the need for high-dose IL2. A first-in-human single-institution study (NCT05470283) evaluating the safety of OBX-115 in metastatic melanoma is enrolling. The current study (NCT06060613) is enrolling pts with solid tumors at multiple US sites using centralized manufacturing. **Methods:** This phase 1/2, single-arm, open-label, nonrandomized, multicenter study will assess the safety, tolerability, and efficacy of the OBX-115 engineered autologous TIL cell therapy regimen in pts with unresectable or metastatic melanoma resistant to ICI and relapsed/refractory metastatic NSCLC (Table). Primary objectives of Phase 1 are to characterize safety and tolerability and identify a recommended Phase 2 dose of OBX-115 + ACZ; Phase 2 will evaluate efficacy (ORR using RECIST v1.1 responses) of the regimen. Cryopreserved OBX-115 is generated from the pt's own tumor tissue procured by surgical excision or core needle biopsy, and is infused after standard- or low-dose (based on clinical status) lymphodepletion (cyclophosphamide and fludarabine). ACZ is administered at cohort-defined doses once daily for up to 10 days, with optional ACZ redosing at Week 6-8 for up to 10 days in pts without radiographic response. No systemic high-dose IL2 is administered. Two sites are open and recruiting, with additional sites being activated.

Key Eligibility Criteria	
Key Inclusion Criteria	**Key Exclusion Criteria**
Age ≥18 years	Uveal melanoma
Histologically confirmed diagnosis of unresectable Stage IIIC, IIID, or Stage IV metastatic melanoma with documented radiographic disease progression after systemic therapy containing a PD-1- or PD-L1-blocking antibody (if adjuvant setting, progression during or within 12 weeks after the last dose) and received a BRAF inhibitor ± MEK inhibitor if *BRAF* V600 mutation-positive *OR* Histologically confirmed diagnosis of metastatic NSCLC previously treated with an approved systemic therapy for metastatic disease (including an ICI-based regimen and/or targeted therapy where applicable) and progressed, no longer deriving benefit, or unable to continue due to treatment intolerance	Active autoimmune disease, including active uveitis or any other medical illness that would pose increased risks for study participation
≥1 lesion suitable for OBX-115 manufacturing, with expected minimum of 1.5-cm diameter; minimally invasive tumor tissue procurement (core needle biopsy) may be considered on a case-by-case basis after discussion with the Medical Monitor	Prior allogeneic organ transplant, allogeneic cell therapy, or genetically engineered cell therapy (not including autologous stem cell or unengineered TIL cell therapy)
≥1 RECIST 1.1-measurable lesion remaining after tumor tissue procurement	Systemic steroid therapy >10 mg/day of prednisone or equivalent
ECOG performance status 0 or 1	
Estimated life expectancy >6 months	

1896. Interim Safety and Efficacy of Anti-CD70 CAR-NKT (CGC729) for Patients with Advanced Renal Cell Carcinoma

Jian Zhang[1], Weina Shen[1], Muyu Kuang[1], Feifei Guo[2], Yuanyuan Qu[1], Hailiang Zhang[1], Guohai Shi[1], Dalong Cao[1], Dong Han[2], Yingmin Zhang[2], Shuyang He[2], Dingwei Ye[1]

[1]Fudan University Shanghai Cancer Center, Shanghai, China,[2]Curegenetics LLC, Suzhou, China

Background: Tumor heterogeneity and suppressive tumor microenvironment (TME) are major barriers to successful chimeric antigen receptor (CAR) T cell therapy in solid tumors. Natural killer T (NKT) cells, a subset of innate-like T cells, have emerged as a novel platform for the treatment of solid tumors owing to NKT's multifactorial antitumor and TME modulation properties. Here we describe interim findings from an investigator-initiated trial (IIT) of

CGC729, an autologous anti-CD70 CAR-NKT product, in patients with advanced clear cell renal cell carcinoma (ccRCC). **Methods:** This is a single-arm, open-label IIT (NCT06182735) using 3 + 3 design to evaluate the safety and efficacy of CGC729 at three dose levels including $5\times10^6/m^2$ (DL1), $1.5\times10^7/m^2$ (DL2), and $4.5\times10^7/m^2$ cells (DL3). As of Jan. 2024, five patients who received at least 2 prior lines of therapy were enrolled and treated with a single infusion of CGC729 following a 3-day lymphodepletion regimen (fludarabine $25mg/m^2$/day; cyclophosphamide $250mg/m^2$/day). At the time of the presentation, at least six patients will have been treated with 3-9 months follow-up. Cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) were graded based on ASTCT criteria, and adverse effects were evaluated according to CTCAE 5.0. **Results:** As of the Jan. 22 data cutoff, four patients (DL1=3; DL2=1) were eligible for safety evaluation. Adverse events were manageable, and no dose-limiting toxicities (DLT) were observed. The most common adverse events were lymphodepletion associated reduction of neutrophils, platelets, and white blood cells; however, no > Grade 2 cytopenia was observed by Day 28. One patient (0102) in DL1 had Grade 2 CRS resolved within 24 hours. The same patient developed Grade 2 ICANS and was resolved with corticosteroids. No CRS or ICANS was observed in the other two patients in DL1 and the one patient in DL2. The overall response rate (ORR) in DL1 at Week 12 was 66.7% (2/3), and the ORR among ccRCC $CD70^+$ patients (0101 and 0103) was 100% (2/2). Those two $CD70^+$ patients had deep tumor reduction of 48.4% at Week 20 and 70.8% at Week 12, respectively (Figure 1A). Notably, both $CD70^+$ patients had low CD70 expression (H-score < 100) in ccRCC indicating CGC729 may have the potential to target CD70-low patients. Patient 0102 in DL1 was ccRCC $CD70^-$ and experienced progress disease (PD) on Day 28. Among four patients evaluable for PK, robust CAR-NKT expansion in the peripheral blood was observed in all patients including Patient 0102 who had negative CD70 expression in ccRCC. CGC729 had peak expansion between Day 14 and Day 28, and it persisted up to Week 20 (Figure 1B). Moreover, $CD70^+$ T cells in the peripheral blood decreased significantly after CGC729 infusion and remained at a low level up to Week 20, indicating a durable activity of CGC729. **Conclusion:** Interim analysis of this first-in-human trial of anti-CD70 CAR-NKT demonstrates a manageable safety profile and promising antitumor activity for the treatment of advanced ccRCC.

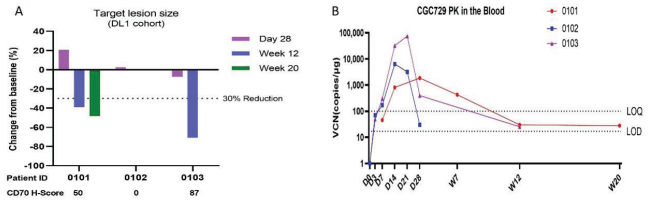


Figure 1: CGC729 had deep anti-tumor activity (A) accompanied with robust expansion and durable persistence (B) in DL1.

1897. Phase I Study of GUCY2C CAR-T Therapy IM96 in Metastatic Colorectal Cancer Patients

Changsong Qi[1], Chang Liu[1], Jian Li[1], Jifang Gong[1], Xicheng Wang[1], Zhenghang Wang[1], Xin'an Lu[2], Ting He[2], Yanping Ding[2], Fei Wu[2], Dongqun Liu[2], Lin Shen[1]

[1]Peking University Cancer Hospital and Institute, Beijing, China,[2]Beijing Imunopharm Technology Co., Ltd, Beijing, China

Background: The clinical outcomes of metastatic colorectal cancer (mCRC) therapies are limited, especially for liver metastasis. Guanylyl cyclase 2C (GUCY2C) is ectopically expressed in all stages of CRC and intestinally-restricted. GUCY2C-targeted CAR-T (IM96) was developed and phase I study was conducted to evaluate the safety and efficacy (NCT05287165). *Methods*: In this open-label, 3+3 dose-escalation study, IM96 was evaluated in GUCY2C-positive mCRC patients (pts) failed to ≥3 lines of therapies. Bridging therapies were allowed. Pts were pre-treated with fludarabine and cyclophosphamide, and received a single infusion of IM96 at the dose of 3×10^8 (DL1), 6×10^8 (DL2), 12×10^8 (DL3), or 20×10^8 (DL4) CAR-T cells. Dose-expansion study was performed at DL3. The primary objectives were safety and toxicity, and the secondary objectives were efficacy and pharmacokinetic profile. *Results*: As of December 2023, 20 pts were enrolled and infused with IM96. The follow-up time was 7-19 months for all pts. The median age was 52.5, and 11/20 cases were male. Liver metastasis was found in 11/20 cases (55%). Genetic testing revealed proficient mismatch repair (pMMR) in 20/20 pts (100%), KRAS mutation in 12/20 pts (60.0%), NRAS mutation in 1/20 pts (5.0%), and BRAF mutation in 3/20 pts (15.0%). Bridging therapies were used in 19 pts. Only 1/20 pt (5.0%) showed neurotoxicity and ≥grade 3 cytokine release syndromes (CRS). Grade 1-2 CRS occurred in 16/20 pts (80.0%) with dramatic increase of interleukin-6. Grade 1-3 rash was observed in 14/20 pts (70.0%). Grade 3 diarrhea occurred in 11/20 pts (55.0%), and grade 1-3 oral mucositis appeared in 7/20 pts (35.0%), only in DL2, DL3 and DL4 groups. Dose-limiting toxicity and maximum tolerated dose were not achieved. Among 19 evaluable pts, the disease control rate (DCR) was 73.7%, and the objective response rate (ORR) was 26.3%. In DL3 group, pts showed an ORR of 40.0%, nevertheless of liver metastasis or not. Six pts in DL3 group were evaluated as stable disease (SD) after treatment for 1 month. Among them, 4/6 pts (66.7%) turned to partial response (PR) and 2/6 pts (33.3%) retained SD. The median progression-free survival time was 7 months in DL3 group. No responding Pts showed disease progression within 6 months. Tumor responses were correlated with significant decreases in CEA levels among all pts. *Conclusions*: This study demonstrated that IM96 has durable efficacy with acceptable safety profile in pMMR mCRC pts, in particularly, showing high therapeutic potential in liver metastasis pts.

1898. Product Attributes of Tumor Associated Antigen (TAA) T Cells May Inform Clinical Responses in Pediatric Patients with CNS Tumors

Stephanie Gomez[1], Ashley Geiger[1], Rachel DiCioccio[1], Melanie Grant[1], Haili Lang[1], Abeer Shibli[1], Anushree Datar[1], Emily Reynolds[1], Christopher A. Lazarski[1], Mariah Jensen-Wachspress[1], Gloria Pezzella[1], Daisy Ayala-Gomez[1], Jay Tanna[1], Fahmida Hoq[1], Chase D. McCann[1], Patrick J. Hanley[1], Lindsay B. Kilburn[2], Brian R. Rood[1], Roger J. Packer[2], Conrad Russell Y. Cruz[1], Catherine M. Bollard[1], Eugene I. Hwang[2]

[1]Center for Cancer and Immunology Research, Children's National Hospital, Washington, DC,[2]Center for Cancer and Blood Disorders, Children's National Hospital, Washington, DC

BACKGROUND: Central nervous system (CNS) tumors are the most common, and deadliest, solid tumor in children, and there is an urgent need for novel therapies. The polyclonal nature of multi antigen-specific T cells may provide a clinical benefit to patients with high-risk brain tumors where intratumoral heterogeneity plays a prominent role in therapeutic resistance. **OBJECTIVE:** To determine safety and feasibility of multi tumor associated antigen T (TAA-T) cells in patients with CNS tumors. **METHODS/DESIGN:** Pediatric patients with CNS malignancies enrolled in a phase I dose escalation study (NCT03652545) were infused with ex vivo expanded, autologous T cells. T cells were pulsed with peptide libraries spanning tumor-associated antigens: WT1, PRAME, and Survivin. TAA-T cell product characterization and preliminary clinical outcomes were examined for patients diagnosed with diffuse intrinsic pontine gliomas (Arm A, n = 8) or recurrent/progressive non-brainstem CNS malignancies without lymphodepletion (Arm B, n = 18). To determine immunophenotype, TAA-T cell products were characterized by flow cytometry. TAA-T cell product specificity was determined by assessing reactivity through IFNg enzyme-linked immunosorbent spot (ELISpot) assay following re-stimulation with TAA or control peptide pools. **RESULTS/DISCUSSION:** In Arm A, the median overall survival (OS) was 14 months, compared to a historical median OS of 10 months. One patient in Arm A had a progression-free survival (PFS) of 11 months and an OS of 32 months. In Arm B median PFS was 7 months, and 3 patients out of 18 treated remain alive with stable disease more than 2 years after infusion. We observed a dose level effect across all patients analyzed, in which increased TAA-T dose was associated with prolonged disease stabilization. Additionally, our results suggest that superior TAA-T cell products (defined as specific by ELISpot and >75% alpha beta T cells) are associated with prolonged PFS. Future directions include testing personalized, neoantigen-specific T cells in patients with CNS malignancies (IMPACT trial).

1899. Case Report of Patient with Castrate Resistant Prostate Cancer Achieving Clinical Benefit PostTGFβRdn on PSMA-Redirected CAR-T Cells

Naomi B. Haas[1], Vivek Narayan[1], Julie Barber-Rotenberg[2], Katie Alexander[1], Vanessa Gonzalez[1], Carly Stein[1], Carl H. June[2], Joseph A. Fraietta[2]

[1]Abramson Cancer Center, Philadelphia, PA,[2]University of Pennsylvania, Philadelphia, PA

Introduction: Transforming growth factor-β (TGFβRdn) on prostate specific membrane antigen (PSMA)-redirected CAR-T cells following lymphodepletion (LD) chemotherapy in our Phase I trial was feasible, generally safe and showed both T-cell proliferation and persistence in 14 treated patients with metastatic castrate resistant prostate cancer (CRPC) (Nat Med. 2022 Apr;28(4):724-734. PMC10308799). However, no patient demonstrated long term control of prostate cancer. Here we report clinical improvement and post-treatment correlative analyses of a patient with metastatic CRPC treated in an expansion cohort of the PSMA-redirected and TGFβRdn co-expressing CAR-T cell trial.The patient is a 77 yo male with stage IV group 4 CRPC and known bone metastases previously treated with a luteinizing hormone-releasing hormone agonist and an androgen receptor secondary inhibitor therapy, followed by sipuleucil therapy and then docetaxel therapy upon progression. He then received LD chemotherapy, consisting of cyclophosphamide (300 mg/m^2) and fludarabine (30 mg/m^2), followed four days later by an infusion of CART-PSMA-TGFβRDN (1 x 10^8 CAR+ PSMA cells). The next day, he experienced Grade 2 Cytokine Release Syndrome (CRS), which was managed with supportive intravenous (IV) fluids. His initial post-treatment course was notable for grade 2 cytokine release syndrome (CRS), managed with IV fluids, tocilizumab and dexamethasone, as well as an unexplained syncopal event. He was discharged from inpatient monitoring on day 6, following clinical stability and improvement in post-LD chemotherapy cytopenias. However, the next day he experienced several syncopal events accompanied by a hemophagocytic-like (HLH) syndrome manifesting with transaminitis, elevated ferritin, and low platelets, requiring rehospitalization and a prolonged dexamethasone taper. Notably his bone pain resolved, and he sustained a PSA decline of 17.7% decrease at 12 weeks and 13.5% decline at D85 accompanied by CAR-T expansion and persistence which continued after recovery from the HLH-like syndrome and steroid wean. CAR-T by PCR analysis was detected in both blood and bone biopsy at day 36. CT imaging was stable at 12 weeks although bone scan showed new uptake. The patient is asymptomatic at 6 months. Methods: Serum samples obtained pre- and post- CART-PSMA-TGFβRDN infusion and cryopreserved at −80 °C were analyzed for cytokine measurements using either a human cytokine magnetic 30-plex panel (Life Technologies) or a 31-plex panel (Millipore Sigma). Multiplex assay for all cytokines were obtained at multiple timepoints (days 1-71). IL-18 soluble proteins were obtained in a separate assay. Bone biopsy at metastatic tumor site was obtained pre and post treatment (day 36). PCR amplification for peripheral blood and tumor samples (QuantStudio 3D digital PCR instrument) was previously described). Results: Cytokines demonstrated 40-50x increase in IL2, IL6 and a 10x increase in interferon-γ following CAR-T infusion. Sustained high IL18 levels accompanied the HLH-like syndrome. CAR-T was detectable in the blood (57265.61 copies/

μg DNA, peak day 28) and bone metastases (36401.59 copies/μg, day 36) by PCR. Conclusion: This is the first clinical improvement using PSMA-redirected and TGFβRdn co-expressing CAR-T in metastatic CRPC. Elevated IL18 and IL-2 levels may be indicative of PSMA TGFβRdn CAR-T response.

1900. Trial in Progress: A Phase 1, First in Human Clinical Trial for T-Plex, a Multiplexed, Enhanced T Cell Receptor-Engineered T Cell Therapy (TCR-T) for Solid Tumors

Justin Moser[1], Brian Pico[2], Brian Henick[3], Rom Leidner[4], Yazan Samhouri[5], James Isaacs[6], Jared Weiss[7], Michael Hurwitz[8], Jaspreet Grewal[9], Jason Luke[10], Shrikanta Chattopadhyay[11], Yun Wang[11], Marlyane Motta[11], Jim Murray[11], Debora Barton[11], Dawn Pinchasik[11], Gavin MacBeath[11], Sajeve Thomas[12]

[1]HonorHealth Research Institute, Scottsdale, AZ,[2]Memorial Healthcare System, Pembroke Pines, FL,[3]Columbia University Medical Center, New York, NY,[4]Providence Cancer Institute, Portland, OR,[5]Allegheny Health Network, Pittsburgh, PA,[6]Cleveland Clinic, Cleveland, OH,[7]University of North Carolina at Chapel Hill, Chapel Hill, NC,[8]Yale School of Medicine, New Haven, CT,[9]Norton Healthcare, Louisville, KY,[10]University of Pittsburgh Medical Center, Pittsburgh, PA,[11]TScan Therapeutics, Waltham, MA,[12]Orlando Health, Orlando, FL

Checkpoint immunotherapies have revolutionized solid tumor treatment yet durably benefit a minority of patients, as they rely on endogenous anti-tumor T cells. A potential solution for patients lacking functional endogenous anti-tumor T cells is engineering their T cells with exogenous T cell receptors (TCRs) to target and kill tumor cells. Initial clinical trials with TCR engineered T cell therapies (TCR-Ts) targeted single tumor antigens on single HLA and produced partial, short-lasting responses. Solid tumors are notoriously heterogenous with highly variable antigen expression and have an immunosuppressive microenvironment. Recent discoveries also identified HLA loss of heterozygosity in up to 40% of solid tumors, allowing tumor cells to evade T cell attack. To overcome this heterogeneity and immune evasion, TScan has developed T-Plex, a multiplexed cell therapy comprising two to three different TCR-Ts, chosen from a collection of TCR-Ts called the ImmunoBank, to target different tumor antigens on different HLA types with confirmed tumor expression. To deepen clinical responses, TCR-T cells are engineered to express CD8α/β co-receptors that, in preclinical experiments, enable CD4+ helper T cells to have >100-fold improved cytotoxicity and cytokine secretion over CD4+ cells expressing the TCR alone. To allow T cell persistence despite immunosuppressive TGF-β in the tumor microenvironment, TCR-T cells also express the dominant negative TGF-β receptor, enabling ~10-fold improved proliferation in the presence of TGF-β compared to T cells expressing the TCR and CD8α/β co-receptors alone. The Phase 1 study utilizes a separate screening protocol to pre-identify patients with head and neck, cervical, anogenital cancers, NSCLC, and melanoma, any time during standard clinical care, enabling rapid enrollment into the treatment protocol upon disease progression. Screening comprises germline HLA testing, and archival tumor testing for antigen expression and HLA loss. As HLA loss is restricted to only half the HLA genes, this strategy enables selection of TCR-Ts that target antigens and HLAs on the genes that are not lost, thereby overcoming common mechanisms of tumor cell escape from immune attack. Treatment includes standard, non-myeloablative lymphodepletion followed by one or two doses of T-Plex infused 28 days apart. Dose escalation under the interval 3+3 design starts with testing single TCR-Ts in dose levels 1 and 2. Thereafter, TCR-Ts are combined and escalated in dose levels 3 and 4. TCR-Ts currently in the master protocol target PRAME, MAGE-A1 or HPV16 on HLA-A*02:01 or MAGE-A1 on HLA-C*07:02. Additional TCR-Ts added to the ImmunoBank and master protocol go through dose levels 1 and 2 as single therapies before becoming available for multiplexed dose levels 3 and 4. Primary endpoints include safety, feasibility and identifying the recommended Phase 2 dose. Secondary endpoints are rates and durations of response, and exploratory endpoints measure T cell activation and persistence. The goal of the ImmunoBank is to enable up to 80% of patients with common solid tumors to qualify for multiplexed TCR-T therapy.

1901. Trial in Progress: A Phase 1 Trial of TSC-100 and TSC-101, Engineered T Cell Therapies That Target Minor Histocompatibility Antigens to Eliminate Residual Disease After Hematopoietic Cell Transplantation

Monzr Al Malki[1], Alla Keyzner[2], Hyung Suh[3], Uday Popat[4], Saar Gill[5], Yi-Bin Chen[6], Melhelm Solh[7], Joseph Uberti[8], Lohith Gowda[9], Erica Buonomo[10], Yun Wang[10], Jim Murray[10], Gavin MacBeath[10], Debora Barton[10], Shrikanta Chattopadhyay[10], Ran Reshef[11]

[1]City of Hope, Duarte, CA,[2]Mount Sinai Hospital, New York, NY,[3]Hackensack University, Hackensack, NJ,[4]MD Anderson Cancer Center, Houston, TX,[5]University of Pennsylvania, Philadelphia, PA,[6]Massachusetts General Hospital, Boston, MA,[7]Northside Hospital, Atlanta, GA,[8]Karmanos Cancer Institute, Detroit, MI,[9]Yale University, New Haven, CT,[10]TScan Therapeutics, Waltham, MA,[11]Colombia University, New York, NY

Engineered T cell therapies such as CAR-T cell therapies have transformed the treatment of B-cell but not non-B cell hematologic malignancies. Allogeneic hematopoietic cell transplantation (HCT) remains the best curative option for hematologic malignancies but ~40% of patients relapse post-HCT with up to 90% mortality due to residual disease post-HCT. A potential solution is targeting minor histocompatibility antigens (MiHAs) that are homogenously expressed on all hematopoietic cells and are genetically mismatched between donors and patients undergoing HCT. These mismatches enable engineered T cells to selectively eliminate residual patient hematopoietic cells, normal or malignant, leaving donor cells untouched. TScan has developed allogeneic donor derived T-cell products TSC-100 and TSC-101, targeting MiHAs HA-1 and HA-2 respectively, both presented on HLA-A*02:01. By choosing HCT patients who are HLA-A*02:01 positive (>98% of whom are either HA-1 or HA-2 positive) and donors who are either HLA-A*02:01 or MiHA negative, TSC-100 or TSC-101 can potentially eliminate all residual patient-derived hematopoietic cells after HCT, to prevent disease relapse. Study NCT05473910 is a multi-center, multi-arm, non-randomized controlled Phase 1 umbrella study evaluating the feasibility, safety and preliminary efficacy of TSC-100 and TSC-101. Inclusion criteria include adults with AML, MDS or

ALL eligible for reduced intensity conditioning-based haploidentical donor transplantation from HLA or MiHA mismatched donors. HLA-A*02:01-positive patients undergo HA-1/ HA-2 testing and are assigned to either TSC-100 or TSC-101 treatment arms in addition to HCT. HLA-A*02:01-negative patients in the control arm receive HCT alone. Upon count recovery after HCT, patients in treatment arms receive either TSC-100 or TSC-101, administered as single or two doses. Primary endpoints include adverse event profiles and dose limiting toxicities. Secondary endpoints include relapse rates, disease-free survival and overall survival. Exploratory endpoints include surrogates of efficacy such as donor chimerism rates and kinetics and minimal residual disease (MRD) rates. Donor chimerism is measured by standard STR-based and novel high-sensitivity NGS-based assays to quantify residual patient-derived hematopoietic cells. MRD is measured before and after HCT using flow cytometry and NGS. Together, these assays measure elimination of residual patient hematopoietic cells, malignant or normal, and could provide early evidence of biological activity.

1902. Clinical Validation and Mechanistic Insights of ThisCART: A Novel Allogeneic Platform Enhancing CAR-T Signaling via Intracellular TCRαβ/CD3 Retention

Jun Li[1], Lei Xue[2], Sujun Li[2], Ling He[1], Kaichun Liu[1], Rong Jin[1], Tao Wang[1], Yinhang Zhang[1], Shanshan Chen[1], Yulian Gao[1], Dan Liu[1], Liyun Yang[1], Xingbing Wang[2]

[1]Fundamenta Therapeutics, Suzhou, China,[2]The First Affiliated Hospital of USTC, AnHui, China

Background: Current allogeneic CAR-T cell therapies face challenges with post-infusion expansion and efficacy. In response, we developed ThisCART, a non-genetic editing, allogeneic platform that significantly enhances CAR-T cell expansion and efficacy. This is achieved through a novel approach of intracellular retention of membrane proteins, reducing surface expression of TCRαβ/CD3 complexes and HLA-I molecules. Our lead product, ThisCART19, has demonstrated superior efficacy compared to conventional AutoCART19 in preclinical and early clinical studies for B-ALL. **Methods:** In the clinical trial NCT04384393, we assessed the safety and efficacy of ThisCART19 in patients with B-cell malignancies. Post-lymphodepletion, participants received infusions of ThisCART19 and AutoCART19, both personalized from the patient's own T cells, in equal proportions. Safety and efficacy were the primary endpoints, evaluated on Day 28 post-infusion and throughout extended follow-up. Mechanistic insights were gained through comprehensive multi-omics studies, including RNAseq, whole-cell proteomics, and phosphoproteomics. **Results:** ThisCART19 showed enhanced expansion and an improved safety profile in vivo. As of 01 January 2024, Two patients diagnosed as relapse/refractory acute B cell leukemia (R/R B-ALL) were enrolled in the clinical trial. Pt1 is a 55yo male with Ph-positive B-ALL (36% blasts) who relapsed after multiagent chemotherapy and TKIs. Pt2 is a 16yo male with CNS leukemia (78.5% blasts) after multiagent chemotherapy. No DLTs or ICANS were observed. Pt1 experienced Grade 1 CRS that resolved without treatment, and Pt2 experienced Grade 2 CRS that resolved after tocilizumab and corticosteroids. Complete remission with MRD-negative status was achieved by Day 28. Pharmacodynamic analysis indicated a significant expansion of CAR-T cells, predominantly in the CAR+TCR- subset, highlighting autonomous CAR signaling enhancement. Multi-omics analysis identified upregulation in JAK-STAT, PI3K-Akt, and NFKB pathways, suggesting improved cell proliferation, survival, anti-apoptotic activity, and effector function. This molecular profiling provides a mechanistic understanding of ThisCART19A's therapeutic efficacy. **Conclusion:** The ThisCART platform marks a transformative advancement in allogeneic CAR-T cell therapy. By enhancing CAR signaling autonomously through a unique intracellular retention of TCRαβ/CD3 complexes, ThisCART has shown promising safety and efficacy in early clinical trials. Its potential in reshaping the treatment landscape for hematological and autoimmune disorders positions ThisCART as a crucial development in cellular therapeutics.

1903. Interim Analysis on Neurological Outcomes in Hurler Syndrome Patients Treated with Autologous *Ex Vivo* Hematopoietic Stem Cell Gene Therapy

Giulia Consiglieri[1], Francesca Fumagalli[1], Francesca Tucci[1], Chiara Filisetti[1], Matilde Cossutta[1], Stefano Scarparo[1], Marina Sarzana[1], Cristina Puricelli[1], Francesca Ciotti[1], Maurizio Barbera[1], Silvia Darin[1], Rossella Parini[1], Cristina Baldoli[1], Bernhard Rudolf Gentner[2], Alessandro Aiuti[3], Maria Ester Bernardo[1]

[1]San Raffaele Telethon Institute for Gene Therapy, Milano, Italy,[2]SR-TIGET, Lausanne, Switzerland,[3]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Milan, Italy

Introduction: Early treatment with allogeneic haematopoietic stem cell transplantation (HSCT) has been demonstrated in many cases to prevent deterioration of cognitive function in Mucopolysaccharidosis type I Hurler (MPSIH) patients. Nevertheless, HSCT is not able to fully address central nervous system (CNS) manifestations, leaving patients with residual disease burden (doi: 10.1016/j.ymgme.2021.03.002). The neurological outcomes of 8 MPSIH patients enrolled in a phase I/II study (NCT03488394) of autologous *ex vivo* hematopoietic stem cell gene therapy (HSC-GT, also known as OTL-203) were evaluated up to 4 years after treatment. *Methods*: IDUA activity, dermatan (DS) and heparan sulphate (HS) levels were measured in cerebral spinal fluid (CSF) at baseline and at different timepoints after HSC-GT. Ages of achievement of motor and language milestones were compared with the window of achievement defined by the World Health Organization (WHO) in healthy children. Gross and fine motor skills were analyzed using the Peabody Developmental Motor Scales, while measurement of cognitive functions was evaluated by the Bayley Scales of Infant and Toddler Development and the Wechsler Preschool and Primary Scale of Intelligence. Typical MRI brain abnormalities were assessed by a specific neuroradiologic scoring system previously developed for MPSIH patients treated with HSCT. *Results*: From 3 months after GT, IDUA activity became detectable in CSF and persisted over time, accompanied by local reduction of both DS and HS with undetectable DS in 7/8 patients at +4 year-follow-up. Motor and language milestones were accomplished in all patients at the latest follow up, either at a normal or delayed time for age, except for one patient who suffers

from behavioral issues preventing the subject from demonstrating his full neurocognitive abilities, determined to be unrelated to treatment. Regarding motor abilities, we observed a stability of normality or a gain of normality in 5/8 patients in terms of total motor quotient (TMQ) and gross motor quotient (GMQ) at last follow-up, while fine motor quotients (FMQ) remained or became normal after treatment in 7/8 patients. After HSC-GT 7/8 patients continued to gain cognitive skills in line with typically developing children. All patients experienced an improvement or stabilization of the brain MRI scores with a significant reduction of the typical brain abnormalities. Compared to baseline, brain MRI images at last follow-up showed a meaningful reduction of multiple enlarged perivascular space and of subarachnoid space enlargement, regression of faint white matter alterations and normal progression of myelination. *Conclusions*: This preliminary neurological analysis suggests biochemical detoxification in the CSF and initial clinical response in terms of motor and cognitive abilities up to 4 years after treatment, except for one patient with behavioral issues preventing him from demonstrating his full neurocognitive abilities, determined to be unrelated to treatment. CSF detoxification suggests migration of gene-modified cells across the blood brain barrier and local engraftment in the CNS. Neuroradiological modifications are consistent with biochemical and clinical outcomes. A longer follow-up and a prospective comparison with HSCT are needed to draw definitive conclusions on the effect of HSC-GT on the typical neurological manifestations of MPSIH.

1904. Non-Neurological, Non-Skeletal Outcomes After Autologous Hematopoietic Stem Cell Gene Therapy in Hurler Patients: Retrospective Comparison with Allogeneic Hematopoietic Stem Cell Transplantation

Francesca Tucci[1], Maria Luz Uria Oficialdegui[2], Giulia Consiglieri[1], Matilde Cossutta[1], Chiara Filisetti[1], Luca Galimberti[1], Mariana Priviero Spiridon[1], Maria Pia Manitto[1], Carla Butera[1], Roberto Santangelo[1], Giulia Danè[1], Chiara Camesasca[1], Silvia Darin[1], Mireia Del Toro[2], Cristina Diaz de Hereida[2], Rossella Parini[1], Alessandro Aiuti[3], Maria Ester Bernardo[1]

[1]San Raffaele Telethon Institute, Milano, Italy,[2]Hospital Universitari Vall d'Hebron, Barcelona, Spain,[3]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Milan, Italy

Introduction: Preliminary results of a phase I/II study (NCT03488394) of autologous *ex vivo* hematopoietic stem and progenitor cells gene therapy (HSC-GT) in 8 Mucopolysaccharidosis type I Hurler syndrome (MPSIH) patients demonstrated superior metabolic correction compared to allogeneic haematopoietic stem cell transplantation (HSCT). Nevertheless, HSC-GT effects on corneal clouding (CC), hearing loss (HL), carpal tunnel syndrome (CTS) and cardiac involvement have not been extensively reported. We analysed for the first time these outcomes after HSC-GT and performed a retrospective comparison with an external cohort of MPSIH patients undergoing HSCT. *Methods:* MPSIH patients who received HSC-GT (N= 8) were compared to retrospectively collected data from MPSIH patients treated with HSCT (N= 9) in Vall d'Hebron Hospital, Barcelona. Patient and transplant characteristics are reported in Table 1. 2/9 HSCT patients experienced primary graft failure and required a second HSCT. CC was assessed using clinical grading score (Fahnehjelm KT, et al. Acta Opthalmol 2012 for HSC-GT), HL was evaluated using brainstem auditory evoked responses. Cardiac function was studied by echocardiography, CTS by median nerve electroneurography. HSC-GT patients were followed-up for a median of 4 years (range: 4-5), while HSCT patients for a median of 15 years (range: 4-21). To enable appropriate comparison, the analysis was performed within the same time window (4-5 years after treatment). *Results:* At baseline, all subjects had evidence of mild to moderate CC. Of HSC-GT patients, 3/8 achieved CC resolution and 5/8 had mild CC at last follow-up. In HSCT cohort, no patient experienced CC resolution with all showing moderate CC at 5-year after HSCT. In HSC-GT cohort at baseline, 50% of patients had mild to severe HL. An improvement in most of the patients was observed with 4/7 showing normal hearing function at last follow-up. In HSCT cohort, 7/9 had mild or moderate HL at baseline with an improvement at 5-year follow-up as 4/9 showed normal hearing function. ENT surgery incidence and timing after treatment was comparable in the 2 cohorts. In HSC-GT-cohort none of the patients developed severe cardiomyopathy or valvular disease, while in HSCT-cohort 2/9 patients experienced progression of valvular insufficiency, not requiring valve replacement. One HSC-GT patient underwent CT surgery before treatment, and another 6 months after HSC-GT due to pre-existing severe damage before treatment. The remaining patients did not develop CTS. In HSCT cohort 7/9 patients developed CTS, requiring surgery at a median of 3.7 years post-HSCT. *Conclusion:* Our results indicate stability or improvement of CC, HL, cardiac manifestations and CTS in MPSIH patients after HSC-GT up to 4-year after treatment. A longer follow-up and a prospective comparison with HSCT patients are required to draw definitive conclusions on the efficacy of HSC-GT in addressing these disease manifestations.

Table 1: Patient and transplant characteristics of the HSPC-GT and HSCT cohorts.

	HSPC-GT	HSCT
Number of patients (M/F)	8 (6/2)	9 (5/4)
Median age at treatment, months (range)	23.5 (14-34)	25 (17-29)
Type of conditioning	Busulfan + Fludarabine§	Busulfan + Cyclophosphamide/Fludarabine
Median enzyme level pre treatment (range)	0.3 (0-3) µmol/L/h	0.02 (0.01-0.08) nmol/h/mg proteins
Median enzyme level post treatment (range)	101.3 (40.7-145.1) µmol/L/h at 4 years after HSPC-GT	5.4 (4.6-11.3) nmol/h/mg proteins at 5 years after HSCT
Median follow-up, years (range)	4 (4-5)	15 (4-21)

§ also received rituximab as part of the pre-transplant regimen
For HSC-GT, reference values for IDUA in DBS stratified by age: 6 months to <6 years of age: 3.81-35.04 µmol/L/h; 6 to 18 years: 2.27-22.56 µmol/L/h. For HSCT patients IDUA on Leukocytes: normal values 1.6-8.7 nmol/hour/mg protein

1905. Lentiviral Haematopoietic Stem Cell Gene Therapy for Late Juvenile Metachromatic Leukodystrophy

Valeria Calbi[1], Vera Gallo[1], Fabiola De Mattia[1], Alberto Zambon[2,3], Salvatore Recupero[1], Elena Sophia Fratini[1], Alessandra Clerici[1], Francesco Morena[4], Sabata Martino[4], Jean Brooks[5], Alan Richardson[5], Laura Cambpell[5], Maria Ester Bernardo[1,3], Alessandro Aiuti[1,3], Francesca Fumagalli[1]

[1]San Raffaele Telethon Institute for Gene Therapy (SR-TIGET), Milano, Italy,[2]Neurology & Neurophysiology Unit, IRCCS San Raffaele Scientific Institute, Milano, Italy,[3]Vita-Salute San Raffaele University, Milano, Italy,[4]Department of Chemistry, Biology and Biotechnologies, University of Perugia, Perugia, Italy,[5]Orchard Therapeutics (Europe) Limited, London, United Kingdom

Background Metachromatic Leukodystrophy (MLD) is a rare, disabling and fatal lysosomal storage disease. Atidarsagene autotemcel (arsa-cel) is a hematopoietic stem cell (HSC) gene therapy (GT) and consists of autologous CD34+ cells transduced ex vivo with a lentiviral vector encoding for the human ARSA gene, infused after intravenous myeloablative busulfan conditioning. Arsa-cel has shown favorable long-term outcomes in early-onset MLD; however, its risk-benefit profile in late onset variants, in particular the Late Juvenile subtype (LJ), has not yet been investigated. **Methods** This ongoing phase III trial (NCT04283227) is investigating the safety and efficacy of arsa-cel in pre-symptomatic or early-symptomatic (able to walk independently and without cognitive decline) LJ-MLD patients (expected or actual symptom onset between 7th and 17th birthday). Here we report preliminary engraftment and safety data from an *ad hoc* interim analysis. **Results** As of January 2024, all 6 patients planned in the protocol have been enrolled and treated. Four patients were pre-symptomatic and 2 were early-symptomatic. Before enrolment in this study, one patient underwent allogeneic HSC transplant; however, she experienced autologous reconstitution with absence of donor chimerism, enabling eligibility for HSC-GT. Median age at GT was 10.4 years (range 2.7-15.5 years). Stem cell source was mobilized peripheral blood. After transduction, the infused drug product median dose was 23.1x10^6 CD34+cells/kg (range 16-28.94x10^6 CD34+cells/kg) with a median vector copy number (VCN)/cell of 3 (range 2-5).All patients received myeloablative conditioning with busulfan with a final cumulative target area under the curve of 85 mg*h/L. After a median follow-up of 15.4 months (range 0.2 to 21.8 months) all patients are alive with no treatment-related serious adverse events. The type and nature of adverse events were consistent with the known safety profile of busulfan. There were no malignancies, no evidence of abnormal clonal expansion or replication-competent lentivirus, and no evidence of immune response against ARSA enzyme. All 5 patients with available post-treatment data achieved normal hematological reconstitution and engraftment of transduced cells in line with previous experience in early-onset MLD. Median time to neutrophil and platelet engraftment was 33.0 days (range 26-42) and 26 days (range 25-46), respectively. At 90 days post-treatment, median VCN in peripheral blood mononuclear cells (PBMCs) and CD15+ cells were 0.49 and 0.82 copies/cell, respectively; the percentage of transduced bone marrow progenitors ranged between 23.81 and 81.25%. ARSA activity was restored and reached supranormal levels in PBMCs and restored to normal levels in cerebrospinal fluid (CNS pharmacodynamic efficacy measure) at 6 months post-treatment. **Conclusion** This analysis demonstrates that the initial results on engraftment, ARSA activity, safety, and drug product characteristics for arsa-cel in LJ-MLD were similar to those reported in early-onset MLD.

1906. Clinical Efficacy and Safety of Gene Therapy for Transfusion-Dependent β-Thalassemia Patients with Quick Engraftment of Genetically Modified Hematopoietic Stem Cells

Zhen Gao[1,2], Jinqi Huang[3], Liwei Fang[1,2], Yuming Zhang[3], Lijun Liu[1,2], Zhexiang Kuang[1,2], Jiali Sun[1,2], Dehui Zou[1,2], Yao Zou[1,2], Qiang Li[1,2], Xiao Yu[1,2], Hong Pan[1,2], Weiwang Li[1,2], Lele Zhang[1,2], Liang Liang[3], Xiaoqian Li[3], Zhonglu Ye[3], Bin Lin[3], Jingzhi Ma[3], Jingyu Zhao[1,2], Anni Wang[1,2], Jing Xu[1,2], Weiwei Wang[1,2], Liyun Li[1,2], Yanjie Liu[1,2], Jin Dong[1,2], Chun Xu[1,2], Youren Tong[4], Haoquan Wu[4], Jun Shi[1,2]

[1]Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College, Tianjin, China,[2]Tianjin Institutes of Health Science, Tianjin, China,[3]Department of Hematology, The Affiliated Hospital of Guangdong Medical University, Zhanjiang, China,[4]Kanglin Biotechnology (Hangzhou) Co., Ltd., Hangzhou, China

Introduction Clinical evaluation of autologous hematopoietic stem cell (HSC)-based gene therapies for β-thalassemia major patients have been well-advanced in the US and Europe but not in other parts of the world. **Methods** We conducted non-randomized, single-dose, open-label clinical trials of HSC-based gene therapy in representative β-thalassemia major patients in China. We treated 17 patients using their own HSCs after transduced *ex vivo* with KL003, a lentiviral vector expressing a functional β-globin gene. We then monitored patients closely for efficacy and safety of KL003 treatment, including stem cell engraftment, hematopoietic reconstitution/recovery, need for blood transfusion, and adverse events. **Results** The median engraftment time for 11 patients with <5,000 μg/L ferritin at enrollment was 13 days for neutrophils (range: 8-15 days) and 19 days (range: 13-28 days) for platelets, respectively. This is a significant improvement compared with much longer engraftment time (i.e. approximately 4 weeks for neutrophils and 6 weeks for platelets) previously reported for autologous HSC-based gene therapies. The median day for platelet counts to reach above 50×10^9/L was 22 days (range: 11-63 days), representing a much quicker platelet recovery compared with 8 weeks reported previously. On the other hand, for a cohort of 6 patients with ≥5,000 μg/L ferritin at enrollment, the median engraftment time was 14.5 days for neutrophils (range: 9-18 days) and 39 days for platelets (range: 19-121 days), respectively. Platelet engraftment was significantly delayed compared with the cohort of patients with <5,000 μg/L ferritin at enrollment (p value = 0.0240). The median day for platelet counts of this group to rebound above 50×10^9/L was 87.5 days (range: 33 days to >231 days), which was significantly delayed (p value = 0.0015) compared with those of patients with <5,000 μg/L ferritin at enrollment. These results may represent a common challenge for treating patients with severe iron overload. Importantly, blood transfusion was no longer needed for 16 of 17 patients with a median day 18 (range:

10-177 days) after treatment, 1 week faster than previously reported. The longest duration without blood transfusion for these patients has been 24 months thus far. The safety profile was consistent with that typical of busulfan-based myeloablation conditioning. There were no severe adverse events associated with KL003 treatment. **Conclusion** Our clinical trial results demonstrate potentially curative outcomes of KL003 in β-thalassemia major patients, including those in poor clinical condition (e.g, with severe iron overload (≥5,000 μg/L ferritin)), which account for the majority of the β-thalassemia major patients in developing countries. In addition, our optimized transduction process of HSCs might have contributed to maintaining stemness of HSCs, which is essential for quick engraftment but often gets lost during *ex vivo* manipulation. This has led to significantly faster engraftment and better efficacy without severe adverse effect. Thus, KL003 could provide a potential functional cure for β-thalassemia major patients.

1907. First-in-Human Use of Platelet-Derived FVIII for Severe Hemophilia a with a History Inhibitors

David A. Wilcox[1,2,3], Lynn M. Malec[1,3], Myriam A. Armant[4], Bryon D. Johnson[5], Lily M. Du[1], Qizhen Shi[1,2,3], Huiqing Xu[5], Lisa J. Duffy[6], Frederic D. Bushman[7], James H. Jerkins[5], Peiman Hematti[8], Ruta Brazauskas[9], Shawn M. Jobe[3], Parmameswaran N. Hari[5], Mary Eapen[5]

[1]Pediatrics, Medical College of Wisconsin, Milwaukee, WI,[2]Children's Research Institute, Children's Wisconsin, Milwaukee, WI,[3]Blood Research Institute, Versiti, Milwaukee, WI,[4]Translab, Boston Children's Hospital, Boston, MA,[5]Medicine, Medical College of Wisconsin, Milwaukee, WI,[6]Indiana University, Gene Therapy Testing Laboratory, Indianapolis, IN,[7]Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA,[8]Medical College of Wisconsin, Milwaukee, WI,[9]Institute for Health and Society, Medical College of Wisconsin, Milwaukee, WI

Introduction: Platelettargeted gene therapy with human factor VIII (FVIII) has established hemostasis in animal models with severe hemophilia A (HEMA) without eliciting inhibitory antibodies. Here we describe feasibility, safety and efficacy in subject 1 treated on a first-in-human phase 1 trial that targets FVIII synthesis and storage within platelet αgranules for delivery at the site of vascular injury for HEMA for longterm hemostasis. **Method:** Eligible are HEMA patients ≥18 years with a history of inhibitors to FVIII (≥0.6 Bethesda Units [BU]/ml). Autologous CD34+ cells transduced with a lentivector encoding the *ITGA2B* gene promoter driving expression of human Bdomaindeleted FVIII in megakaryocytes are infused after reducedintensity cytoreduction with fludarabine and melphalan. Primary endpoints of feasibility and safety are defined as 1) feasibility of the cell manufacturing procedure by the availability of ≥4 x 10^6/kg transduced CD34+ cells with viability ≥70% and undetectable microbial contamination and 2) safety defined as hematopoietic recovery ≤28 days of infusion and absence of ≥ grade 3 toxicity. **Results:** Subject 1 is a 29 year old male with HEMA and a history of inhibitors (2.6 BU/ml). Immune tolerance to FVIII was established with a no inhibitor titer at enrollment. Hemostatic prophylaxis was maintained with emicizumab and recombinant FVIII for breakthrough bleeding. The cell product yielded 6.73 x 10^6/kg viable CD34+ cells post-transduction with a vector copy number (VCN) of 1.16 copies/cell. Biologically active FVIII:C levels were 101.32 mU/10^6 megakaryocytes. ***Primary Outcomes*** showed excellent tolerance of the treatment plan with a cell product meeting release criteria with neutrophil recovery (≥0.5 x 10^9/L) and platelet transfusion independence (≥50 x 10^9/L) achieved at 15 days post-infusion sustained >18 months. Hospitalization duration was 21 days. There was no breakthrough bleeding during collection and hospitalization and there was no unexpected toxicity ≥ grade 1. Emicizumab was discontinued 3.6 months postinfusion after detecting significant VCN in whole blood. ***Secondary Outcomes*** at 18 months follow up showed 1) absence of replication competent lentivirus and inhibitory antibodies, 2) gene integration site analysis with highly polyclonal insertion with no notable integration near oncoassociated genes, 3) whole blood VCN ≈0.27 from 3-18 months posttransplant consistent with FVIII:C detected stabilized at ≈10 mU/10^8 platelets, 4) normal platelet count/function, and 5) *ex vivo* blood clotting parameters observed complete transition from emicizumab to platelet FVIII maintaining hemostasis throughout. Of note, the patient's joint health mobility score improved from 11 pre- to 9 post-transplant, and annualized bleeding rate decreased from 9.6 pre- to 0.0 for entire 18 months post-infusion. **Conclusion:** This report demonstrates feasibility, safety and efficacy in subject 1 with HEMA and a history of inhibitors who received lentivector gene therapy to induce megakaryocytes to synthesize and store FVIII in platelets. These findings extend the potential to treat HEMA patients ineligible for current FDA approved gene therapy.

1908. Outcomes Post Infusions of Autologous HIV-Specific T Cells Targeting Conserved HIV Epitopes in Individuals with HIV on Antiretroviral Therapy (NCT03485963)

Danielle K. Sohai[1], Michael D. Keller[1], Patrick J. Hanley[1], Fahmida Hoq[1], Divyesh Kukadiya[1], Anushree Datar[1], Emily Reynolds[1], Dennis C. Copertino[2], Christopher Lazarski[1], Chase D. McCann[1], Jay Tanna[1], Abeer Shibli[1], Haili Lang[1], Pamela A. Chansky[1], Cecilia Motta[1], Tan T. Huynh[2], Bridget Dwyer[3], Andrew Wilson[3], Rebecca Lynch[3], Talia Mota[2], Winiffer D. Conce Alberto[2], Zabrina L. Brumme[4], Natalie N. Kinloch[4], Conrad Russell Y. Cruz[1], Lynsay Ehui[5], Sarah Henn[5], R. Brad Jones[2], Catherine M. Bollard[3]

[1]Children's National Hospital, Washington, DC,[2]Weill-Cornell Medicine, New York, NY,[3]The George Washington University, Washington, DC,[4]Simon Fraser University, Burnaby, BC, Canada,[5]Whitman-Walker Health, Washington, DC

There are nearly 40 million people living with HIV (PLWH) worldwide. Antiretroviral therapy (ART) suppresses HIV replication to clinically undetectable levels, but ART is not curative because it does not eliminate the latent HIV reservoir. During untreated HIV, infected cells are cleared by an active CD8+ T cell response, which suggests that T cell responses are critical to HIV control. However, viral immune escape mechanisms, including mutations in immunogenic T cell epitopes, can reduce antiviral immune responses. Thus, T cell therapy targeting conserved HIV epitopes is a promising cure strategy for PLWH. In a phase 1 clinical trial, we evaluated the safety, virologic,

and immunologic responses of autologous HIV-specific T cells that were expanded to target conserved epitopes in HIV Gag and Pol as well as HIV Nef (HST-NEETs) in PLWH on ART. Six participants (4M, 2F; 33-57 years) received two infusions ($2x10^7$ cells/m^2 BSA/dose) of autologous HST-NEETs. HSTNEET infusions were well tolerated with seven grade 1 and one grade 2 adverse events that were possibly product related in only 2 participants following infusions. HST-NEET products were predominantly CD3+CD8+ effector memory T cells with variable expression of activation markers. Epitope mapping of the products identified 13 CD8-restricted epitopes, including repeated Nef epitopes in 3/6 participants. Peripheral blood mononuclear cells (PBMCs) were isolated at each follow-up timepoint to evaluate changes in HIV-specific T cell responses, anti-HIV antibody responses, size of the HIV reservoir in CD4+ T cells, and expansion and persistence of HIV-reactive T cell clones pre versus post infusion. HIV-specific T cell responses increased in 4/6 participants post infusions as assessed by IFNγ ELISpot. Anti-HIV gp120 envelope-specific antibody responses were maintained in all participants. Following infusions, we observed decreases in genome-intact HIV proviruses in 3/6 participants and decreases in the defective HIV proviruses in 2/6 participants. TCR profiling of HST-NEET products and followup PBMCs was used to evaluate the expansion and persistence of HIV-reactive T cell clones associated with the HST-NEET products in the participants. TCR profiling of sorted HIV-reactive T cells from the HST-NEET products and participant PBMCs demonstrated polyclonality (median, 9 clones; range, 1-137 clones/sample), where ≤4 T cell clones that were present in the HST-NEETs showed expansion and persistence in participant PBMCs for >45 weeks post-infusion. Two HST-NEET products showed Nef specificity, with expansion and persistence of Nef-reactive T cell clones in participants post infusion. T cell responses targeting a conserved Gag epitope (SPRTLNAWV) in one participant coincided with the expansion and persistence of a high frequency Gag/Pol reactive T cell clone: CASRPGTTYNEQFF. In sum, we report that administrations of HST-NEETs to PLWH without prescribed lymphodepletion were safe. HIV-specific T cell expansion was observed in the peripheral blood of all participants post infusion with decreases in genome-intact HIV proviruses in 3/6 participants. This promising therapeutic approach presents opportunities for combination therapies for HIV cure strategies.