

Investigating pseudotyped lentiviral vectors for gene delivery to the lung

**Thesis submitted in partial fulfilment of the requirements for the degree
of Doctor of Philosophy**



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Abstract

Recombinant lentiviral vectors (LV) are an attractive vehicle for gene delivery to the lung due to their ability to achieve stable, long-term, expression and the opportunity to add glycoprotein pseudotypes for efficient lung cell-targeting. Two LV pseudotypes derived from sialic acid-binding Sendai virus and influenza virus, referred to as F/HN and Rostock respectively, can facilitate efficient *in vivo* transduction of the murine airway and were compared alongside a new influenza-based pseudotype (Shanghai) which, in contrast to F/HN and Rostock, is based on a strain that infects humans. Lectin staining confirmed a sialylated receptor distribution that is different between murine and human lung, with a greater abundance of $\alpha 2,6$ and relative lack of an $\alpha 2,3$ subtype in the human airway, such that transduction of mouse lung is likely a poor predictor of performance in humans. *Ex vivo* transduction of murine precision cut lung slices (PCLS) recapitulated results from *in vivo* experiments. However, PCLS from resected human lung did not contain airways, and therefore cultures of human airway epithelial cells, maintained at an Air-Liquid Interface (ALI), were used to model transduction of the polarised airway epithelium. Different cellular source materials and ALI culture protocols showed differences in the abundance of human sialylated receptor subtypes available, as well as subsequent transduction efficiency by F/HN pseudotyped LV. Importantly, transduction of human ALI cultures was more efficient using vectors based on human as opposed to simian immunodeficiency virus. A greater transduction efficiency was observed for Shanghai pseudotyped LV compared with Rostock but, overall the F/HN pseudotype demonstrated superior efficiency, transducing all human airway cell types characterised. This was consistent with the greater affinity of the F/HN pseudotype for the $\alpha 2,3$ sialylated (N-acetyl)lactosamine) receptor present in all models investigated, and also explained the broad range of pulmonary cell-types (including the repopulating/progenitor cells of murine airway) transduced in all models investigated. These data support the strong translational potential of the F/HN pseudotype.

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Declaration

This thesis has been composed by myself and not used in any previous degree application. All the results presented were obtained by myself and any contributions made by others have been acknowledged in the text. All information from published or unpublished work has been acknowledged by reference or note of personal communication.

List of Abbreviations

γ	Gamma	DMEM	Dulbecco's modified eagle's medium
δ	Delta	DNA	Deoxyribonucleic acid
ε	Epsilon	dNTPs	Deoxynucleoside triphosphates
3'	Three prime end of DNA/RNA	dsDNA	Double-stranded DNA
5'	Five prime end of DNA/RNA	EDTA	Ethylenediaminetetraacetic acid
α	Alpha	EF1α	elongation factor 1 α promoter
AAV	Adeno-associated virus	EGFP	Enhanced fluorescent green protein
ABCD1	ATP-binding cassette-subfamily D	EIAV	Equine infectious anaemia virus
AcMNPV	<i>Autographa californica</i> multicapsid nucleopolyhedrovirus	EMA	European medicines agency
ADA	Adenosine deaminase	EMEM	Eagle's minimum essential medium
AIDS	Acquired immune deficiency syndrome	EVLV	<i>Ex vivo</i> lung perfusion
ALI	Air-liquid interface	F	Fusion glycoprotein
ALL	Acute lymphoblastic leukemia	F/HN	Pseudotype of F and HN glycoproteins from Sendai virus
Amp	Ampicillin	FACS	Fluorescence-activated cell sorting
APC	Antigen presenting cell	FBS	Foetal bovine serum
APOBEC3	Apolipoprotein B editing catalytic polypeptide 3	FDA	U.S Food & Drug Administration
ARSA	Arylsulphatase A	FITC	Fluorescein isothiocyanate
ASL	Airway surface liquid	FIV	Feline immunodeficiency virus
Asn	Asparagine	FPV	Fowl plague virus
ATCC	American type culture collection	FPV/Rostock	H7N1 (A/Fowl Plague Virus/Rostock/8/1934)
ATI	Alveolar type 1	GFP	Green fluorescent protein
ATII	Alveolar type 2	Gluc	Gaussia luciferase
ATP	Adenosine triphosphate	GM	Gene Medicine
AUC	Area under the curve	GP	Glycoprotein
Aw	Airway	H441	Human lung adenocarcinoma cell line
β	Beta	HA	Hemagglutinin glycoprotein
B-ALI	Bronchial ALI cultures derived in-house from HBEC (Lonza)	HBEC	Human bronchial epithelial cells
BLI	Bioluminescence imaging	HBSS	Hank's balanced salt solution
BMS	Biomedical services (unit)	hCEF	CpG-free human CMV enhancer and CpG-free human EF1α promoter
bp	Base pairs	HEK293	Human embryonic kidney 293 (cells)
BSA	Bovine serum albumin	HEK293F	HEK293 cells commercially adapted to FreeStyle media
BST-2	Bone marrow stromal antigen 2	HEK293T	HEK293 cells containing the SV40 T-antigen
BV	Blood vessel	HIV	Human immunodeficiency virus
CAG	Chicken β-actin promoter plus human CMV immediate early enhancer	HN	Hemagglutinin-neuraminidase glycoprotein
CAR	Chimeric antigen receptor	HPAIV	High pathogenic avian influenza virus
Cas9	CRISPR-associated protein 9	hPIV	Human parainfluenza virus
CBA	Chicken β-actin gene promoter	hrs	hours
CC10	Club cell 10-kDa protein	HSC	Hematopoietic stem cells
CD	Cluster of differentiation	HTA	Human tissue act
cDNA	Complementary DNA	hygB	Hygromycin B
Cer	Ceramide	IAV	Influenza A virus
CF	Cystic fibrosis	IDREC	Interdivisional research ethics committee
CF10	10-layer Cell Factory system	IFN	Interferon
CFTR	Cystic fibrosis transmembrane conductance regulator	IHC	Immunohistochemistry
cGAS	Cyclic GMP-AMP synthase	IRES	Internal ribosome entry site
CLL	Chronic lymphocytic leukemia	Kan	Kanamycin
CMP-Sia	Cytidine-5'-monophosphate-sialic acid	LacNAc	N-acetylglucosamine
CMV	Cytomegalovirus (promoter/enhancer)	lacZ	β-galactosidase
CNS	Central nervous system	LB	Lysogeny broth
CO	Codon-optimised	LBB	Lectin blocking buffer
CpG	CpG oligodeoxynucleotides	LDL	Low-density lipoprotein
cPPT	central PPT (sequence)	LMP	Low melting point
CRISPR	Clustered regularly interspaced short palindromic repeats	LPAIV	Low pathogenic avian influenza virus
DAPI	4',6-diamidino-2-phenylindole	LTR	Long terminal repeat

Lux	Firefly luciferase	rSIV	Recombinant SIV-based LV
LV	(Recombinant) Lentiviral vector	RSV	Rous sarcoma virus (promoter)
M1	Matrix-1 capsid protein from IAV	RT	Room temperature (20-25°C)
M2	Matrix-2 proton channel from IAV	SALI	Surfactant ALI
MAI	<i>Maackia amurensis</i> lectin I	SEM	Standard error of the mean
MAII	<i>Maackia amurensis</i> lectin II	Ser	Serine
MDBK	Madin-Darby bovine kidney	ShanghaiN1	Pseudotype of including HA from H7N9 A/Shanghai/2/2013, M2 from FPV/Rostock, and NA from H1N1 A/Puerto Rico/8/1934
MFD	Maximum feasible dose	ShanghaiN9	Pseudotype of including HA and NA from H7N9 A/Shanghai/2/2013, and M2 from FPV/Rostock
miRNA	Micro RNA	Sialidase	Sialic acid-cleaving enzyme (referencing to exogenous in this thesis)
mirT	miRNA target (sequence)	Sialidase A : SA	Sialidase from <i>Arthrobacter ureafaciens</i>
MLD	Metachromatic leukodystrophy	Sialidase S : SS	Sialidase from <i>Streptococcus pneumoniae</i>
MLV	Murine leukaemia virus	SIAT1	a2,6-sialyltransferase 1 cDNA
mRNA	Messenger RNA	SIV	Simian immunodeficiency virus
MucilAir	HBEC-derived ALI cultures from Epithelix Sarl (bronchial)	SIVagm	African green monkey SIV
MX2	IFN-induced myxovirus resistance 2 (MXB)	SIVcpz	Chimpanzee SIV
NA	Neuraminidase glycoprotein	SIVmac	Macaque SIV
NDV	Newcastle disease virus	SIVsm	Sooty mangabey SIV
Neo	Neomycin	SmallAir	HBEC-derived ALI cultures from Epithelix Sarl (bronchiolar)
Neu5Ac	N-acetylneuraminic acid (Sialic acid)	SNA	<i>Sambucus nigra</i> lectin
Neu5Gc	N-glycolylneuraminic (Sialic acid; 'non-human' isoform)	ssDNA	Single-stranded DNA
NGS	Normal goat serum	STING	Stimulator of interferon genes
NONO	Non-POU domain-containing octamer-binding protein	TAE	Tris-acetate-EDTA
NPC1	Niemann-Pick C1 (cholesterol transporter)	TBS	Tris buffered saline
NVAMD	Neovascular age-related macular degeneration	tdTomato	Tandem dimer 'tomato' red fluorescent protein
O/N	Overnight	TEER	Trans epithelial electrical resistance
OBM	Oxford Biomedica batch	TFF	Tangential flow filtration
OCT	Optimal cutting temperature (compound)	Thr	Threonine
OD	Optical density	TIM1	T-cell Immunoglobulin and Mucin domain 1
ORB	Oxford Radcliffe Biobank	TRIM	Tripartite motif-containing protein
AXB	Oxford Biomedica	tRNA	Transfer RNA
PA	<i>Pseudomonas aeruginosa</i>	TSSM	Buffer (20mM Tromethamine, 100mM NaCl, 10mg/mL sucrose, and 10mg/mL mannitol)
PAMP	Pathogen associated molecular patterns	TU	Transducing units
PBS	Phosphate Buffered Saline	UbC	Human ubiquitin C gene promoter
PCLS	Precision cut lung slices	UK	United Kingdom
PCR	Polymerase chain reaction	UKCFGTC	UK Cystic Fibrosis Gene Therapy Consortium
pDNA	Plasmid DNA	USA	United States of America
PDOC	Polidocanol	UV	Ultraviolet
PEI	Polyethyleneimine	v/v	volume: volume
PFA	Paraformaldehyde	vGM	Gene Medicine vector
PPT	polypurine tract (sequence)	VSV	Vesicular stomatitis virus
qPCR	Quantitative PCR	VSVg	Glycoprotein from VSV
rAAV	Recombinant AAV vector	w/v	weight: volume
RBC	Red blood cells	WAS	Wiskott-aldrich syndrome
RD114	Glycoprotein from feline endogenous virus	WAVE	Cellbag bioreactor
rEIAV	Recombinant EIAV-based LV	Wave	GM LV production (batch)
rHIV	Recombinant HIV-based LV (rHIV-1)	WPRE	Woodchuck hepatitis virus (sequence)
RLB	Reporter lysis buffer	WT	Wild-type
RLU	Relative light units	X-ALD	X-linked adrenoleukodystrophy
RNA	Ribonucleic acid	Yrs	Years
ROI	Region of interest	α2,3St	α2,3-sialyltransferase from <i>Pasteurella multocida</i>
Rostock	Pseudotype of including HA and M2 from FPV/Rostock, and NA from H1N1 A/Puerto Rico/8/1934	α2,6St	α2,6-sialyltransferase from <i>Photobacterium damsela</i>
RPM	Revolutions per minute	ΔU3	deletion of the U3 region
RPPH1	Ribonuclease P RNA component H1 (gene)	ψ : PSI	Packaging signal
RRE	Rev response element (sequence)	Ω	Ohm

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1. Chapter 1- Introduction

1.1. Gene therapy

Gene therapy involves the transfer of genetic material into cells either to replace a function that has been lost due to mutation (such as in cystic fibrosis; CF) or give the cell a new function (such as CAR-T therapy; 1.1.2.2). This can be achieved by the delivery of material to inhibit (knock-down), edit, or otherwise influence the expression of genes native to the cell, most traditionally an exogenous gene is introduced into somatic cells (gene addition) to achieve protein expression for therapeutic or prophylactic purposes. This is a particularly attractive option for genetic diseases, to tackle the underlying cause of the disease rather than just the symptoms. The aim is to cure disease with a one-off treatment. With many successful, well-advanced, strategies for introducing genes now available, the specific gene delivery approach needs to be carefully selected. In broad terms, delivery is achieved either using a recombinant virus to vector DNA to cells, or nucleic acid is vectored by physical force such as direct injection, electroporation, or complexing DNA with synthetic (e.g. polymers such as Polyethyleneimine; PEI) or natural (e.g. lipid) compounds (Dunbar et al., 2018; Foldvari et al., 2016).

1.1.1. Non-viral approaches

Non-viral approaches offer some significant advantages over viral systems. The low expense and ease of manufacturing nucleic acid sequences for transfer, combined with the relative safety of physical or chemical delivery methods provide an appealing strategy that mitigates the immunogenic and toxic risks associated with viral systems. Further, these approaches offer the possibility for repeated therapeutic application, a potentially fundamental requirement in the treatment of patients with chronic or unpredictable conditions.

Despite these advantages, compared with viral approaches, success is hampered by low efficiency of delivery and transience of expression. This is despite the many different methods

developed to improve efficacy (reviewed (Ramamoorth & Narvekar, 2015)). Although many possible reasons contribute to this restricted longevity of expression, ranging from degradation of the exogenous DNA to silencing of the promoter, ultimately the DNA transferred typically exists as episomal in the nucleus of transfected cells, and unless an opportunity is provided for genome integration or replication, episomal DNA will be diluted in dividing cells. To overcome this limitation, non-viral approaches are being developed in conjunction with systems such as the sleeping beauty transposon system (Hudecek et al., 2017). Facilitating integration of DNA to achieve longevity, this approach additionally reduces potential for repeated array (concatemer) induced silencing through promotion of monomeric DNA integration reducing the potential for extra-chromosomal concatemer formation (Grabundzija et al., 2010).

The strategies developed towards efficient transfection of DNA and complexes are also benefiting from the now well-established field of gene editing by enhancing the safety and efficiency of delivery of CRISPR/Cas9 editing components (reviewed in L. Li, Hu, & Chen, 2018). However, with limited opportunity to manipulate tropism and enhance delivery, non-viral approaches perhaps remain best suited to applications incorporating concentrated and controlled (*ex vivo*) exposure to the gene transfer agent.

1.1.2. Recombinant viral vectors

Recombinant viral vectors by contrast are considered far more efficient at mediating gene transfer, exploiting the evolutionary mechanisms of wild type (WT) virus to promote uptake by cells and using intracellular machineries for trafficking the delivered gene(s). Many different types of viral vectors have been developed, but the two most commonly adopted in the field of gene therapy are based on Adeno-associated virus (AAV) and Retrovirus.

A small non-enveloped virus with an icosahedral capsid, AAV infects humans, but is unable to replicate without co-infection and initiation of lytic cycle by another virus, and so does not typically result in pathogenesis, and tends to provoke only very mild immune responses.

Infecting both dividing and non-dividing (quiescent) cells, WT AAV has the ability to integrate its genome into AAVS1, a 2kb region on human chromosome 19. Vectors based on AAV are a popular choice particularly for *in vivo* approaches, with many (>13) naturally-occurring capsid serotypes demonstrating alternative cell type tropisms to choose from in order to target disease relevant cells (Zincarelli, Soltys, Rengo, & Rabinowitz, 2008). The broad range in tropism of the various serotypes have been further exploited through shuffling of capsid sequences, generating vectors with alternative efficiencies and tropism (Burger et al., 2004; Koerber, Jang, & Schaffer, 2008). Unlike WT AAV however, the DNA transferred by recombinant AAV (rAAV) vectors does not specifically integrate into AAVS1, but rather persists in the majority of cells as an episome in the nucleus (Kearns et al., 1996). This can result in persistent transgene expression in cells that are not proliferating, though interestingly, this is achieved in part by concatemer formation (Duan et al., 1998). However, a major limitation of rAAV vectors is that the length of DNA that can be delivered is limited to ~5kb. Additionally, though rAAV vectors have a record for generating lower immune response versus many viruses currently used as vectors, pre-existing immunity is a recognised limitation in a considerable percentage of the population (Verdera, Kuranda, & Mingozzi, 2020). Furthermore, without integration, long-term expression, as with most non-viral approaches, is limited to the life span of the cells targeted. One advantage of this non-integrating approach is that transgene expression remains uninfluenced by the chromatin structure and transcriptional activity of the targeted cell and; furthermore, this also mitigates the reciprocal risks of influencing cellular transcription upon integration. Overall, rAAV remains an attractive vector where transgene sequence length allows, and cells to be targeted are post mitotic.

In stark contrast to rAAV, retrovirus-based vectors offer the potential for integration of a transgene (up to 8kb) facilitating transmission to all daughter cells of those targeted. The retroviridae family includes seven genera of enveloped viruses carrying two single stranded copies of a positive sense RNA genome; α , β , γ , δ and ϵ retroviruses, lentivirus and spumavirus.

Upon infection, the genome is reverse transcribed into DNA in the cytosol before transport to the nucleus where the genome is integrated. Although the generation of vectors based on α retrovirus and Foamy virus (spumavirus) has generated some research interest (Everson, Hocum, & Trobridge, 2018; Kaufmann et al., 2013), Υ -retrovirus and lentivirus-based vectors have been most widely investigated.

1.1.2.1. Gamma (Υ) retroviral vectors

Initial developments towards use of a retrovirus-based vector for gene therapy focused on Υ -retrovirus and treatment for conditions such as severe combined immunodeficiency (SCID); this can be caused by genetic mutation in a number of genes, most commonly IL2RG on the X-chromosome (hence this variant is known as X-linked SCID, ~40% cases) encoding the common gamma (Υ) chain vital for the activity of many interleukin receptors. Alternatively, SCID results from mutation in the adenosine deaminase (ADA) gene (~15% cases) resulting in deficiency of this enzyme and accumulation of toxic purine degradation waste products most potently impacting lymphocytes (Flinn & Gennery, 2018). Clinical trials beginning in the early 1990s focused on delivering the ADA gene *ex vivo* to patient T-cells, this was largely successful with some patients no longer requiring ADA enzyme replacement therapy, and no harmful effects of the treatment recorded at follow up (Aiuti et al., 2009). This therapy was subsequently approved by the European Medicines Agency (EMA) in 2016 for patient use and is now marketed under the name Strimvelis™. Recently however, one patient developed leukaemia 4 years following treatment, prompting re-evaluation for safety of this therapy (Orchard Therapeutics, 2020).

Trials to deliver the gene for cytokine receptor Υ to hematopoietic stem cells (HSC) to combat X-linked SCID, did not proceed so smoothly. Although early studies demonstrated a high success rate, uncontrolled T-cell lymphoproliferation was observed in 6 out of 20 patients enrolled, a syndrome that led to the eventual death of one patient that did not respond to treatment for this experimentally induced leukaemia (Check, 2005). It was confirmed by

integration site analysis that the patients that developed lymphoproliferation had done so as a direct result of retroviral vector insertion near proto-oncogenes such as LMO2 (Hacein-Bey-Abina et al., 2003). This exemplifies the risks associated with transgene integration and concerns over the unintended activation of an oncogene, or inhibition of a tumour suppressor gene, have severely hampered use of Υ -retrovirus-based vectors (Bushman, 2007). These findings, prompted further study into the integration site profiles of retrovirus-based vectors, and it is now understood that there are distinct differences between retroviruses in their integration site preferences, with Υ -retrovirus integration concentrated near transcriptional start sites of genes in active transcription (DeRijck et al., 2013; Roth, Malani, & Bushman, 2011). Recognition of this genotoxic risk prompted a shift in focus to alternative lentivirus-based vectors as well as a drive towards reducing the risk of oncogenesis and improving overall safety of the vectors.

1.1.2.2. Lentiviral vectors (LV)

Lentivirus based vectors, although still characterised to integrate with preference for active transcription units, do not target the 5' end of these units and are therefore considered a safer alternative to Υ -retrovirus-based. Instead, recombinant lentiviral vectors (LV) randomly integrate into transcribed regions (Neschadim, McCart, Keating, & Medin, 2007; Schröder et al., 2002). Given the general excess of intronic over exonic sequences in transcriptional units, random integration into transcribed regions by LVs actually manifests itself as a preference to integrate into non-coding intronic sequences (Van Haasteren, Munis, Gill, & Hyde, 2020). This difference between LVs and Υ -retrovirus is attributed to the alternative interactions their respective integrase proteins have with host factors that guide integration (reviewed in Kvaratskhelia et al., 2014). Additionally, LVs, unlike their Υ -retroviral relatives, do not tend to target cell-cycle genes (Montini et al., 2006). LVs also offer stable transduction of both actively dividing and non-dividing cells (Naldini et al., 1996) whereas vectors based on Υ -retrovirus, such

as murine leukaemia virus (MLV), require the targeted cells to undergo mitosis in order to integrate (Roe, Reynolds, Yu, & Brown, 1993).

Crucially, LV have additionally demonstrated potential for repeated administration *in vivo* without inhibition by immune responses (Alton et al., 2017; P. L. Sinn, Arias, Brogden, & McCray, 2008). This is unexpected given that anti-LV antibodies are detected after a single administration, and these are able to neutralise LV *in vitro*. It is unclear how LV evades the neutralisation by these antibodies *in vivo*, however successful transduction on repeated administration could, for example, result from either insufficient antibody levels present in the relevant organ or that the kinetics of LV transduction bypass antibody interaction and inhibition.

LVs have now been established in the clinic for *in vivo* delivery towards the treatment of conditions such as Parkinson's disease (Palfi et al., 2018) and age-related macular degeneration (reviewed in Pecun & Kaiser, 2015). The most success in using these vectors in clinical trials, however, has been in the *ex vivo* transduction of autologous hematopoietic stem cells allowing them (and their progeny) to exert a therapeutic effect upon delivery and potential engraftment back to the patient. Success in the treatment of genetic diseases, such as X-linked adrenoleukodystrophy (Cartier et al., 2009; Eichler et al., 2017), is due to delivery of a functional copy of the mutated gene. Additionally, this approach is also successful in the delivery of a novel, synthetic transgene encoding Chimeric Antigen Receptor (CAR); T-cells modified with such transgenes are able to treat diseases such as acute and chronic lymphoid leukaemia (Frey et al., 2020; Porter, Levine, Kalos, Bagg, & June, 2011). This latter approach relies on the engineered CAR to recognise a protein specific to the cancerous cells and the modified T-cells are thus able to recognise and destroy the cancerous cells. The success of LV in this *ex vivo* approach is facilitated by the controlled exposure to target cells outside the body, allowing efficient and measurable transduction before transfer back into the patient minimising the chances of off target transgene expression or immune response to the vector. Where the cells to be targeted

cannot be treated *ex vivo* the challenge is largely targeting the correct cells efficiently enough *in vivo* to achieve appropriate expression levels of the transgene.

1.2. Development of LV

1.2.1. Human immunodeficiency virus (HIV)

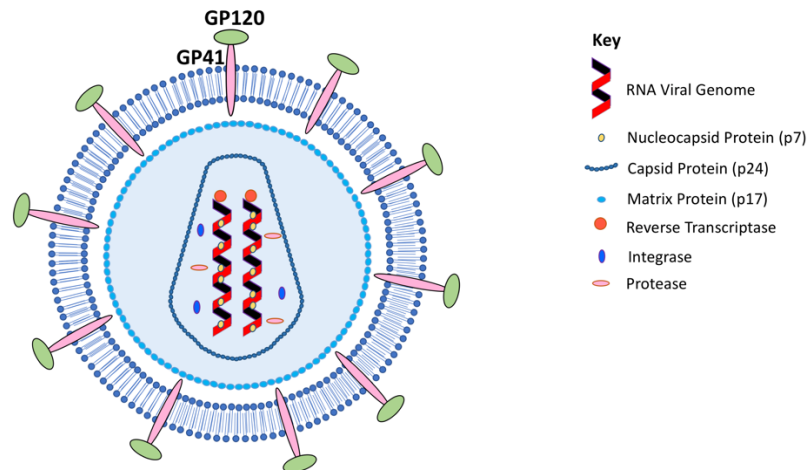


Figure 1- HIV-1 particle schematic. Schematic illustrating the content of HIV. The virus is enveloped by lipid bilayer derived from the host cell which incorporates the viral surface envelope glycoprotein GP120 anchored by non-covalent interaction to the viral transmembrane envelope glycoprotein GP41. These glycoproteins are produced in the host cell via the cleavage of the viral protein GP160 by the cellular protease Furin in the endoplasmic reticulum (Hallenberger et al., 1992). The viral envelope surrounds two copies of the viral RNA genome which is bound to nucleocapsid proteins (p7), encased by a capsid composed of p24 and finally surrounded by matrix proteins (p17). The viral enzymes reverse transcriptase, integrase and protease are also packaged along with a transfer RNA (tRNA) and regulatory and accessory proteins.

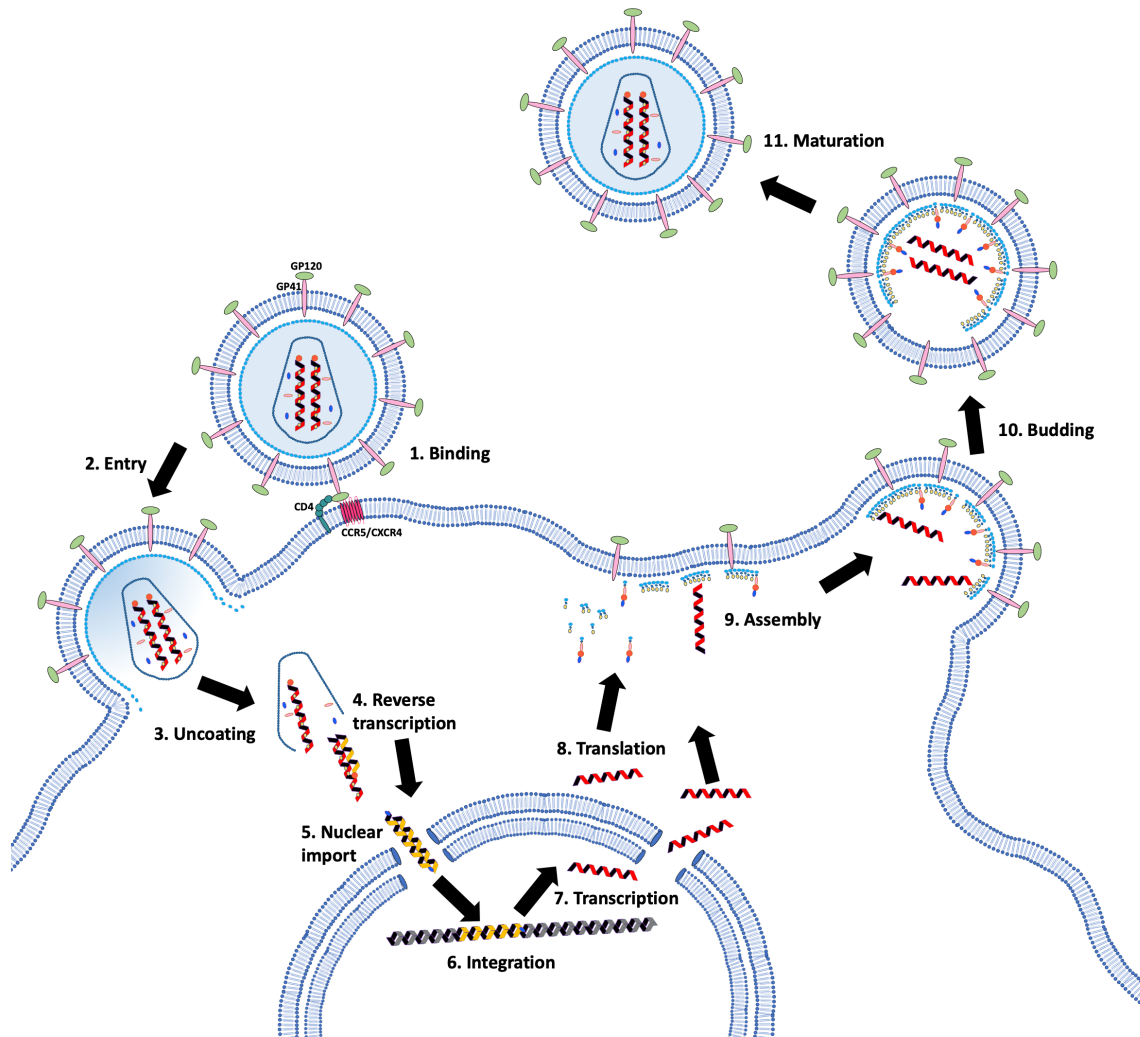


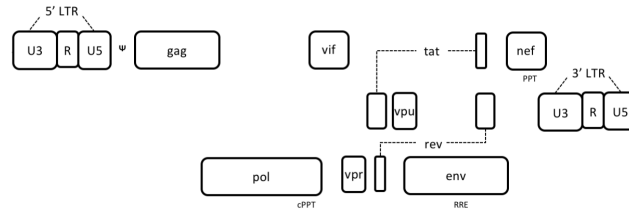
Figure 2- HIV-1 life cycle. The HIV life cycle begins with the binding of the surface envelope glycoprotein (GP120) to the host cell receptor CD4 and co-receptor CXCR4 (1). This interaction facilitates the fusion of the HIV and cellular membrane (2; Entry) and the capsid undergoes uncoating within the cytosol (3). The viral reverse transcriptase then converts the RNA genome to proviral cDNA (4), which is then actively transported (Bukrinsky et al., 1993) (unlike γ -retrovirus which relies on active mitosis (Lewis & Emerman, 1994)) in a pre-integration complex to the nucleus (5; nuclear import) where viral integrase acts to integrate the proviral cDNA into the host genome (6). From the integrated provirus DNA, viral RNA is transcribed (7), is alternately spliced and translated to produce multiple viral proteins and polyproteins within the cell (8), unspliced RNA accumulates and associates with the viral proteins before migration and accumulation at the cell membrane and assembly into new virions (9). Budding of ‘immature’ virions then takes place (10) before viral proteases cleave the viral polyproteins to generate a mature infectious particle (11).

LVs were initially developed from the human immunodeficiency virus (HIV-1), which is a human pathogenic lentivirus that targets the CD4⁺ cells of the immune system and ultimately can cause acquired immune deficiency syndrome (AIDS) through their destruction. Targeting of CD4⁺ cells by HIV is achieved through the attachment of viral glycoprotein GP120 (Figure 1). The life cycle of this lentivirus is illustrated in Figure 2. Being a retrovirus, lentiviruses package two identical copies of the viral RNA genome, each encoding three large genes flanked by the viral long terminal repeat (LTR) promoter sequence. These genes - gag, pol (gag-pol) and env - encode the structural capsid proteins, enzymes and envelope proteins that coat the virus, respectively (Figure 3 & Table 1)(Escors & Breckpot, 2010). As a 'complex' retrovirus however, lentiviruses also encode regulatory and accessory proteins. In the instance of HIV these are tat and rev (regulatory), and vpr, vpu, nef and vif (accessory).

1.2.2. 4th Generation LV

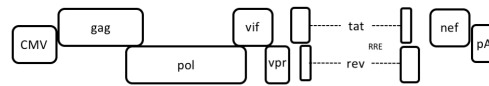
As a result of the huge wealth of research and knowledge regarding HIV-1 and its role in the onset of AIDS, LVs based on HIV-1 were the first to be developed. Following initial development of replication-deficient HIV-1 vectors for use to study the life cycle of the WT virus (Page, Landau, & Littman, 1990), LVs were first described in 1996 (Naldini et al., 1996) for gene delivery, demonstrating the key advantage over γ -retroviral vectors in transduction of non-dividing cells.

HIV-1 genome:

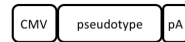


First generation :

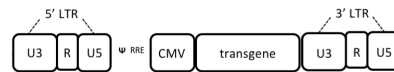
Packaging:



Pseudotype:

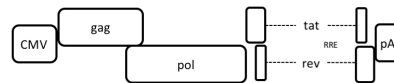


Genome:

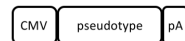


Second generation :

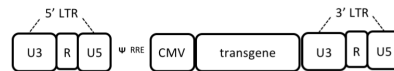
Packaging:



Pseudotype:



Genome:



Third generation :

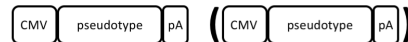
Packaging:



Rev:



Pseudotype:



Genome:

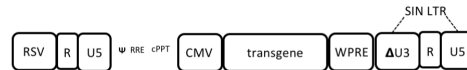


Figure 3- HIV-1 genome and plasmids used to produce first, second and third generation lentiviral vectors (LV). The HIV proviral genome is flanked by two long terminal repeat (LTR) sequences consisting of three segments (U3, R and U5) essential for initiating replication, integration and expression from the transcript. Packaging signal (Ψ ; PSI) facilitates RNA incorporation into particles. A polypurine tract (PPT) and central PPT (cPPT) act as initiation sites for reverse transcription. The gag gene encodes a polyprotein encoding the structural proteins

(p7, p24 and p17) (Figure 1). The pol gene encodes a polyprotein encoding enzymes necessary for various processes in the HIV-1 life cycle (reverse transcriptase, integrase and protease). The env gene encodes the GP160 glycoprotein processed into the surface and transmembrane glycoproteins facilitating entry of HIV-1 (Figure 2). The rev and tat genes both encode 'regulatory proteins'. The tat protein is involved in enhancing LTR transcription. The rev protein binds to the Rev response element (RRE) sequence within the env gene, a step essential for export and accumulation of both unspliced and incompletely spliced RNA transcripts to the cytosol. Accessory proteins encoded by vif, vpr, vpu and nef, support viral replication and counteracting restriction factors in infected cells. In first-generation LVs, genome components are split into three plasmids: The packaging plasmid; encoding transcription of the viral proteins except for those from env and vpu is achieved via the use of a constitutive promoter (the human cytomegalovirus immediate early enhancer and promoter (CMV) or the Rous sarcoma virus promoter (RSV)). The pseudotype plasmid; facilitating expression of the glycoprotein to be enveloped on the LV (pseudotype). Finally, the genome plasmid; encoding the transgene, its promoter and the HIV-1 genome elements required for packaging and processing. In second-generation LVs, a similar three plasmid system is employed except for the accessory genes (vif, vpr and nef) were removed from the packaging plasmid. In third-generation LVs, a constitutive promoter replaces the 5' LTR U3 in the viral genome plasmid, the tat gene is thus no longer required and removed from the system, whilst rev is expressed from a separate plasmid and deletions are made to inactivate the enhancer/promoter with the 3' LTR U3 (Δ U3) of the genome plasmid – this deletion (which is ultimately propagated into both the 5' and 3' LTR sequences found in the provirus of cells transduced by the vector) abolishes LTR transcriptional activity. Additional elements to enhance transduction and expression such as regulatory element of woodchuck hepatitis virus (WPRE) and cPPT sequence are also incorporated to third generation genome plasmids.

In the development of LVs based on HIV-1 (Figure 3), the transgene was first supplied using a genome plasmid adopting the HIV-1 genome elements required to signal packaging and processing. LVs were produced using two co-transfected plasmids; one encoding gag, gag-pol, accessory and regulatory proteins, plus another encoding envelope proteins (pseudotype) (Naldini et al., 1996). In the second generation of LVs the accessory proteins were deleted to reduce the risk of generating a pathogenic recombinant virus *in vivo* as these proteins are

essential for viral replication (Zufferey, Nagy, Mandel, Naldini, & Trono, 1997). In the third generation, safety was further enhanced through engineering the vector LTR to be self-inactivating through incorporation of deletions in the U3 region of the 3' LTR as well as deletion of the regulatory tat gene that is required for activation of the 5' LTR and high level vector RNA expression (Dull et al., 1998; Kim, Mitrophanous, Kingsman, & Kingsman, 1998). Since the proviral 5'LTR is produced by copying the 3'LTR during the viral reverse transcription process, deletion of the U3 from the 3'LTR abolishes promoter activity in both the 5' and 3' proviral LTRs. This reduces risk of any promoter activity on downstream oncogenes from the 3'LTR following integration (Herman & Coffin, 1987) as well as reducing the possibility of recombination events with the LTRs of WT HIV to generate replication competent virus. The removal of tat is facilitated by addition of a constitutive promoter to the genome plasmid (Figure 3) in place of the U3 of the 5' LTR, which in turn facilitate viral RNA expression by the genome plasmid in the absence of tat. Regulatory protein rev is provided using a separate plasmid as it is still required for sufficient mRNA transcript accumulation. The continued optimisation of LV safety profile has been carried out in parallel with efforts to improve efficacy. For example, inclusion of the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE) boosts transgene expression (Zufferey, Donello, Trono, & Hope, 1999), while incorporation of the cPPT sequence enhances vector reverse transcription and thus improves transduction efficiency (Arhel, Souquere-Besse, & Charneau, 2006).

In the fourth generation of LVs, codon optimisation is utilised to minimise homology between the packaging and genome plasmids whilst at the same time aiming to improve translation efficiency/transcript stability of viral gag/pol packaging components, in turn making the vector (at least in some configurations) independent of the rev regulatory protein for sufficient transcript accumulation (Kotsopoulou, Kim, Kingsman, Kingsman, & Mitrophanous, 2000). Additionally, codon optimisation of viral components is widely employed to improve the translation of viral transcripts and enhance titres (Ikeda et al., 2003).

1.2.3. LV from non-pathogenic virus

Despite the number of safety measures implemented in the third and fourth generation LV, mitigating the risks of replication competent or pathogenic virus emerging, interest in the development of vectors based on viruses that are less or non-pathogenic to humans persists, potentially offering added safety and acceptance for progression to the clinic (Munis, 2020). Developed in parallel with HIV-1, these include LV systems based on HIV-2 (E. Poeschla et al., 1998), SIV (Simian immunodeficiency virus) (Nakajima et al., 2000), EIAV (Equine infectious anaemia virus) (Olsen, 1998) and FIV (Feline immunodeficiency virus) (E. M. Poeschla, Wong-Staal, & Looney, 1998). Importantly, each WT lentivirus has adapted to the alternative species/cells in which the respective virus propagates and, as such, the accessory proteins these viruses utilise to combat the restriction factors specific to those cells and facilitate viral replication, differ (Table 1).

Table 1- Simple and complex retrovirus genes.

Genus	Virus	Genes		
		Structural	Regulatory	Accessory
γ-retrovirus (simple)	MLV	gag pol env		
Lentivirus (complex)	HIV-1 /SIVcpz	gag pol env	tat rev	vpr vpu nef vif
	HIV-2 /SIVsm	gag pol env	tat rev	vpr vpx nef vif
	SIVagm	gag pol env	tat rev	vpr nef vif
	EIAV	gag pol env	tat rev	s2
	FIV	gag pol env	rev	orf2 vif

LVs derived from SIV are potentially most useful as the most closely related lentivirus to HIV-1, and due to this homology are next most widely researched. Although we know of at least six different lineages of SIV according to host species, it is clear from phylogenetic analysis that HIV-1 and HIV-2 likely arose from the transmission of chimpanzee SIV (SIVcpz) (F. Gao et al., 1999) and sooty mangabey SIV (SIVsm) (Hirsch, Olmsted, Murphey-Corb, Purcell, & Johnson, 1989) to humans, respectively. Unlike HIV, SIV is non-pathogenic in its natural host; although it

is known to cause disease in alternative simian species. SIV from two simian species have been developed as third generation LVs, one from Macaque (SIVmac - same lineage as SIVsm) (Hanawa et al., 2004) and a second, from African green monkey (SIVagm) (Kobayashi, Iida, Ueda, & Hasegawa, 2003). EIAV and FIV-based LV systems have also been developed, also produced using the split genome approach in the absence of accessory proteins, and confirmed to transduce non-dividing cells (Curran, Kaiser, Achacoso, & Nolan, 2000; Mitrophanous et al., 1999). Use of vectors derived from viruses not adapted to infect humans, to ultimately deliver genes to human cells, may have implications. The respective adaptation of each virus to succeed for efficient replication and transmission in their respective host (reflected by the diversity in accessory genes expressed), should be considered.

1.2.3.1. Restriction factor implications for use of LV

In order for a lentivirus to become established with its host, it has to either adapt to co-exist with its natural host, or generate defence mechanisms to counteract host actions to inhibit the virus. Such adaptations may be specific to its host species and thus the virus may remain vulnerable to 'host restriction' by similar factors in alternate species; these differences in part explain the host range of the native virus. Certain features of the retrovirus and its life cycle are distinct from that of the host cell and thus are prone to be recognized as pathogen associated molecular patterns (PAMP), features including viral processes associated with reverse transcription and particle budding, as well as the specific viral proteins/nucleic acids both delivered to and manufactured within the host cell. Much of the ability of the host to restrict, or defend itself from, lentiviral infection depends on its ability to recognise these processes and associated molecules.

One important example is the family of apolipoprotein B editing catalytic polypeptide 3 (APOBEC3) enzymes, cytidine deaminases found exclusively in mammals that protect themselves from viral infections. They act by interfering with the process of reverse transcription

(F. Guo, Cen, Niu, Saadatmand, & Kleiman, 2006; Mbisa et al., 2007). Resistance to APOBEC3 is facilitated by the accessory protein vif that act to reduce incorporation into viral particles. Alternatively, bone marrow stromal antigen 2 (BST-2) restriction factor, also known as tetherin, inhibits the release of newly formed viral particles from the surface of the cell membrane, such that 'tethered' particles are endocytosed and degraded (Neil, Zang, & Bieniasz, 2008). To overcome this, viral accessory proteins act to degrade or downregulate tetherin expression (vpu (HIV-1) or nef (SIVagm/SIVmac) (F. Zhang et al., 2009)). Both APOBEC3 and tetherin act during, or are dependent on, lentiviral assembly and release, and so although their effects may be applicable to the production of LV, they are unlikely to restrict LV gene delivery. Further, APOBEC3 dependency on packaging, implicated by vif mediated viral escape, indicates irrelevance as a potential barrier to efficient transduction by LV, with an absence of APOBEC3 in the HEK293T cells commonly used for LV production (Piroozmand et al., 2007). These cells are also identified as lacking in expression of BST-2 (Sato et al., 2009).

Another lentiviral restriction factor is SAMHD1 that acts non-specifically to antagonize the reverse transcription process by depleting the dNTP pools required for proviral DNA synthesis (Goldstone et al., 2011). Again, expression of accessory proteins (vpr or vpx) can counter this antiviral restriction (Lim et al., 2012). Conversely, Tripartite motif 5 (TRIM5) protein (and its related family) are examples of cellular restriction factors known to directly interact with viral proteins (Sayah, Sokolskaja, Berthoux, & Luban, 2004; Stremlau et al., 2004). In the case of TRIM5, the viral nucleoprotein complex (VNC) is revealed upon lentiviral particle cellular entry, and TRIM5 interacts with the viral capsid proteins and interferes with the uncoating process. Interestingly, HIV-1 appears to be only weakly restricted by human TRIM5 α (Ganser-Pornillos & Pornillos, 2019; F. Zhang, Hatzioannou, Perez-Caballero, Derse, & Bieniasz, 2006) possibly indicative of HIV-1 adaptation to escape recognition. However, such restriction factors may determine the host specificity of WT virus as they may more potently block a virus from an alternate host species. For example, unlike HIV-1, SIVmac (closely related to SIVsm), HIV-2 and

EIAV are all efficiently blocked by human TRIM5 α (F. Zhang et al., 2006), while HIV-1 is efficiently blocked by simian TRIM5 α (Stremlau et al., 2004). As the expression of some host restriction factors could result in resistance to gene delivery by LV, differences between the lentiviral components used in each vector and the expression of restriction factors in the targeted cells (ultimately human) should be considered in LV development and evaluation. Indeed, known species-specific retrovirus restriction by TRIM5 α was confirmed to translate to restriction of gene delivery using the respective vectors derived (P. L. Sinn et al., 2012). The implications of these restriction factors for LV applications is complicated by the removal of the viral accessory proteins from the third-generation LVs. The great diversity in sequences between primate accessory proteins compared with the structural proteins highlights their importance in host range (McCarthy & Johnson, 2014). Even though PAMPs associated with WT virus infection may be absent or reduced in the process of gene transfer by replication-deficient LV, efficient gene delivery can be restricted in a species-specific manner (Geis et al., 2017; Morrison et al., 2020), with many restriction factors involved, only a few of which are mentioned here.

1.2.3.2. Innate immune response implications for use of LV

The innate inflammatory response of cells to lentivirus may also be involved in the restriction of LV. The cGAS-STING PAMP pathway recognises cytosolic DNA and initiates a cascade of cellular signalling resulting in the expression of type 1 interferons (IFN-I) and inflammatory cytokines. Following LV infection, cGAS-STING recognises, in a sequence specific manner, unpaired guanosine residues within the secondary structure of the stem loop cDNA formed by HIV-1 (Herzner et al., 2015). It has also been shown that cGAS mediates activation of these pathways upon infection of mouse and human cell lines with other retroviruses (MLV and SIV) (D. Gao et al., 2013). Thus, the ability to escape from cGAS-mediated restriction may be another factor that determines the host species range. Different mechanisms for escape may be employed, for instance, vpx accessory protein (HIV-2 and SIVmac only) antagonizes cGAS-STING-mediated NF-

kB signalling by interacting with a specific domain of STING (Su et al., 2019). Furthermore, immune activation in dendritic cells by cGAS against HIV has been identified as co-dependent on Non-POU domain-containing octamer-binding protein (NONO) interaction with a conserved region of the HIV capsid, an interaction that is of higher affinity for HIV-2 (low pathogenicity) than HIV-1 (Lahaye et al., 2018). Such a difference in affinity could contribute to HIV-1 escape from cGAS-mediated restriction in human dendritic cells.

The downstream restriction factors stimulated by the innate immune response are also known to confer virus specificity. For example, activation of interferon signalling results in restriction of virus infection/propagation by upregulating expression of a range of antiviral genes. Specifically, IFN-I signalling restricts SIV in macaques by upregulating expression of numerous restriction factors such as APOBEC3 and IFN-induced myxovirus resistance 2 (MX2, also referred to as MXB)(Sandler et al., 2014). The action of human MX2 is to suppress the lentiviral post entry stage and is dependent on viral capsid protein interaction - demonstrated by an HIV-1 gag sequence mutation/replacement which results in the loss of MX2 sensitivity (Goujon et al., 2013). Such induced restriction factors could also become a determinant of host range restriction, with virus capsid proteins under selection pressure to escape recognition by such factors. In this instance, however, with maximal HIV-1 inhibition and partial inhibition of HIV-2 and SIVmac/SIVagm identified, and further no restriction of EIAV, FIV or MLV, it appears that human MX2 is specific to human and related lentivirus.

With reduced PAMP exposure during LV gene delivery versus WT lentivirus infection, the mounting of an innate immune response to restrict LV transduction may seem unlikely, although, pathways such as IFN-I can act to restrict LV gene delivery (Brown et al., 2007). This innate immune response further complicates and hinders the ability to predict successful translation of an LV for human gene delivery without investigating its effects in human cells. Furthermore, as cell and tissue type-specific expression patterns of restriction factors may be implicated in the cell- and tissue-specific tropism of WT lentivirus they could clearly be

important for LV delivery. For instance, the IFN-I restriction on gene delivery with HIV-based LV observed by Brown et al.,(2007) appeared to be hepatocyte-specific with no restriction observed in related and adjacent kupffer cells. Taken together, such observations on host restriction factors highlights the need to investigate LV delivery in human cell types specific to the proposed therapeutic application.

1.3. LV in the clinic

Approximately 200 active clinical trials are currently using LV (clinicaltrials.gov, 2020), with the majority encompassing *ex vivo* therapeutic approaches utilizing the most established HIV-1 based LV platform (Table 2). These include Kymriah (tisagenlecleucel; CTL019) approved by U.S Food & Drug Administration (FDA) in 2017, whereby T-cells are delivered a gene encoding a CAR to target CD19 common to B-cells in order to treat B-cell acute lymphoblastic leukemia (ALL). The limited number of *in vivo* LV applications reaching the clinic (at least compared with rAAV), represents unfulfilled potential, but also reflects the challenge of use of LV *in vivo*.

Table 2- Exemplar LV clinical trials and products. rHIV-1: recombinant HIV-1 based LV. rEIAV: recombinant EIAV based LV. X-ALD: X-linked adrenoleukodystrophy. WAS: Wiskott-Aldrich syndrome. MLD: metachromatic leukodystrophy. CLL: chronic lymphocytic leukemia. ALL: acute lymphoblastic leukemia. NVAMD: Neovascular Age-related Macular Degeneration. ABCD1: adenosine triphosphate (ATP)-binding cassette-subfamily D. ARSA: arylsulphatase A. CAR-T: chimeric antigen receptor T-cell. FDA: U.S Food & Drug Administration. EMA: European Medicines Agency.

Vector type	Disease	Transgene	Clinical phase	Route of administration	References	Clinical trial/Product name
rHIV-1	β -thalassemia	β -globin	Phase III	<i>Ex vivo</i> (HSCs)	(Thompson et al., 2018)	NCT03207009
rHIV-1	X-ALD	ABCD1	Phase II/III	<i>Ex vivo</i> (HSCs)	(Eichler et al., 2017)	NCT01896102
rHIV-1	WAS	WAS	Phase I/II	<i>Ex vivo</i> (HSCs)	(Ferrua et al., 2019)	NCT01515462
rHIV-1	MLD	ARSA	Phase III	<i>Ex vivo</i> (HSCs)	(Sessa et al., 2016)	NCT01560182, NCT04283227
rHIV-1	CLL	CAR-T	Phase I	<i>Ex vivo</i> (T cells)	(Porter et al., 2011)	NCT01029366
rHIV-1	ALL	CAR-T	FDA and EMA approved	<i>Ex vivo</i> (T cells)	(Frey et al., 2020).	Kymriah (tisagenlecleucel; CTL019)
rEIAV	Parkinson	Tyrosine hydroxylase, AADC, cyclohydrolase 1	Phase I/II	Intracranial	(Palfi et al., 2018)	NCT00627588
rEIAV	NVAMD	Endostatin, angiostatin	Phase I	Subretinal	(Campochiaro et al., 2017))	NCT01301443

1.3.1. Challenges for *in vivo* applications of LV

The use of LV for *in vivo* therapeutic applications requires not only efficient delivery to the relevant cell types, but restricting delivery or expression in off-target cells, avoiding host restriction and minimising immunogenicity against LV components. The highly successful *ex vivo* gene delivery achieved by LV is owed in part to the ease of controlled exposure to the relevant cells, whereas, systemic or even localised *in vivo* delivery typically achieves only transient

exposure of the vector to the desired cells. Achieving efficient *in vivo* delivery may often require large volumes of highly concentrated LV that, regrettably, often also results in significant transduction of off-target cell types. These limitations have encouraged the development and use of LV pseudotypes with efficient and specific cellular tropism.

For treatment of some conditions the therapeutic protein can only be manufactured to be functional in particular cells (Weaver, 1998), as appropriate cellular machinery for post-translational modification or cleavage is required. Although expression of the protein in off-target cell types could be benign, the potential impact is more often unknown and expression of any transgene in excess of the needs of the condition unnecessarily increases the risks of immune response against the transgene product. Thus, methods to restrict delivery or expression in off-target cell and tissue types without compromising targeting efficiency are highly desirable.

The other significant hurdle to *in vivo* delivery is potential for innate or adaptive immune responses to the LV PAMPs introduced, which may result in restriction or neutralisation of the LV. In addition, pre-existing immunity against LV components should be considered beyond limiting application or repeated administration potential; it is speculated that pre-existing acquired immunity may have resulted in the severe inflammatory reaction and death of a patient receiving adenovirus based gene therapy in 1999 (Cichon et al., 2001).

1.3.2. Possible solutions

1.3.2.1. Localised delivery

The few LV clinical trials that involve *in vivo* use of LV have taken advantage of localised delivery approaches. Clinical trials involving EIAV-based LV delivery directly to the central nervous system (CNS) and eye have demonstrated safety, although clinical benefit was modest in the outcomes measured for treatment of Parkinson's disease (Palfi et al., 2018) and neovascular age-related macular degeneration (NVAMD)(Campochiaro et al., 2017).

1.3.2.2. Cell and tissue specific promotion and silencing

Despite potential for localised application *in vivo*, this is not possible for some conditions, where target cells are inaccessible from local routes of administration. Since first generation LV development (Naldini et al., 1996), use of the constitutive promoter/enhancer from cytomegalovirus (CMV) (Foecking & Hofstetter, 1986) has been a popular choice with high levels of expression demonstrated in cells derived from various mammalian species and tissue types. Promoter selection, however, is an opportunity to use a cell type-specific promoter to control/restrict expression to disease-relevant cells. For instance, the creatine kinase gene promoter has been used to selectively drive transgene expression in muscle cells that readily express the transcription factors to interact with this promoter (Himeda, Chen, & Hauschka, 2011).

Another safeguard against off-target expression that could be used in combination with a cell- or tissue-specific promoter is the incorporation of a microRNA target sequence to restrict expression at a post-transcriptional level. Typically added to the 3' untranslated region of the transgene cassette, such sequences are ideally targeted by microRNAs only present in cells in which transgene expression is undesirable. This approach is regularly adopted for the prevention of transgene expression in antigen presenting cells (APCs), and is achieved by incorporating a sequence complementary to mir-142-3p within the LV transgene cassettes (Brown, Venneri, Zingale, Sergi, & Naldini, 2006). Preventing expression in APCs following *in vivo* systemic delivery appears key to avoiding an unwanted immune response against the transgene product (De Geest, Linthout, & Siré Collen, 2003).

1.3.2.3. Pseudotyping

A potentially powerful solution to the challenge of using LV for *in vivo* delivery is the ability of these enveloped vectors to be interchangeably pseudotyped – allowing selection of LVs with broad or targeted cell tropism and efficiency. Pseudotyping has been confirmed as a powerful

tool to direct tropism of LV in various settings such the CNS (Watson, Kobinger, Passini, Wilson, & Wolfe, 2002) and airway (S. Y. Chan, Speck, Ma, & Goldsmith, 2000). WT HIV targets CD4+ T cells using the gp160 which is cleaved to yield the surface (gp120) and transmembrane (gp41) subunit glycoproteins (Figure 1). It was established early in the development of LV that the env gene glycoproteins can be exchanged for heterologous glycoproteins, creating a pseudotype with alternative tropism (Naldini et al., 1996); this flexibility in choice of pseudotype offers the opportunity to target LV to specific cells with greater efficiency. LV is perhaps most commonly pseudotyped using the G glycoprotein from VSV (VSVg) likely due to its stability in withstanding LV particle concentration processes such as ultracentrifugation (Burns, Friedmann, Driever, Burrascano, & Yee, 1993); as well as its broad and efficient tropism for cells due to its use of the ubiquitous cellular LDL receptor for transduction (Finkelshtein, Werman, Novick, Barak, & Rubinstein, 2013). VSVg pseudotyping of LV facilitates reliable production of high titre and concentrated LV preparations ideal for both *ex vivo* and *in vivo* applications and when combined with control measures such as cell-specific promoters and mirT sequences, high cell type-specificity of transgene expression can be achieved.

Pseudotyping LV in order to direct targeting of particles *in vivo* is a potentially powerful tool, exploiting the diversity in WT virus evolution to bind specific cellular targets (reviewed in Joglekar & Sandoval, 2017). However, efficient pseudotyping of retroviral vectors with heterologous glycoproteins is not guaranteed, even when using glycoproteins from within retroviridae. For example, while pseudotyping with feline endogenous virus RD114 glycoprotein is efficient on MLV-based vectors (Cosset, Takeuchi, Battini, Weiss, & Collins, 1995), it is very inefficient with SIV-based LV (Nègre & Cosset, 2002; Sandrin et al., 2002). The observation that the MLV glycoprotein can efficiently pseudotype SIV led to the construction of an MLV/RD114 glycoprotein hybrid (Sandrin, Muriaux, Darlix, & Cosset, 2004), where the MLV cytoplasmic tail facilitates active endosomal recruitment and efficient pseudotyping.

How and where retrovirus particle components assemble is not fully understood and, in particular, how the pseudotyping glycoproteins are incorporated. One proposed mechanism relies on passive incorporation of glycoproteins into the LV particle as a pseudotype. This passive approach is supported by 'natural' pseudotyping that is observed upon dual infection of cells with alternative enveloped viruses (Zavada, 1982). This passive approach is assisted by enrichment of envelope glycoproteins on lipid raft microdomains, which can occur by serendipity at the site of virus budding, similar to the proposed mechanism explaining seemingly nonspecific interactions between envelope and core virus proteins (Briggs, Wilk, & Fuller, 2003). Importantly, truncated retroviral glycoproteins shortened at the cytoplasmic tail incorporate to HIV-1 particles, supporting this passive incorporation model as long as the cytoplasmic domain does not interfere with particle assembly (Mammano, Kondo, Sodroski, Bukovsky, & Göttlinger, 1995). Interestingly, It has also been shown that several enveloped viruses assemble their envelope glycoproteins by microdomain concentration (Pickl, Pimentel-Muiños, & Seed, 2001).

1.4. Pseudotypes for lung cell targeting

1.4.1. Lung as a target for LV gene delivery

The lung facilitates gaseous exchange for respiration, which takes place in the lung parenchyma, at the end of the conducting airways. During breathing, air enters the lung through the (proximal) trachea and passes through the branching airways (bronchi, bronchioles and the smallest, most distal terminal bronchioles) before reaching alveoli, the site of gas exchange. The proximal airway of the trachea and bronchi comprise a pseudostratified, ciliated epithelium of many different specialised cell types, often categorised by their ciliary, secretory or progenitor roles (Figure 4). The many ciliated cells are interspersed with secretory cells such as goblet cells, expressing mucins that make up the mucus lining. Basal cells, often distributed at the basement membrane of the epithelium, act as the primary progenitor cell type with the pluripotency to differentiate upon stimulus, such as injury, to replenish the epithelium (Rock et al., 2009).

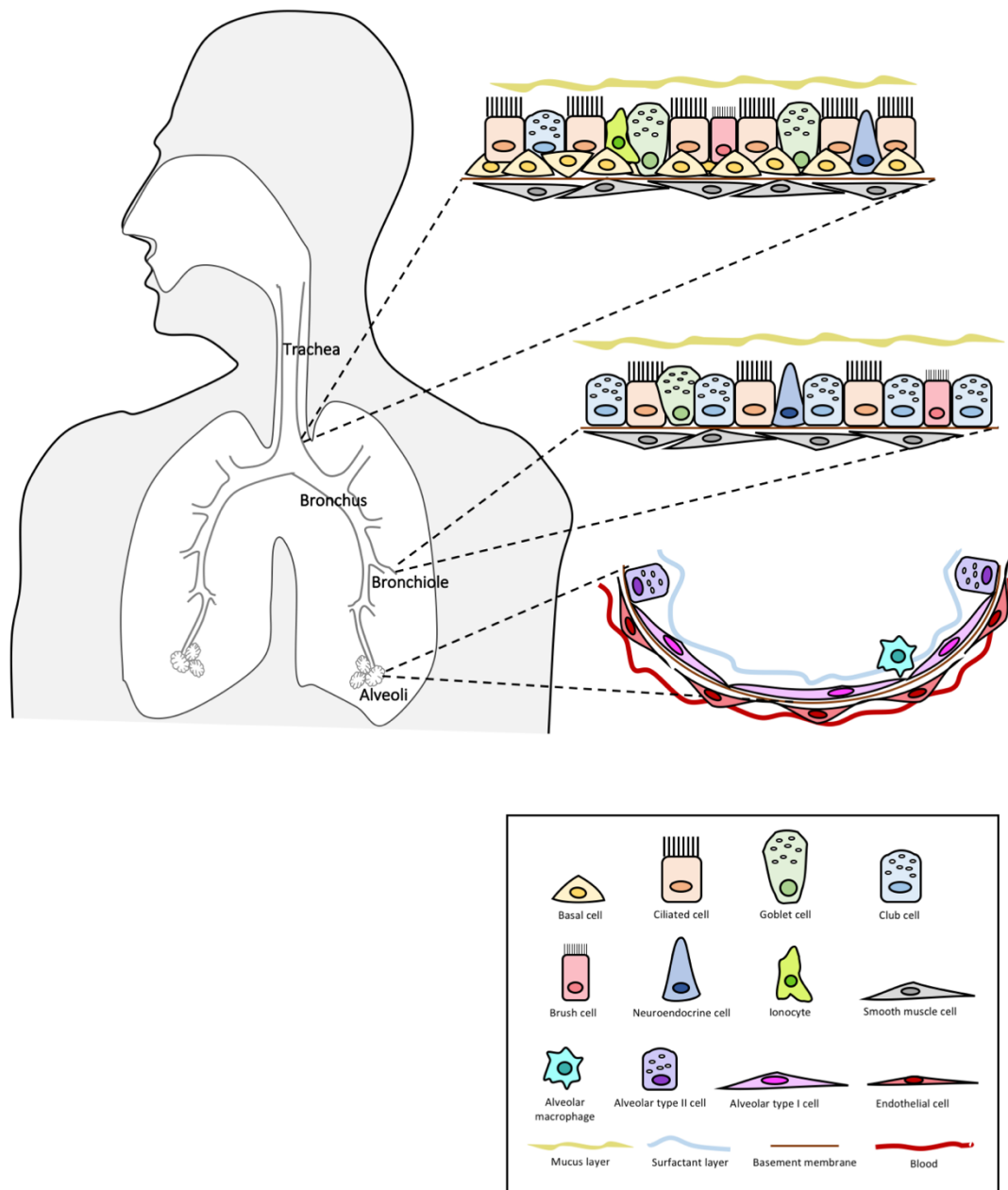


Figure 4- Pulmonary epithelium composition from proximal conducting airways to distal alveoli. Lung cell types may vary in composition and abundance in different compartments of the lung to reflect and change to reflect requirements of air conduction, maintenance, defence and gas exchange at each site.

In the more distal conducting airways, basal and goblet cell frequency is reduced (Figure 4), and replaced by a greater frequency of an alternative secretory cell type named club cells (Boers, Ambergen, & Thunnissen, 1999), previously known as Clara cells (Winkelmann & Noack,

2010). These cells, most abundant in the terminal bronchioles, secrete components of the airway surface liquid, and are involved in a variety of functions such as immunomodulation, degradation of harmful products and toxins, and surface tension regulation. Club cells have also been identified as multipotent, displaying a similar role in regeneration and repair in the small airways, just as basal cells do in the larger airways. Club cells are able to differentiate into both ciliated and non-ciliated cells. Numerous subsets of these airway epithelial cells have been further characterised across the airway epithelium, each with unique expression profiles. Of particular interest, are some rare cell types with distinct roles, including the brush (tuft) and neuroendocrine cells, which mediate inflammation and secrete hormones; and the pulmonary ionocyte, which regulates osmolarity (Montoro et al., 2018).

The predominant cells lining the parenchymal alveoli are the alveolar type 1 (ATI) and 2 (ATII) cells, specialised to facilitate gas exchange and secrete surfactant proteins to the lining of the alveoli respectively. According to lineage tracing experiments, ATII cells also serve as the progenitor to terminally differentiated ATI cells (Treutlein et al., 2014). Lining the alveoli basolateral to the pulmonary epithelium basement membrane, the endothelial cells make up the alveolar-capillary barrier and facilitate gas exchange to circulating red-blood cells. Also resident in the parenchyma are alveolar macrophages which are responsible for the detection of pathogens and mediating the host immune response. The cells lining the respiratory tract all the way from the nares to the site of gas exchange are all therefore accessible by inhalation; and as a candidate organ for *in vivo* LV gene therapy, the lung has long been considered ideal with potential to use localised, non-invasive inhalation delivery to directly access cells relevant to numerous hereditary and acquired lung conditions. There are a number of challenges in targeting these cells, however, as the lung epithelium has evolved to clear incoming particulates and to resist viral infection. Specifically, the specialised mucociliary phenotype of the airway epithelia captures and removes material in the airway lumen, preventing infection by opportunistic pathogens, a natural defence mechanism restricting apical airway transduction.

This protective barrier is particularly challenging in cystic fibrosis (CF), a rare genetic disorder resulting from defects in the cystic fibrosis transmembrane conductance regulator (CFTR) chloride ion channel leading to a thick, immobile mucus barrier.

Another innate host defence mechanism is the abundance of glycosylated proteins in airway mucus that also hinder infection by viruses such as influenza (Cohen et al., 2013) and adenovirus (Stonebraker et al., 2004). The glycocalyx provides abundant “decoy” receptors to reduce lung cell infection as mucus competes for virus interaction with glycosylated residues. The role of mucus is complex, with speculation that the mucus environment could also serve to colonize growth of certain pathogens (Zanin, Baviskar, Webster, & Webby, 2016). For example, *Pseudomonas aeruginosa* (PA) bacteria are thought to modify mucin glycosylation (with pyocyanin) to favour its own growth, facilitating repeated cycles of infection. This hypothesis correlates with the challenge faced by CF patients who typically suffer from persistent PA re-infection due to their inability to clear stagnant mucus deposits harbouring bacterial reservoirs (Jeffries et al., 2016). The glycocalyx, also plays an important role in the protection of the epithelium from chemical and physical challenge as well as enabling immune discrimination of self- versus non-self recognition, reviewed in Springer & Gagneux (2013).

The cells of the pseudostratified airway epithelium also demonstrate a polarised, asymmetrical expression of structural protein components to form a barrier between the airway lumen and the basolateral membrane by tight junctions. The significant impact of this polarised expression on the cellular receptors accessible across the epithelium is exemplified by the inefficiency of the VSVg pseudotype mediated gene delivery at the apical surface versus basolateral (Wang et al., 1998). This is presumed to be a result of a lack in LDL receptors (the receptor to which VSVg binds to allow LV binding and entry into cells) distributed on the apical surface of the airway epithelium. Although this barrier can be overcome with the addition of adjuncts, such as lysophosphatidylcholine, to open tight junctions (Cmielewski, Anson, &

Parsons, 2010), such strategies also compromise the epithelial barrier, risking damage and systemic infection (Larry G. Johnson, Vanhook, Coyne, Haykal-Coates, & Gavett, 2003).

Pseudotyping to target LV for apical lung cell delivery has therefore been an important focus to overcome these challenges. Applications for gene delivery to the pulmonary epithelium are being explored towards both for correction of hereditary diseases directly affecting the cells of the lung, and for use as a target for systemic delivery of secreted proteins. Fortunately, there is a diverse abundance of WT enveloped viruses naturally evolved to target the airway epithelium which offer potential as pseudotypes for LV.

1.4.2. Pseudotypes developed towards pulmonary gene transfer

In recognising the need for pseudotypes efficient for targeting the apical respiratory epithelium, and with such a diverse list of candidate glycoproteins to investigate, initial focus has been on glycoproteins representing the typical retroviral envelope type 1 transmembrane proteins that assemble into homotrimers. This type of glycoprotein offers the advantage of requiring expression of only a single protein from producer cells, simplifying LV pseudotyping.

1.4.2.1. Ebola Zaire virus GP

The first LV pseudotype to demonstrate efficient transduction of airway epithelia, with apical rather than basolateral, transduction preference was Zaire strain Ebola virus glycoprotein (Kobinger, Weiner, Yu, & Wilson, 2001). Following initial promising studies, efforts were made to mitigate the risks associated with a using glycoprotein from such a pathogenic WT virus by minimising the envelope to remove regions associated with toxicity of the WT virus (Medina et al., 2003). Despite several attempts, and some success to optimise pseudotyping of LV with modified filoviral glycoproteins, production of sufficient functional transducing units (TU) of Ebola pseudotypes was challenging with titres obtained much lower (~70 fold) than those obtained for VSVg pseudotyped LV; this has severely limited their use (Medina et al., 2003; P. L.

Sinn et al., 2003). The cellular receptors mediating infection by the Zaire strain Ebola virus glycoprotein are thought to be TIM1 (T-cell Immunoglobulin and Mucin domain 1, receptor for phosphatidylserine) (Kondratowicz et al., 2011) and NPC1 (Niemann-Pick C1- cholesterol transporter)(Côté et al., 2011). The tropism for airway epithelial cells is therefore speculated to result from TIM1 expression on ciliated cells (Kondratowicz et al., 2011), however, there is some evidence that this is not the sole cell surface receptor involved in attachment to mediate efficient targeting. Enhanced cellular entry is also reported via several alternate membrane proteins including the Tyrosine kinase receptor- Axl (Shimajima, Ikeda, & Kawaoka, 2007), and Folate receptor- α (Stephen Y. Chan et al., 2001).

1.4.2.2. Influenza D (Thogoto virus) GP75

Apart from targeting of membrane proteins, many respiratory viruses utilise the glycocalyx on the apical surface of the epithelium, binding to glycosylated residues found on lipids and membrane proteins. Specific saccharide sequences corresponding to those found in the glycocalyx such as heparan sulphate (a glycosaminoglycan of repeating disaccharides of mostly glucuronic acid linked to N-acetylglucosamine) and glycans that are terminally sialylated (sialic acid linked to galactose) are known common targets. Initial attempts at pseudotyping LV using influenza virus A Hemagglutinin (HA) glycoproteins to target sialylated glycans proved challenging, presumably due to the need to include additional influenza A glycoproteins. Investigation into pseudotyping with influenza D (Thogoto virus) glycoprotein 75 GP75 (a homotrimeric viral receptor mediating both binding and fusion with a single glycoprotein) was pursued and demonstrated some successful pseudotyping of FIV-based vectors (P. L. Sinn, Burnight, Hickey, Blissard, & McCray, 2005). Although promising apical preference for transduction of airway epithelial cells was confirmed for Thogoto GP75, as seen with Ebola Zaire the LV yields were modest.

1.4.2.3. Baculovirus AcMNPV GP64

Baculovirus glycoprotein GP64 shares significant amino acid sequence homology with influenza D (Thogoto virus) GP75 (Morse, Marriott, & Nuttall, 1992), hypothesised to result from ancestral horizontal gene transfer (Pearson & Rohrmann, 2002). Highly efficient pseudotyping of HIV-based LV using GP64 from baculovirus *Autographa californica* multicausid nucleopolyhedrovirus (AcMNPV) was demonstrated with titres comparable to VSVg (Kumar, Bradow, & Zimmerberg, 2003). Additionally, GP64 (from AcMNPV) pseudotyped LV demonstrated similarly broad tropism to VSVg across cells *in vivo* and cell lines *in vitro*, although with restricted dendritic cell-targeting reported (Schauber, Tuerk, Pacheco, Escarpe, & Veres, 2004). As a relative of GP75, the Baculovirus GP64 pseudotype was investigated for apical airway transduction when pseudotyped on an FIV-based LV (P. L. Sinn, Burnight, et al., 2005). Very efficient pseudotyping was achieved with three-log higher production titres than Thogoto pseudotypes; an apical transduction preference on airway epithelia was demonstrated in primary human cultures, along with efficient transduction of murine nasal epithelia when administered in conjunction with ciliastatic agent. As a glycoprotein from a baculovirus (naturally evolved to target insect cells) the GP64 pseudotype is surprisingly effective for mammalian airway delivery. Although an alternative receptor interaction mediates infection of insect cells, mammalian cell transduction by GP64 is dependent on interaction with heparan sulphate, which is abundant in the glycocalyx of human lung (Duisit et al., 1999; C. Wu & Wang, 2012). Although use of ciliastatic/viscoelastic adjuncts has been investigated and used to improve efficiency of GP64 in targeting airway epithelia (P. L. Sinn, Shah, Donovan, & McCray, 2005), co-administration of an additional agent, such as methylcellulose, is a potential bottleneck for translation to clinical use. As such, directed evolution approaches have recently been applied to identify novel GP64 variants with improved efficiency in transducing human airway epithelia (P. Sinn et al., 2017).

1.4.2.4. Sendai F/HN

In pursuit of a pseudotype for efficient apical transduction of respiratory epithelia, without the need for adjuvant or pre-treatment, a pseudotype based on Sendai virus fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins was developed. Sendai virus, a member of the Paramyxoviridae that is highly infectious of the rodent respiratory tract via binding of sialic acid residues, was itself originally developed as an efficient vector for gene transfer (Hasan et al., 1997) and further shown to target the airway efficiently (Yonemitsu et al., 2000). However, the powerful but very short-lived cytoplasmic expression observed with this vector and resultant need for repeated administration to achieve duration of expression, highlighted this candidate for LV pseudotyping (Mitomo et al., 2010). Sendai viral vector tropism was identified across a wide range of animal cells beyond airway epithelium – ranging from the retinal pigment epithelium (Murakami et al., 2008) to neurons (H.-O. Li et al., 2000), and also to hematopoietic stem cells (Nishimura et al., 2011), presumably as result of the ubiquitous distribution of sialylated receptors on cells.

As a pseudotype for LV, efficient incorporation of both glycoproteins to an SIV-based platform was achieved following modifications to the glycoproteins; truncating the cytoplasmic tail of WT Sendai F, and addition of the cytoplasmic tail of the WT SIVagm envelope glycoprotein to the N-terminus of Sendai HN protein (Kobayashi et al., 2003). Both pseudotyping models could potentially explain the enhanced efficiency of the chimeric HN and truncated F incorporation to SIV-based LV with active recruitment of HN by the chimeric SIV tail, interacting with SIV core matrix proteins and passive incorporation of truncated F (Kobayashi et al., 2003). Despite the HN pseudotype glycoprotein development incorporating the SIVagm cytoplasmic tail, successful F/HN pseudotyping of HIV-1 based LV using the same modified F and HN molecules has also been demonstrated (Gélinas, Davies, Gill, & Hyde, 2017). The truncation of paramyxovirus F glycoprotein required to facilitate incorporation to LV is also observed in the case of measles virus (Funke et al., 2008). Additionally, use of a chimeric glycoprotein

pseudotype to enhance incorporation has also been exemplified in attempts to pseudotype LV with rabies virus glycoprotein for CNS gene transfer, where the cytoplasmic portion was replaced with that of VSVg (Carpentier et al., 2012).

This F/HN pseudotype consisting of these two modified glycoproteins, supplied in separate pseudotype plasmids, was adopted by the UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC) and developed as LV (SIVagm based) showing efficient transgene delivery to the airway and lung of mice *in vivo* without the requirement for preconditioning or adjuvants, and achieving long-term duration of expression, essentially for the lifetime (~2yrs) of the mouse (Griesenbach et al., 2012; Mitomo et al., 2010). Further, although robust efficacy was demonstrated after a single dose, re-administration was also shown to be effective with a third monthly dose to mice *in vivo* resulting in similarly efficient transgene expression detection in the nose and lung to that of a single (first) dose (Griesenbach et al., 2012; Mitomo et al., 2010). This repeat dosing is considered to be a likely requirement for the treatment of lifelong conditions in the lung, such as CF. Despite the advantage of LV genome integration potentiating long term expression, particularly if progenitor cells can be targeted, the majority of the cells comprising the airway epithelium and lung that are accessible are thought to be terminally differentiated. For example, ciliated cells of murine airways are suggested to have a half-life of approximately 17 months (Rawlins & Hogan, 2008). The ability to readminister LV pseudotyped with F/HN is therefore a potentially significant advantage, contrasting the lack of successful transduction following repeated rAAV administration (Halbert, Standaert, Wilson, & Miller, 1998; Sumner-Jones, Gill, & Hyde, 2007).

In addition to success in murine models, successful transduction of differentiated primary human airway cells grown at an Air-Liquid Interface (ALI) as well as human precision cut lung slices (PCLS) was demonstrated (Griesenbach et al., 2012; Mitomo et al., 2010). Although the cell types targeted and the dose of LV required to achieve efficient transduction were undetermined in these human models, transduction of a wide range of cell types in murine

airway and parenchyma were confirmed following *in vivo* delivery (Alton et al., 2017). Interestingly, when transduction of polarised epithelium was compared for apical versus basolateral preference in rat tracheal epithelial cells, no apparent preference was observed (Kobayashi et al., 2003).

1.4.2.5. Influenza A Rostock FPV H7

Another pseudotype to utilise sialic acid was developed by McKay *et al.* for lung gene delivery using the hemagglutinin (HA) glycoprotein from an avian influenza H7^(Rostock)- fowl plague virus (FPV) H7N1 (A/Fowl Plague Virus/Rostock/8/1934)(McKay, Patel, Pickles, Johnson, & Olsen, 2006). This glycoprotein, from highly pathogenic avian influenza virus (HPAIV) FPV was selected due to previous successful incorporation in retrovirus-based vectors. (V. Bosch, Kramer, Pfeiffer, Stärck, & Steinhauer, 2001; Hatzioannou, Valsesia-Wittmann, Russell, & Cosset, 1998; Sandrin et al., 2002). In a bid to generate a pseudotype utilising this hemagglutinin for incorporation and production of functional EIAV and HIV-1 based LV, the other WT influenza envelope glycoproteins, namely neuraminidase (NA) and M2 proton channel, were also included. Although the M2 pseudotype was derived from the same Rostock strain as the hemagglutinin, the neuraminidase glycoprotein is from the widely used H1N1 A/Puerto Rico/8/1934 N1) (N1^(Puerto Rico)) strain as this neuraminidase was thought to confer broad sialic acid cleavage activity (McKay et al., 2006). Using a single plasmid to express both H7^(Rostock) and N1^(Puerto Rico) pseudotype proteins, and another plasmid expressing M2^(Rostock), enhanced LV production yields three-fold (Patel, Giddings, Sechelski, & Olsen, 2013).

This pseudotype, herein referred to as Rostock, efficiently transduced the murine airway epithelium (approximately 40% of large airway surface), without the requirement for adjuvant or preconditioning (Patel et al., 2013), unlike GP64. Preference for apical transduction of polarised epithelium was also investigated in murine tracheal epithelial cells. Although some apical preference was observed (in contrast to no preference by F/HN transducing rat tracheal

epithelial cells (1.2.1.4)), the relative efficiency to the basolateral surface was similar (McKay et al., 2006). Both the Rostock and F/HN pseudotypes, similarly derived from hemagglutinating viruses that bind sialic acid, mediate efficient transduction *in vivo* without the requirement for preconditioning or adjuvant. Further investigation into pseudotypes that use sialic acid to mediate efficient gene transfer may provide an opportunity to develop alternatives.

1.5. Sialic acid as a viral receptor in the lung

Sialic acid is a 9-carbon monosaccharide found most ubiquitously terminating the branches of N-(nitrogen) and O-(oxygen) linked glycan conjugates of glycolipids and glycoproteins expressed on the surface of, and secreted by, cells. In the respiratory tract, sialic acid is found enriched in the glycocalyx covering the epithelial surface. The most predominantly expressed, and studied, sialic acid residue in humans is N-acetylneuraminic acid (or Neu5Ac)(A. Varki & Schauer, 2009). The function(s) of sialic acid, though not fully characterised, are considered to range from cell signalling and adhesion, discrimination of self, versus non-self, immunity, as well as biophysical influences, in the form of attraction of water by negative charge, to repel unwanted cellular interactions (A. Varki & Gagneux, 2012; A. Varki, Schnaar, & Schauer, 2017). The vital role of this monosaccharide is exemplified by the resultant lethality in mice after gene editing to inactivate the gene for the enzyme responsible for the initial stages of sialic acid biosynthesis (Schwarzkopf et al., 2002).

A mammalian 'non-human' isoform, N-glycolylneuraminic acid (or Neu5Gc), cannot be synthesised in humans, although it is commonly synthesised in non-human mammals, such as horses and pigs with a similar predominance to Neu5Ac. Interestingly, viruses such as influenza A appear selective, with exclusive binding of either Neu5Gc or Neu5Ac characterised indicating a species barrier that could explain the lack of known Neu5Gc binding virus circulating in the wild (Broszeit et al., 2019). A further important determinant for sialic acid binding by hemagglutinating viruses is the glycosidic linkage to the penultimate galactose of the glycan

conjugate. Though most commonly linked to the 3rd or 6th carbon of galactose in terminal sialylation (α 2,3 or α 2,6 respectively; Figure 5), sialic acid can also be found linked to the 8th carbon (α 2,8) in an internal position of glycans – in such cases commonly bound in tandem di-sialyl linkages. Reviewed by De Graaf & Fouchier (2014), this glycosidic linkage is considered a key host species determinant for influenza A virus infectivity, which is the best characterised virus interaction with sialylated receptors. In accordance with the observed binding of human versus avian influenza A viruses, the human upper respiratory tract and large airways are rich in α 2,6 sialylated residues. Conversely, avian influenza A virus binds preferentially to α 2,3 sialylated residues, consistent with the rich distribution of these residues in the respiratory and gastrointestinal tract of aquatic birds. There is growing consensus, however, that viral specificity to just the glycosidic linkage is an oversimplification (Stencel-Baerenwald, Reiss, Reiter, Stehle, & Dermody, 2014). There are many different variations of α 2,3 and α 2,6 terminally linked sialylated acid molecules available in the glycocalyx, each of which can be diverse in the complexity, length, topology and the monosaccharides they comprise (Figure 6).

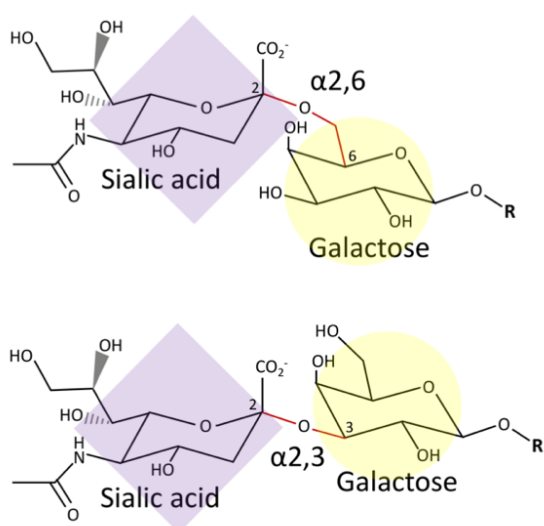


Figure 5- Structure of common galactose sialylations. Sialic acid (N-acetylneuraminic acid or Neu5Ac) and galactose bound by either an α 2,6 (top) or α 2,3 (bottom) glycosidic linkage. This disaccharide is often found terminating glycan branches (R).

penultimate galactose are more common on O-glycans or glycolipids (e.g. Ganglio-series, see Figure 6).

Receptor specificity for the glycan structure is also implicated, for example, one study identified length of α 2,6 sialylated glycans (longer residues conferring an umbrella topology) to play an important role in determining human adaptation of influenza rather than preference between the α 2,3 and α 2,6 linkage (Chandrasekaran et al., 2008).

Although largely it is still considered that sialylated glycans, N- or O- linked to proteins as well as O-linked to lipids, can be utilised as receptors by sialic acid binding viruses (Y. Suzuki, Suzuki, & Matsumoto, 1983; Y. Suzuki, Suzuki, Matsunaga, & Matsumoto, 1985), there is some speculated dependence of influenza on N-linked glycans, evidenced by lack of endocytosis by Chu & Whittaker (2004), however disputed by De Vries et al. (2012), evidencing N-linked glycan requirement for infectivity is only in the presence of serum. This alludes to the inhibitory decoy activity of soluble sialylated residues such as those in serum or mucus, whilst additionally highlighting potential preferred use of N-linked glycans. A selection pressure against the mostly O-linked glycosylation on mucins that would act as inhibitory decoys to transmission may result in an N-linked glycan preference.

It is conceivable that residues on membrane glycoproteins and residues conjugated as glycolipids play alternative roles for viruses in attachment and facilitating fusion. Both may be important given variable accessibility of receptors on the glycocalyx. Sialylation on glycoproteins may facilitate attachment with greater accessibility as they are located further from the cell membrane; while sialylated glycolipid interaction may be required to facilitate entry (for example to stimulate endocytosis or facilitate fusion). The confirmed N-glycan use by influenza for mediating endocytosis would suggest residues conjugated to proteins are indeed capable of facilitating virus entry/endocytosis of influenza. Additionally, mouse cells deficient in glycolipids were readily infected with human IAV (H3N2) confirming glycolipid binding is not a requirement (Abian, Rawat, Blumenthal, & Puri, 2001). The potentially discriminating roles of sialylated

receptors in mediating attachment as opposed to transduction are apparent in contrasting findings observed for binding and transduction. For example, influenza virus that binds Neu5Gc (non-human) sialic acid was less efficient at transducing human epithelial cells supplemented to express this sialic acid; a finding thought to be due to a decoy binding effect competing with Neu5Ac functional binding to mediate cell entry (Takahashi et al., 2014).

1.5.1. Sialic acid mediated transduction by paramyxovirus

The F/HN pseudotype is derived from Sendai virus, belonging to the family Paramyxoviridae; negative strand enveloped RNA viruses that cause disease both in humans and animals. Subdivided into Paramyxovirinae of five genera and Pneumovirinae of two, members of this family are enveloped with glycoproteins facilitating viral infectivity through receptor binding and membrane fusion, and in some cases also receptor 'destroying' activities. One potential evolutionary advantage of these viruses is the separation of the glycoprotein mediating attachment (for Sendai HN) from the glycoprotein mediating fusion (for Sendai F); allowing the two functions to evolve separately. This may help explain the wide variety of hosts and receptors infected by this family of viruses. Following attachment to the cell surface, the homotrimeric F glycoprotein facilitates fusion of the viral and cellular membrane, delivering the viral load to the cytoplasm in a pH-independent manner. There is some evidence for alternative use of an endocytic pathway for Newcastle disease virus (NDV) entry (San Román & Muñoz-Barroso, 1999). Although the F fusion glycoprotein is separate from the attachment glycoprotein, it is widely considered that attachment glycoprotein interaction with its cellular receptor is required for the F glycoprotein to be active and to fuse membranes (Azarm & Lee, 2020). Upon binding of the attachment glycoprotein, a conformational change reveals a site for interaction between the attachment and F glycoproteins, which in turn leads to activation of the F protein and subsequent fusion. For sialic acid binding paramyxovirus, it is understood that this revealed site is on the stalk domain of the attachment protein (Connolly, Leser, Jardetzky, & Lamb, 2009;

Tsurudome et al., 1995)(Figure 7). This is strengthened by studies substituting residues in the stalk domain to confirm influence on fusion activity (Melanson & Iorio, 2004). Further, the conformational change in HN resulting in exposure of these residues on the stalk involves separation of the receptor-binding head domain from the stalk. By mutation to cleave the receptor binding head domain and generating a 'headless' attachment glycoprotein, fusogenic activity of particles is maintained despite being unable to mediate attachment (S. Bose, Song, Jardetzky, & Lamb, 2014; Sayantan Bose et al., 2012). Whilst this strengthens consensus on the mechanism behind F activation, studies of 'headless' paramyxovirus also evidenced the importance of a receptor dependent conditional reveal of the activating stalk region; headless particles were fusogenic; however, premature F activation is hypothesised to render particles non-infectious (Sayantan Bose et al., 2012; Brindley et al., 2013; Q. Liu et al., 2013).

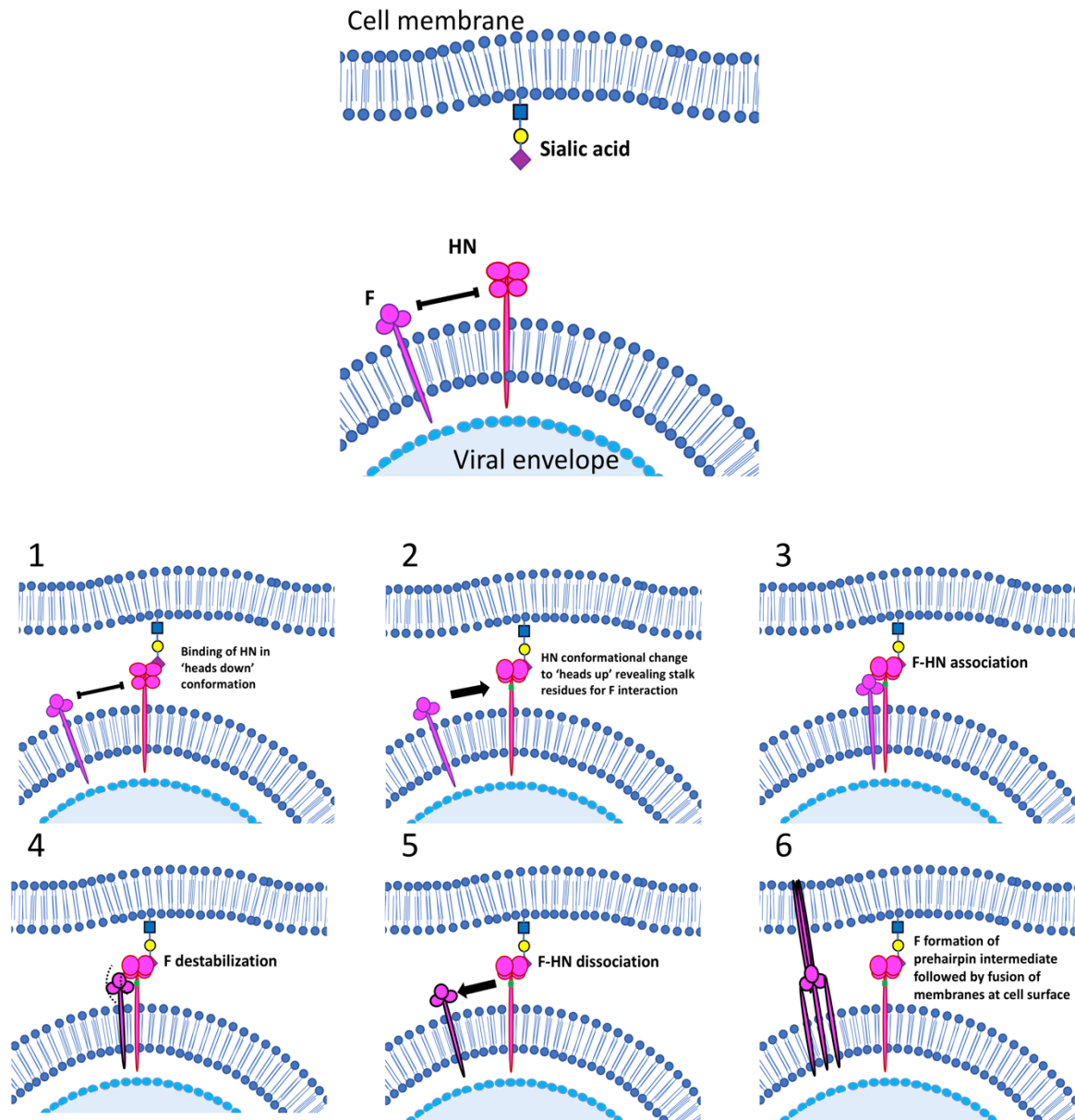


Figure 7- Proposed association model for paramyxovirus F and HN glycoprotein mediated fusion. Sialic acid binding (1) results in change of HN tetramer from 'heads down' to 'heads up' confirmation (2) facilitating F trimer interaction with HN stalk residues (green) exposed in the 'heads-up' conformation. (3) Association of F and HN results in destabilization of F (4) resulting in dissociation (5) and F trimer formation of pre-hairpin intermediate conformation (6) facilitating fusion of cell and viral membrane.

The Fusion glycoprotein is typically synthesised in a form that requires cleavage into two disulphide linked subunits to become active. For some paramyxovirus, such as Sendai virus, a monobasic cleavage site exists that requires cleavage by an extracellular protease (such as

trypsin secreted by club cells) in order to mediate fusion (Tashiro et al., 1992). This requirement for extracellular protease activation could play an important role in transmission; limiting infectivity to tissues expressing relevant proteases, and reducing the potential for immediate host cell reinfection. Conversely, a polybasic site allowing F glycoprotein cleavage by intracellular proteases such as Furin is found in some paramyxovirus such as measles virus (M. Watanabe et al., 1995). Cleavage of measles F during processing in the trans-golgi network, prior to its trafficking to the membrane surface for viral incorporation, results in particles budding with an immediate ability to promote fusion; thus measles particles have potentially a greater chance of fusion, but at the cost of a greater chance of immediate cell reinfection, a feature that may ultimately limit the spread and transmission of the virus.

Although the attachment proteins of paramyxovirus can confer binding to a variety of cellular receptors, those of the Paramyxovirinae subfamily and Respirovirus (e.g. Sendai), Rubulavirus (e.g. Mumps) and Avulavirus (e.g. NDV) genera, also confer a receptor destroying activity. This is reflected in the glycoprotein being named as hemagglutinin-neuraminidase (HN) describing the dual activities of this glycoprotein in both binding of sialic acid (which can experimentally cause erythrocyte agglutination, also termed hemagglutination), as well as its ability to cleave sialic acid residues with sialidase (also termed neuraminidase) activity. Paramyxovirus belonging to Morbillivirus family (i.e. Measles) conversely only possesses binding activity (Hemagglutinin). In contrast, the two Pneumovirus genera do not cause hemagglutination, or possess any sialidase activity, instead they employ alternative cellular receptors for attachment (Villar & Barroso, 2006). While the ability to hydrolyse and cleave sialic acid residues from the glycan conjugates might seem counterproductive to binding and attachment, it is thought that this function plays a vital role both in facilitating exit of viral particles from host cells, by preventing particles remaining attached, and by hydrolysing non-infectious interactions of the virus thought to act as decoy (Mikhail Matrosovich & Klenk, 2003).

Though the interaction between sialic acid residues and hemagglutinin is considered to be of relatively low affinity (Taroni, Jones, & Thornton, 2000), the requirement for viral neuraminidase activity to facilitate transmission (cleave viral interactions with non-functional receptors such as sialylated residues on mucin) reflects the strength of interaction facilitating attachment. It is possible that multiple (cooperative) glycoprotein-sialic acid interactions may be required to facilitate attachment sufficient to achieve fusion, triggering the conformational change in the attachment glycoprotein from 'heads down' to 'heads up' and revealing the F-activating stalk residues.

The dual activity of HN glycoprotein, facilitating both attachment and receptor cleavage, offers a potentially significant advantage for pseudotyping as it is becoming clear that a balance in these activities is important for efficient infection (Benton, Martin, Wharton, & McCauley, 2015). Pseudotyping LV particles with separate glycoproteins to carry out these linked processes leaves particles vulnerable to an imbalance between these activities; for example, viral particles may become ineffective by failing to exit host cells, or failing to enter new cells, by cleaving functional receptor interaction before fusion can take place. Such an imbalance in activities could occur if insufficient separate neuraminidase or hemagglutinin glycoproteins are incorporated to a viral particle. For HN, however, possessing both activities in a single glycoprotein means this balance is likely to be unaffected by the efficiency of glycoprotein incorporation.

1.5.1.1. Respirovirus sialylated receptor preferences

The F/HN pseudotype derived from Sendai virus has been shown to depend on sialylated residues, as expected for a pseudotype derived from a member of the Respirovirus genus. As mentioned above, however, there are several levels of diversity to sialylated receptors, which likely influences their use by hemagglutinating viruses (Herrler, Hausmann, & Klenk, 1995). Sendai virus is highly infectious in the respiratory tract of rodents, which may in part be due to

the specific sialylated receptors used. The Sendai virus receptor specificity for sialic acid was initially characterised using gangliosides adsorbed to a solid phase to investigate binding, or incorporated into cell membranes to investigate infection (Holmgren, Svennerholm, Elwing, Fredman, & Strannegård, 1980; M. A.K. Markwell, Svennerholm, & Paulson, 1981). Subsequently, Madin-Darby bovine kidney (MDBK) cells were used, whereby the sialylated oligosaccharides expressed could be manipulated, using exogenous sialic acid-cleaving enzymes (neuraminidase/sialidase) and sialylating enzymes (sialyltransferases) (M. A.K. Markwell & Paulson, 1980). Together, these studies showed that Sendai preferred the use of α 2,3 sialylated receptors. Further studies elucidated receptor preference beyond the sialic acid glycosidic linkage to galactose, using assays of binding (to specific receptors adsorbed on solid phase (T. Suzuki et al., 2001) or to asialo-erythrocytes (Y. Suzuki et al., 1985)), or assessing infectivity of cells (Mary Ann K. Markwell, Moss, Hom, Fishman, & Svennerholm, 1986). It was concluded that Sendai virus specifically uses the terminal sialic acid Neu5Ac, and can utilise and/or bind receptors of both the ganglio- as well as the neolacto-series (T. Suzuki et al., 2001)(Figure 6). The data also suggest little or no discrimination of the third monosaccharide (N-acetylgalactosamine or N-acetylglucosamine), although a preference may exist. These studies also confirmed a *preference* for α 2,3 over α 2,6 sialylation; however, affinity of Sendai virus for α 2,6 neolacto-series receptors was detected in contrast to related human parainfluenza virus type 1 (hPIV1) which did not appear to bind (T. Suzuki et al., 2001).

Sendai virus (also known as murine parainfluenza virus type 1) is a respirovirus closely related to the human respiroviruses (parainfluenza virus type 1 and 3; hPIV1 & hPIV3) adapted to respiratory infection of humans. Given alternative distributions between the hosts and known influence of subtypes on influenza hemagglutinin binding preferences, it is unsurprising that the binding preferences of these respiroviruses differentially adapted for rodents and humans are also different. Binding of both hPIV1 and hPIV3 was shown to differ from that of Sendai, with specificity for the neolacto-series and (unlike Sendai) no binding of ganglio-series receptors. This

suggests a selectivity of these human respiroviruses for N- acetylglucosamine in the third position (sialylated LacNAc) (T. Suzuki et al., 2001). Interestingly, hPIV3 binding of neolacto-series receptors was not specific to of the α - linkage, in contrast to the α 2,3 specificity of hPIV1. This inability of hPIV1 to bind α 2,6 sialylated neolacto-series receptors may contribute to the restricted pathogenesis of hPIV1 versus hPIV3 in human lung. Importantly, hPIV1 transmission in humans demonstrates that α 2,6 binding is not a requirement to mediate infection, despite the pattern of human respiratory viruses such as influenza indicating importance of α 2,6 binding for human adaptation (Gambaryan et al., 1997). The reason why Sendai virus, with similar affinity for both α 2,3 and α 2,6 neolacto-series receptors versus hPIV3 (and also broader specificity with additional affinity for ganglio-series), is not transmissible in humans, remains unclear. The answer to this difference could relate to differences in the F glycoprotein, such as the requirement for extracellular protease cleavage in the case of Sendai virus (Tashiro et al., 1992), versus hPIV3 ability to be cleaved by intracellular Furin (Ortmann et al., 1994).

1.5.2. Sialic acid mediated transduction by influenza A

Influenza A virus (IAV), belonging to the Orthomyxoviridae family, is the most virulent influenza genera in humans, and has been responsible for significant outbreaks of transmission and multiple global pandemics leading to many millions of human deaths. The major reservoir for circulating IAV is in the aquatic bird gastrointestinal tract, where infection and transmission is often asymptomatic (Webster, Bean, Gorman, Chambers, & Kawaoka, 1992). Because of their major human health implications, these viruses are the most studied of the sialic acid-binding virus genera. Despite being a distant relation to respiroviruses (hPIV and Sendai), IAV shares a negative sense RNA genome and an envelope of glycoproteins to mediate sialic acid receptor binding, cleavage and fusion to facilitate entry. The glycoproteins and their function however are different to respirovirus. The influenza hemagglutinin (HA) mediates sialic acid binding as well as fusion in a single homotrimeric glycoprotein, while the influenza neuraminidase (NA)

glycoprotein confers sialidase activity. Whereas respirovirus fusion can occur directly at the plasma membrane following sialic acid binding, IAV HA requires a pH-dependent conformational change to expose its fusion active surface, and thus release of the influenza viral nucleic acid load occurs after particle endocytosis (Figure 8). This pH-dependent entry within the endosome is also reliant on proton internalisation by the viral particle itself as viral matrix protein electrostatic interactions must be weakened by acidification to allow capsid uncoating (dissociation of the matrix protein (M1)) to occur (Helenius, 1992; Martin & Helenius, 1991). Thus, IAV membrane envelope contains a proton channel (M2), both facilitating the fusion of IAV as well as playing a role in subsequent IAV particle assembly, by stabilising pH-sensitive HA conformation in the golgi (Takeuchi & Lamb, 1994).

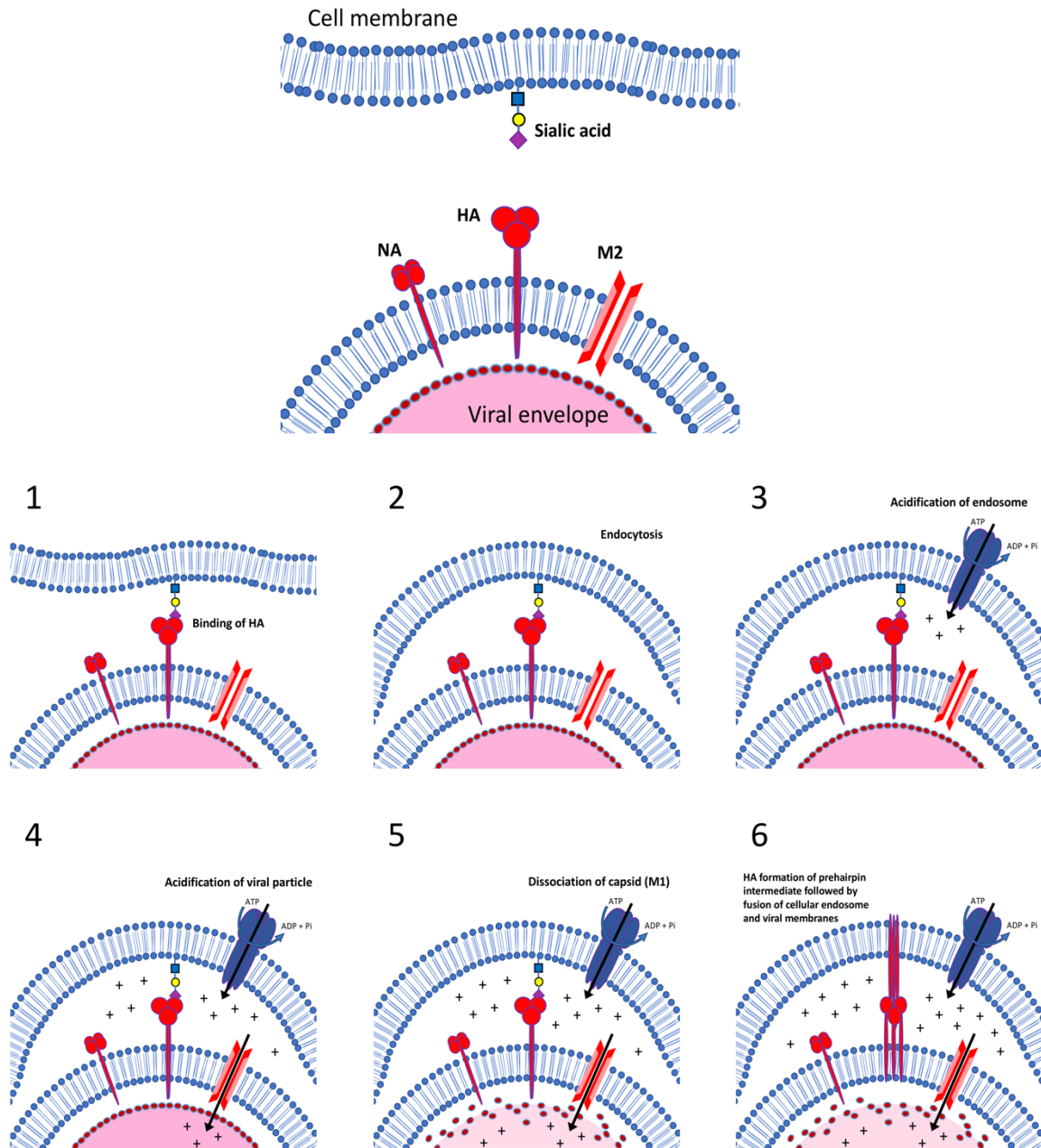


Figure 8- Proposed pH dependent model for influenza HA glycoprotein mediated fusion. Sialic acid binding (1) by HA triggers (typically clathrin- mediated) endocytosis of viral particle (2) and as early endosomes traffic particles to late endosomes, the active acidification of the endosomes by Vacuolar-type ATPase proton transport (3) results in passive proton transport into the viral particle via viral M2 glycoprotein (4). Acidification within the particle dissolves M1 capsid interactions (5) and finally the pH required to drive HA fusion peptide insertion into the endosomal cellular membrane in an extended pre-hairpin intermediate is achieved within the endosome (6)(Akole & Warner, 2019). Fusion of these membranes then facilitate release of influenza particle contents to the cytoplasm.

Differences lie, as they do between different paramyxovirus, between IAV in their dependency on extracellular protease cleavage of the glycoprotein mediating fusion (HA). The HA glycoprotein is a homotrimer that is required to be cleaved (to HA1 and HA2) in order to facilitate IAV infectivity (H. D. Klenk, Rott, Orlich, & Blödorn, 1975). The nature of this cleavage site is recognised as an important determinant of pathogenicity in the avian reservoir host of IAV (F. X. Bosch, Orlich, Klenk, & Rott, 1979). Although cleavage occurs at a conserved arginine-glycine bond, the amino acid sequence varies between different IAV HA, and these variations determine the proteases that can potentially perform the cleavage (Steinhauer, 1999). A monobasic site found in low pathogenic avian influenza virus (LPAIV) is thought to be restrictive on virus transmission, as it confers a site that requires (like Sendai) cleavage by trypsin-like extracellular proteases, thus limiting infectivity to sites that express the required protease activity (such as avian respiratory and gastrointestinal tract). Conversely, a polybasic site facilitates cleavage by intracellular proteases, including the ubiquitous furin, which allows cleavage during particle maturation within the host cell. The polybasic site is found in high pathogenic avian influenza virus (HPAIV) and is thought to explain the unrestricted spread of HPAIV infections to alternative organs (Böttcher-Friebertshäuser, Garten, Matrosovich, & Klenk, 2014). One such HPAIV is that from which the HA and M2 glycoproteins of the Rostock LV pseudotype were derived. As a result, cleavage by trypsin-like proteases following pseudotyped LV production is not a requirement for the Rostock pseudotypes, contrasting with the Sendai F/HN pseudotype (V. Bosch et al., 2001; Kobayashi et al., 2003; McKay et al., 2006). Mammalian IAV however conserves the LPAIV monobasic HA cleavage site; possibly explaining the rarity of dissemination of influenza in humans to organs beyond the respiratory tract, since they lack necessary proteases. Beyond restriction to the respiratory tract, further localisation/containment of infection is understood to determine pathogenesis. Seasonal human influenza strains replicate in the upper respiratory tract, causing transient mild disease, however instances in which infection spreads to distal lung (bronchioles and alveoli), can

become severe and sometimes result in fatal pneumonia (Kuiken & Taubenberger, 2008). These distinctive infection patterns are hypothesised to be at least in part as a result of different sialylated receptor expression and use by these viruses. Indeed, although transmission of avian influenza to humans occurs infrequently, these zoonotic infections (such as HPAIV H5N1 (Beigel et al., 2005) or LPAIV H7N9 (S. Liu et al., 2013)) more frequently result in severe disease, consistent with pathogenicity/infection beyond the upper respiratory tract, facilitated by these viruses' alternative receptor adaptation.

1.5.2.1. Influenza A sialylated receptor preferences

The use of sialylated receptors by IAV HA is well characterised and underpins much of our knowledge relating to the use of sialic acid use as a receptor for viral entry. It is studies of IAV HA from alternative species that provide the bulk of evidence that variation in subtypes of sialic acid residues are a key factor limiting host range (reviewed (Mikhail N. Matrosovich, Gambaryan, & Klenk, 2008; Viswanathan et al., 2010)). Most significantly, avian IAV HA has been shown to bind and infect using α 2,3 sialylated residues whereas human IAV binds preferentially to α 2,6 (Gambaryan et al., 1997). Binding of HPAIV FPV, the HA is used in the Rostock LV pseudotype, has been investigated for sialylated receptor use by binding assays, both to solid phase and competitive for free sialyloligosaccharides. As an avian IAV, strong preference for α 2,3 over α 2,6 was observed in line with the vast majority of other avian strains investigated (M N Matrosovich et al., 1997).

Along with an α 2,3 preference, FPV/Rostock H7N1 also shares in common with Sendai virus, specificity for Neu5Ac sialic acid (not Neu5Gc), and an affinity to bind sialylated residues irrespective of the third monosaccharide. This is demonstrated through assaying GD1a ganglioside (representing a N-acetylgalactosamine) and Sialyl-3-paragloboside (neolacto-series; representing N-acetylglucosamine) binding in solid phase (Figure 6).

Interestingly, the relative affinity of FPV/Rostock H7N1 for binding GD1a compared to Sialyl-3-paragloboside contrasted that characterised for Sendai. A greater affinity for GD1a suggesting preference for ganglio-series (N-acetylgalactosamine) was identified, versus Sendai virus with greater affinity for Sialyl-3-paragloboside (neolacto-series; N-acetylglucosamine)(M N Matrosovich et al., 1997; T. Suzuki et al., 2001).

In contrast to the strong α 2,3 preference of most avian IAV, such as FPV/Rostock, binding and infectivity of the LPAIV H7N9 causing the highly pathogenic zoonotic infections in human (S. Liu et al., 2013), have been thoroughly characterised across many elaborate studies identifying enhanced α 2,6 binding alongside α 2,3 use (Belser et al., 2013; Xiong et al., 2013; Zhou et al., 2013). Additionally, solid phase glycan microarray binding suggests these human pathogenic H7N9 isolates are able to bind α 2,3 sialylated glycans conferring either N-acetylgalactosamine or N-acetylglucosamine as the third sugar (T. Watanabe et al., 2013; Yang, Carney, Chang, Villanueva, & Stevens, 2013).

1.5.3. Sialylated receptor distribution between hosts.

Differences in the distribution of sialylated receptors between tissues and species are characterised using staining techniques (Figure 9), glycan isolation and mass spectrophotometry, or inferred through viral attachment patterns. A rich abundance of α 2,3 sialylated residues in the upper respiratory tract of mice correlates with the known preference of Sendai virus for these receptors (J. P. Guo et al., 2011; Ibricevic et al., 2006; Ning et al., 2009). Furthermore, an abundance in the respiratory and gastrointestinal tract of chickens could explain the α 2,3 sialylation preference of FPV/Rostock (Costa et al., 2012). Despite the preferences of human viruses for α 2,6 sialylated residues, evidence for an abundance of α 2,3 sialylated residues (conferring N-acetylglucosamine not N-acetylgalactosamine as the third sugar) in the upper human respiratory tract, in addition to α 2,6, is becoming more widely recognised (Jia et al., 2020; Nicholls, Bourne, Chen, Guan, & Peiris, 2007; Walther, Karamanska,

Chan, Chan, & Jia, 2013); this questions the reality and mechanism behind the understood necessary adaptation for $\alpha 2,6$ linkage specificity. There is some uncertainty, however, because although IAV binding preferences largely adhere to a requirement for $\alpha 2,6$ binding to permit transmission to the upper respiratory tract of human, $\alpha 2,6$ binding may not be required for respiratory virus infection, such as by hPIV1 (T. Suzuki et al., 2001). That said, there are significant differences in the receptor subtypes characterised between these host species.

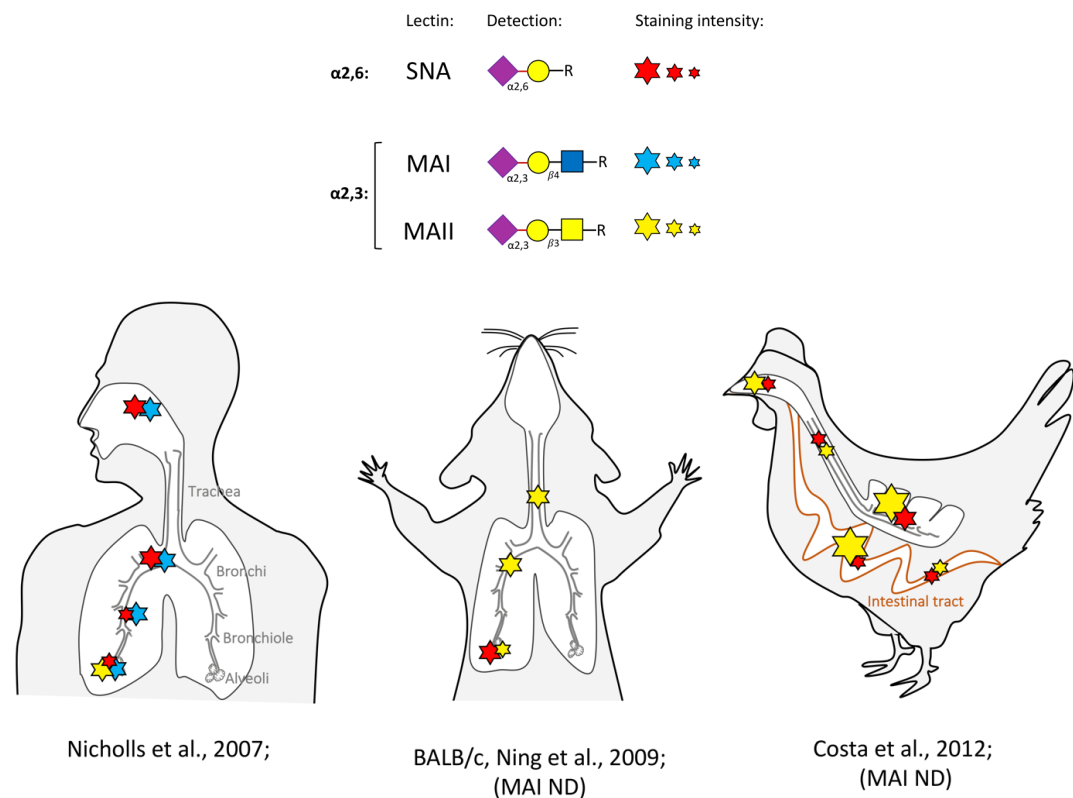


Figure 9- Distribution of Sialylated receptor subtypes as determined using lectins across host respiratory and gastrointestinal tract. Top: consensus saccharide sequences recognised by lectins used to stain for sialylated receptors (reviewed in Geisler & Jarvis, 2011) Each shape corresponds to a monosaccharide (see Figure 6). Bottom: distribution of sialylated receptors as determined using lectins isolated from *Sambucus nigra* agglutinin (SNA (red stars) to detect $\alpha 2,6$) and *Maackia amurensis* (MAI (blue stars) and MAII (yellow stars) to detect $\alpha 2,3$), in the respiratory epithelium of humans (Nicholls et al., 2007; SNA+MAI+MAII), mice (BALB/c, Ning et al., 2009; SNA+MAII only) and chickens, and the large and small intestinal tract of chickens (Costa et al., 2012; SNA+MAII only). Size of the symbol corresponds to the relative staining intensity or assumed receptor abundance. ND; Not determined.

1.6. Aims

The efficient transduction by F/HN pseudotyped LV in a range of pulmonary models, clearly highlights the potential of this pseudotype for efficient lung and airway gene delivery. Similarly, Rostock pseudotyped LV has potential for efficient airway delivery in the absence of airway conditioning *in vivo*. The receptor preferences characterised for the viruses from which these pseudotypes were derived, however, contrast with the α 2,6 sialylated receptor preference by most human-adapted alternatives. It is conceivable that the efficiency of these pseudotypes, in the context of sialic acid binding, may be not optimal for human lung gene transfer. In addition to the unknown, potentially unique, receptor preferences for hemagglutinin-based pseudotypes, the impact of testing transduction in models from species that may not represent the receptor distribution and availability in the human lung adds to the challenge in predicting transduction of human pulmonary cells. Although both the F/HN and Rostock pseudotypes have been shown to facilitate efficient *in vivo* transduction of the murine airway, without the need for adjuvant, the translational potential of each, as well as the relative efficiency, is unknown. Further, a new pseudotype based on human pathogenic LPAIV (2nd isolate from the 2013 outbreak in Shanghai) with enhanced α 2,6 binding, could offer a greater potential for transduction of airway/lung models best representing this receptor availability in humans.

In this thesis, the aim was to determine the relative potential of these pseudotypes to mediate gene delivery in the human lung. Firstly, a direct comparison of these pseudotypes *in vivo* was required to resolve the relative efficiency and cell types targeted. Subsequently, the objective was to evaluate these pseudotypes in alternative models to understand the impact on transduction and cell-type tropism for each pseudotype. Through evaluating the potential of each model to predict aspects of human lung gene transfer in parallel, it was further hoped that the characterisation of the differences between pseudotypes and models could inform their relative translational potential. Finally, by understanding the distribution and use of sialic acid receptors in the various models, the aim was to better understand whether differences in

receptor use by each pseudotype determined transduction profile and efficiency. In this way, it was hoped to better understand the potential of these pseudotyped LV to treat human lung disease.

2. Chapter 2- Methods

2.1. Reagents

Solutions were prepared using Milli-Q reverse osmosis water (Merck, Feltham, UK), water for injection (B.Brain Medical, Sheffield, UK), or nuclease-free water (Promega, Southampton, UK). Absolute ethanol was obtained from VWR (Lutterworth, UK). Sterile Phosphate Buffered Saline (PBS; 1x) was either prepared using tablets as directed (Sigma-Aldrich; Sigma, Gillingham, UK) and autoclaving, or obtained from Life Technologies (dPBS; Paisley, UK). All chemicals were obtained from Sigma unless stated otherwise. Where required, sterilisation was achieved by autoclaving for 15-30 minutes at 121°C or by filtration through a surfactant-free 0.22 µm cellulose acetate filter (Thermo Fisher Scientific, Cramlington, UK). Prepared solutions were stored at room temperature (RT; 20-25°C) unless otherwise stated. Solutions prepared to a percentage refer to weight:volume (w/v) ratios for solid dilution and volume:volume (v/v) for liquids. For all fixation steps a solution of 4% PFA (paraformaldehyde; Sigma) was prepared by dissolving in PBS at 60°C and adjusting to pH 7; this methanol free solution was stored at -20°C until use.

2.2. Cell culture

Live cell counts were determined either with 0.2% trypan blue staining and manual counting with a Neubauer haemocytometer (Sigma), or by automated counting using NucleoCounter NC-200 (ChemoMetec, Allerød, Denmark) and Via1-Cassettes (ChemoMetec) according to manufacturers' instructions.

2.2.1. Adherent cells

Adherent cells were maintained in a 5% CO₂ humidified incubator at 37 °C and passaged once confluency of 70-90% was reached. For each passage, media was replaced and cells were diluted

according to requirements: 1:6 for adherent Human Embryonic kidney (HEK) 293T cells (HEK293T/17 CRL-11268; American Type Culture Collection (ATCC), Manassas, USA); 1:3 for Madin-Darby Canine Kidney (MDCK (NBL-2) CCL-34; ATCC); 1:8 for Madin-Darby Canine Kidney cells stably transfected with human α 2,6-sialyltransferase 1 (SIAT1) cDNA (MDCK-SIAT1; ECACC 05071502); and 1:3 for Madin-Darby Bovine Kidney (MDBK (NBL-1) ATCC CCL-22). Where necessary for detachment of cells, passage involved brief washing in dPBS (Life Technologies) and incubation with TrypLEselect (recombinant trypsin analogue; Life technologies) at 37 °C until detachment. All adherent cells were maintained in either Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies) (HEK293T & MDCK-SIAT1) or Eagle's Minimum Essential Medium (EMEM; ATCC) (MDCK & MDBK), supplemented with 10% Foetal Bovine Serum (FBS; Sigma or ATCC; heat inactivated at 60 °C for 1hr), 100 U/mL penicillin plus 100 mg/mL streptomycin (Sigma) and 2mM L-Glutamine (Life Technologies).

2.2.2. Suspension cells

Suspension cells were maintained in an 8% CO₂ humidified incubator at 37 °C on an orbital platform (1.9cm orbital diameter) agitating cultures at 135 revolutions per minute in polycarbonate Erlenmeyer flasks (Corning). Adherent HEK293T cells that have been previously adapted to serum free suspension growth using sequential reduction of serum (gift from Lee Davies; former Gene Medicine group member) or obtained serum-free and suspension-adapted from ATCC (HEK293T/17 SF ACS-4500; ATCC) were used. HEK293F cells (R79007; Gibco; Thermo Fisher Scientific, Cramlington, UK) were also maintained in suspension and used to determine functional titres of LV where indicated. Viral Production Cells (LV-MAX cells, A35347; Gibco; Thermo Fisher Scientific) - a derivative of the HEK293F cell line - were also used to produce and titrate LV preparations as indicated. Cells were passaged when a density of approximately 4-5e6 cells/mL was reached; diluted into pre-warmed fresh media to achieve 4-5e5 cells/mL or as required. HEK293T and HEK293F cells were maintained using FreeStyle 293 Expression medium

(FreeStyle; Life Technologies) and LV-MAX cells using LV-MAX production medium (Thermo Fisher Scientific), always in the absence of serum or antibiotics.

2.3. Plasmids and DNA

All plasmids used or generated in these studies are detailed in Table 3. All LV genome plasmids were based on third generation LV and include WPRE and cPPT sequences. Plasmids for Oxford Biomedica (OXB) HIV LV platform are derivatives of those previously published (HIV3G; (Siapati et al., 2005). Gene Medicine (GM) SIV LV plasmids use the SIV platform (Kobayashi et al., 2003) based on SIVagm (Fukasawa et al., 1988). All plasmids indicated to express Sendai virus F and HN genes (OXB and GM) refer to sequences derived from (Kobayashi et al., 2003), with cytoplasmic F tail truncation and the SIVagm envelope tail addition to HN as described (1.4.2.4). Influenza virus Rostock (FPV) M2 and H7 genes as well as Puerto-rico N1 are derived from published sequences (McKay et al., 2006) and described (1.4.2.5). Influenza Shanghai H7 and N9 genes are derived from the second isolate of Shanghai human pathogenic H7N9 from (A/Shanghai/2/2013) (W Chan et al., 2013), an isolate of the zoonotic LPAIV (1.5.2 and 1.5.2.1). Shanghai polybasic H7 refers to a modified Shanghai H7 sequence as to incorporate the polybasic cleavage site (KKRKRR; from site II of respiratory syncytial virus F (Rawling, García-Barreno, & Melero, 2008)) and mediate cleavage by intracellular Furin protease.

Table 3- Plasmids used. UbC: human ubiquitin C gene promoter. Lux: firefly luciferase. OtdTomato: codon optimised tdTomato. CMV: cytomegalovirus enhancer/promoter (Boshart et al., 1985). EGFP: enhanced fluorescent green protein. hCEF: combination of CpG-free human CMV enhancer and CpG-free human elongation factor 1 α promoter (Hyde et al., 2008). EGFLux: EGFP fused to firefly luciferase (cDNA from pEGFP-Luc, Promega). sogLux: soGLux: codon-optimised CpG- depleted Gaussia luciferase (Griesenbach et al., 2011). RSV: Rous sarcoma virus promoter (C. M. Gorman, Merlino, Willingham, Pastan, & Howard, 1982). CBA: chicken β -actin gene promoter. CO: codon-optimised. CAG: chicken β -actin promoter plus human cytomegalovirus immediate early enhancer (Jun-ichi et al., 1989). Amp: Ampicillin. Kan: Kanamycin. Neo: Neomycin. hygB: Hygromycin B.

Type	Name	Source	Enhancer/ Promoter	Features	Antibiotic resistance
Expression	pUb.Lux	Bayou Biolabs, LA,USA	UbC	Luciferase cDNA	Amp
	OtdTomato GeneArt String	GeneArt (Invitrogen)	-	Codon optimised tdTomato cDNA	-
OXB HIV LV Genome	pHIV.CMV.TdTomato (pGM564)	These studies	CMV	HIV genome (CMV- TdTomato transgene)	Kan
	pHIV.CMV.Lux (pGM554)	These studies	CMV	HIV genome (CMV- Luciferase transgene)	Kan
	pHIV.CMV.EGFP (pOXB-HVCG)	OXB	CMV	HIV genome (CMV- EGFP transgene)	Kan
	pHIV.hCEF.EGFP (pOXB-hCEF-GFP)	OXB	CMV	HIV genome (hCEF- EGFP transgene)	Neo
GM SIV LV Genome	pSIV.hCEF.EGFP (pGM357)	GM	CMV	SIV genome (hCEF- EGFP transgene)	Kan
	pSIV.CMV.EGFP (pGM311)	GM	CMV	SIV genome (CMV- EGFP transgene)	Kan
	pSIV.hCEF.EGFLux (pGM426)	GM	CMV	SIV genome (hCEF- EGFLux transgene)	Kan
	pSIV.hCEF.sogLux (pGM358)	GM	CMV	SIV genome (hCEF- sogLux transgene)	Kan
GM HIV LV Genome	pHIV.CMV.EGFLux (pGM290)	GM	RSV	HIV genome (CMV- EGFLux transgene)	Amp
OXB HIV LV Rev	pCMV.HIV.Rev (pOXB-REV)	OXB	CMV	HIV rev gene	Kan
GM SIV LV Rev	pSIV.CMV.Rev (pGM299)	GM	CMV	SIV rev gene	Kan
OXB HIV LV Packaging	pHIV.CMV.GagPol (pOXB-HSGP)	OXB	CMV	HIV gag-pol gene	Kan
GM SIV LV Packaging	pSIV.CBA.GagPol (pGM297)	GM	CBA	SIV gag-pol gene	Kan
	pSIV.CBA.coGagPol (pGM691)	GM	CBA	SIV CO gag-pol gene	Kan
OXB LV Pseudotype	pCMV.SeV.Fct4 (pOXB-CMV-Fct4)	OXB	CMV	Sendai virus F gene	Kan
	pCMV.SeV.EnvctHN (pOXB-CMV-coHN)	OXB	CMV	Sendai virus HN gene	Kan
	pCMV.Rostock.M2 (pRKH-M2)	OXB	CMV	Influenza virus Rostock M2 gene	Kan
	pCMV.Rostock.H7.Puerto-rico.N1 (pViro2-H7rN1pr)	OXB	CMV	Influenza virus Rostock H7 and Puerto-rico N1 genes	HygB
	pCMV.Shanghai.H7Polyb.Puerto-rico.N1 (pViro2-H7shN1pr Polyb)	OXB	CMV	Influenza virus Shanghai polybasic H7 and Puerto-rico N1 genes	HygB
	pCMV.Shanghai.H7Polyb.Shanghai.N9 (pViro2-H7shN9sh Polyb)	OXB	CMV	Influenza virus Shanghai polybasic H7 and N9 genes	HygB
	pCMV.VGVg (pOXB-VSVG)	OXB	CMV	Vesicular Stomatitis Virus glycoprotein gene	Kan
GM LV Pseudotype	pCAG. SeV.Fct4 (pGM301)	GM	CAG	Sendai virus F gene	Kan
	pCAG.SeV.EnvctHN (pGM303)	GM	CAG	Sendai virus HN gene	Kan

2.3.1. DNA quantification

DNA concentrations within the range of 0.002-15 mg/mL were determined in triplicate using a NanoDrop Spectrophotometer 2000 (Thermo Fisher Scientific), with the average used to calculate final concentration. By measuring UV light absorption of DNA samples at 260nm, the concentration is determined given that the optical density of a 50 µg/mL double-stranded DNA (dsDNA) solution is 1.0 at 260nm (OD₂₆₀). DNA purity was confirmed by measuring sample OD₂₆₀/OD₂₈₀ ratio, with values of <1.8 indicating contamination.

2.3.2. Plasmid generation

Two HIV-1 based genomes were generated by sub-cloning (firefly) luciferase or tdTomato transgenes into a HIV a genome plasmid obtained from Oxford Biomedica (OXB). The tdTomato cDNA sequence was obtained from Invitrogen (Paisley, UK; GeneArt™), codon optimised using Invitrogen Gene Optimizer Process. The sequence was further modified to include a Kozak consensus sequence, BamHI restriction site and GC clamp at the 5' end as well as NotI restriction site and GC clamp at the 3'. Luciferase transgene was cloned from expression plasmid pUblux (see Table 3). Primers ordered from Sigma were used to anneal at the 5' and 3' ends of both transgenes and amplify transgene insert sequences by polymerase chain reaction (PCR) (2.3.2.1). OXB genome plasmid (pOXB-HVCG; Table 3) was also propagated as described below (2.3.3), ahead of digestion (2.3.2.3) of both genome plasmid and transgene inserts with NotI-HF and BamHI-HF restriction enzymes (New England Biolabs; NEB, Hitchin, UK). Each insert was ligated (2.3.2.5) into the genome following DNA fragment gel extraction (2.3.2.4) to purify digested genome and inserts. Ligated plasmids were then propagated and purified. To confirm successful, mutation-free plasmid generation, a preliminary diagnostic restriction digest and fragment gel electrophoresis confirming expected fragment sizes was followed by Sanger sequencing of plasmids; 7µL of plasmid DNA at 100ng/µL was provided as a sequencing template along with the relevant primers at 3.2pmol /µL to Source BioScience (Nottingham, UK).

2.3.2.1. Polymerase Chain Reaction (PCR)

PCR to amplify sequences in plasmid DNA was performed using GeneAmp PCR system 2700 thermal cycler (Thermo Fisher Scientific). For each reaction, template pDNA at 100ng/μL, Phusion HotStart II DNA Polymerase and Phusion Buffer (Thermo Fisher Scientific), 10mM dNTPs (NEB) and 10μM of forward and of reverse primer were combined and made up to 50μL reaction volume using nuclease-free water. Thermal cycling conditions were: 5 minutes at 96 °C followed by 30 cycles of; 1 minute at 98°C, 1 minute at the temperature determined for annealing of primers using MacVector software v15 (MacVector, Cary, NC, USA) and 2 minutes at 72°C, with final cycle ending on 72°C for 7 minutes.

2.3.2.2. Gel electrophoresis

Size fractionation of DNA by gel electrophoresis was carried out using 0.7% agarose gels prepared using Tris-acetate-EDTA (TAE) buffer (40mM Tris(hydroxymethyl)aminomethane (Tris)-acetate and 1 mM EDTA (ethylenediaminetetraacetic acid) at pH 8.3). The agarose (Life Technologies) was dissolved in TAE buffer by heating in a microwave (1-2 minutes) before addition of SYBRgreen (Invitrogen) and mould pouring. DNA was mixed with Gel Loading Dye (NEB) to a final volume of 10-20μL and electrophoresed (10-15 V/cm) until sufficiently resolved alongside 10μL of the appropriate DNA ladder (100bp (range 100-1517 bp; NEB) or one kb (range 500 bp to 10 kb; NEB). Bands were visualised under UV light (312 nm) using GeneFlash Gel Documentation System (Syngene, Cambridge, UK) or using an iBright FL1500 imager (Thermo Fisher Scientific).

2.3.2.3. Restriction enzyme digest

Restriction enzyme digests were performed using NEB high fidelity restriction enzymes in the recommended buffer (CutSmart; NEB) at the recommended temperature for 1-2 hours. Typically, a 20μL reaction using 2.5μg plasmid DNA was incubated at 37 °C.

2.3.2.4. DNA fragment gel extraction

Following digestion, DNA was gel electrophoresed as above (2.3.2.2) and bands visualised using a UVL-56 hand-held illuminator (UVP Inc., California, USA). The desired DNA band was excised using a scalpel and DNA fragment was purified using QIAquick Gel Extraction Kit (Qiagen, Crawley, UK), eluted into 30µl nuclease free water.

2.3.2.5. DNA fragment ligation

DNA fragments were ligated using T4 DNA ligase and the recommended buffer (NEB) according to manufacturer's instructions: typically 1 µl of T4 DNA ligase (400 U) was combined with 100ng vector (backbone) and 700ng insert DNA (total volume of 10 µl) and incubated for 1-2 hours at 18°C. Product was stored at -20°C if not immediately used for bacterial transformation.

2.3.3. Plasmid propagation

Lysogeny broth (LB) for selective growth of transformed bacteria was prepared to 1% NaCl (Sigma), 0.5% yeast (Merk) and 1% tryptone (Sigma) in Milli-Q water, sterilised by autoclaving. LB-Agar was made by further addition of Select Agar to 1.5% (Life Technologies) and the relevant filter sterilised antibiotic. Antibiotics were obtained from Sigma and stored at 4 °C and Kanamycin, Ampicillin or Hygromycin B prior to addition to achieve a final concentration of 50 µg/mL (Kanamycin) or 100 µg/mL (Ampicillin and Hygromycin B) in LB media or agar.

2.3.3.1. Bacterial transformation of plasmid and inoculation into growth media

For propagation, plasmid DNA was transformed into *E.coli* Stbl3 chemically competent cells (Invitrogen) according to the manufacturer's instructions. Transformed cells were grown using an orbital shaking incubator (diameter of 2.54 cm; Innova 4300, New Brunswick Scientific,

Edison, NJ, USA). Typically, 5µl of plasmid DNA was mixed with 25µl of competent cells, incubated on ice for 30 minutes and then heat-shocked by placing at 42 °C for 45 seconds and then back on ice for at least 5 minutes. Super Optimal Broth (Thermo Fisher Scientific) was then used to recover cells - 125µl incubated at 225 rpm for 1h at 37 °C - and various dilutions were then spread on LB agar plates containing the relevant antibiotic to select for plasmid transformed cells. Plates were incubated at 37 °C overnight and single colonies then picked for subsequent amplification using an inoculating loop and deposited in LB (5mL or 400mL) containing the relevant antibiotic; colonies were grown at the desired scale overnight at 37 °C and 225 rpm.

2.3.3.2. Extraction and purification of propagated plasmid

Plasmid DNA was purified using either the Wizard Plus SV MiniPrep DNA Purification System (Promega; 5mL cultures), or EndoFree Plasmid Mega Kit (Qiagen; 400mL cultures) according to manufacturer's instructions, and eluted in nuclease free water.

2.4. LV production

Recombinant third generation LV based on HIV-1 or SIVagm were produced either using adherent HEK293T cells in Cell Factories (Nunc; Thermo Fisher Scientific), using suspension HEK293T cells in a bioreactor (WAVE; Cellbag bioreactor; GE Healthcare, Little Chalfont, UK) or using LV-MAX cells from the LV-MAX Lentiviral Production system (Gibco; LV-MAX). All productions relied on transient transfection of these cells with the plasmids listed (Table 4 A), in the mass ratio indicated.

Table 4- Vectors used and produced. (A) Plasmids transfected and mass ratio used for production of each vector. For details of all plasmids see Table 3. (B) Methods and titre achieved in production and purification of each vector batch. TU: Transducing Units, CF10: 10-layer Cell Factory system. TFF: Tangential Flow Filtration. ND: Not determined. O/N: Overnight (minimum 18 hours). HEK293T 10-day, HEK293F 3-day and LV-MAX 3-day: Integrative titre determined at OXB or GM (see 2.6.1). LV-MAX 3-day Luciferase assay: see 2.6.4. Activation with TrypLEselect refers to treatment of LV containing cell supernatant crude harvests with 0.05x TrypLEselect recombinant trypsin analogue (Life technologies; 15 minutes at 37°C) prior to purification. rHIV.VSVg.CMV.EGFP (DJ001) was gifted from OXB. All purified vectors were formulated in TSSM buffer (20mM Tromethamine, 100mM NaCl, 10mg/mL sucrose, and 10mg/mL mannitol)(Whitelaw et al., 2004) and stored in aliquots of 10-100µl at -80°C prior to use. (A & B) Shading indicates pseudotype of LV: F/HN (pink), Rostock (red), ShanghaiN1 (orange), ShanghaiN9 (purple) and VSVg (white).

Table 4 A						
LV name	Plasmids transfected					Plasmid mass ratio for transfection (Rev:Packaging:Genome:Pseudotype1:Pseudotype2)
	Rev	Packaging	Genome	Pseudotype 1	Pseudotype 2	
rHIV.F/HN.CMV.EGFP (vGM182)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.EGFP (pOXB-HVCG)	pCMV.SeV.Fct4 (pOXB-CMV-Fct4)	pCMV.SeV.EnvctHN (pOXB-CMV-coHN)	110:140:450:90:90
rHIV.F/HN.CMV.tdTomato (vGM181)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.TdTomato (pGM564)	pCMV.SeV.Fct4 (pOXB-CMV-Fct4)	pCMV.SeV.EnvctHN (pOXB-CMV-coHN)	110:140:450:90:90
rHIV.F/HN.CMV.EGFP (vGM188)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.EGFP (pOXB-HVCG)	pCAG.SeV.Fct4 (pGM301)	pCAG.SeV.EnvctHN (pGM303)	110:140:450:90:90
rHIV.H7rN1pr.CMV.EGFP (vGM222)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.EGFP (pOXB-HVCG)	pCMV.Rostock.H7.Puerto-rico.N1 (pVitro2-H7rN1pr)	pCMV.Rostock.M2 (pRKH-M2)	200:830:830:500:80
rHIV.H7shN1prPb.CMV.EGFP (vGM221)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.EGFP (pOXB-HVCG)	pCMV.Shanghai.H7.Polyb.Puerto-rico.N1 (pVitro2-H7shN1pr Polyb)	pCMV.Rostock.M2 (pRKH-M2)	50:100:900:150:18
rHIV.H7shN9shPb.CMV.EGFP (vGM220)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.EGFP (pOXB-HVCG)	pCMV.Shanghai.H7.Polyb.Shanghai.N9 (pVitro2-H7shN9sh Polyb)	pCMV.Rostock.M2 (pRKH-M2)	50:100:900:150:18
rHIV.F/HN.hCEF.EGFP (vGM193)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.hCEF.EGFP (pOXB-hCEF-GFP)	pCMV.SeV.Fct4 (pOXB-CMV-Fct4)	pCMV.SeV.EnvctHN (pOXB-CMV-coHN)	110:140:450:90:90
rHIV.H7rN1pr.hCEF.EGFP (vGM194)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.hCEF.EGFP (pOXB-hCEF-GFP)	pCMV.Rostock.H7.Puerto-rico.N1 (pVitro2-H7rN1pr)	pCMV.Rostock.M2 (pRKH-M2)	200:830:830:500:80
rHIV.H7shN1prPb.hCEF.EGFP (vGM191)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.hCEF.EGFP (pOXB-hCEF-GFP)	pCMV.Shanghai.H7.Polyb.Puerto-rico.N1 (pVitro2-H7shN1pr Polyb)	pCMV.Rostock.M2 (pRKH-M2)	50:100:900:150:18
rHIV.H7shN9shPb.hCEF.EGFP (vGM192)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.hCEF.EGFP (pOXB-hCEF-GFP)	pCMV.Shanghai.H7.Polyb.Shanghai.N9 (pVitro2-H7shN9sh Polyb)	pCMV.Rostock.M2 (pRKH-M2)	50:100:900:150:18
rHIV.VSVg.CMV.EGFP (DJ001)	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.EGFP (pOXB-HVCG)	pCMV.VGVg (pOXB-VSVg)	N/A	110:140:450:70
rHIV.H7rN1pr.CMV.EGFP Lux	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.EGFP Lux (pGM290)	pCMV.Rostock.H7.Puerto-rico.N1 (pVitro2-H7rN1pr)	pCMV.Rostock.M2 (pRKH-M2)	200:830:830:500:80
rHIV.H7shN9shPb.CMV.EGFP Lux	pCMV.HIV.Rev (pOXB-REV)	pHIV.CMV.GagPol (pOXB-HSGP)	pHIV.CMV.EGFP Lux (pGM290)	pCMV.Shanghai.H7.Polyb.Shanghai.N9 (pVitro2-H7shN9sh Polyb)	pCMV.Rostock.M2 (pRKH-M2)	50:100:900:150:18
rSIV.F/HN.CMV.EGFP (vGM010)	pSIV.CMV.Rev (pGM299)	pSIV.CBA.GagPol (pGM297)	pSIV.CMV.EGFP (pGM311)	pCAG.SeV.Fct4 (pGM301)	pCAG.SeV.EnvctHN (pGM303)	6:9:20:6:6
rSIV.F/HN.CMV.EGFP (vGM216)	pSIV.CMV.Rev (pGM299)	pSIV.CBA.coGagPol (pGM691)	pSIV.CMV.EGFP (pGM311)	pCAG.SeV.Fct4 (pGM301)	pCAG.SeV.EnvctHN (pGM303)	6:9:20:6:6
rSIV.F/HN.hCEF.EGFP (vGM107)	pSIV.CMV.Rev (pGM299)	pSIV.CBA.GagPol (pGM297)	pSIV.hCEF.EGFP (pGM357)	pCAG.SeV.Fct4 (pGM301)	pCAG.SeV.EnvctHN (pGM303)	6:9:20:6:6
rSIV.F/HN.hCEF.EGFP Lux (vGM141)	pSIV.CMV.Rev (pGM299)	pSIV.CBA.GagPol (pGM297)	pSIV.hCEF.EGFP Lux (pGM426)	pCAG.SeV.Fct4 (pGM301)	pCAG.SeV.EnvctHN (pGM303)	6:9:20:6:6
rSIV.F/HN.hCEF.sogLux (vGM124)	pSIV.CMV.Rev (pGM299)	pSIV.CBA.GagPol (pGM297)	pSIV.hCEF.sogLux (pGM358)	pCAG.SeV.Fct4 (pGM301)	pCAG.SeV.EnvctHN (pGM303)	6:9:20:6:6

Table 4 B						
LV name	Production method & scale	Batch	Purification method(s)	Activation with TrypLEselect	Titration method	Integrative titre: TU/ mL
rHIV.F/HN.CMV.EGFP (vGM182)	CF10	OBM335	O/N centrifugation (6000g) & Ultracentrifugation (68000g)	Yes	HEK293T 10-day	1.25E+08
	CF10	OBM337	TFF & Ultracentrifugation (68000g)	Yes	HEK293T 10-day	3.90E+07
	WAVE (5L)	Wave 96	Anion exchange & TFF	Yes	HEK293F 3-day	7.98E+08
	LV-MAX (500mL)	Wave 187	Anion exchange & TFF	Yes	LV-MAX 3-day	2.61E+08
rHIV.F/HN.CMV.tdTomato (vGM181)	CF10	OBM336	O/N centrifugation (6000g) & Ultracentrifugation (68000g)	Yes	HEK293T 10-day	1.74E+08
	CF10	OBM338	TFF & Ultracentrifugation (68000g)	Yes	HEK293T 10-day	2.75E+07
	WAVE (5L)	Wave 95	Anion exchange & TFF	Yes	HEK293F 3-day	6.15E+09
rHIV.F/HN.CMV.EGFP (vGM188)	WAVE (5L)	Wave 105	Anion exchange & TFF	Yes	HEK293F 3-day	5.45E+08
rHIV.H7rN1pr.CMV.EGFP (vGM222)	CF10	OBM368	O/N centrifugation (6000g)	N/A	HEK293T 10-day	1.33E+08
	LV-MAX (500mL)	Wave 193	Anion exchange & TFF	N/A	LV-MAX 3-day	2.34E+08
rHIV.H7shN1prPb.CMV.EGFP (vGM221)	CF10	OBM369	O/N centrifugation (6000g)	N/A	HEK293T 10-day	1.51E+06
	LV-MAX (500mL)	Wave 192	Anion exchange & TFF	N/A	LV-MAX 3-day	4.27E+06
rHIV.H7shN9shPb.CMV.EGFP (vGM220)	CF10	OBM370	O/N centrifugation (6000g)	N/A	HEK293T 10-day	1.13E+07
	LV-MAX (500mL)	Wave 191	Anion exchange & TFF	N/A	LV-MAX 3-day	4.65E+07
rHIV.F/HN.hCEF.EGFP (vGM193)	LV-MAX (500mL)	Wave 113	Anion exchange & TFF	Yes	LV-MAX 3-day	6.76E+08
rHIV.H7rN1pr.hCEF.EGFP (vGM194)	LV-MAX (500mL)	Wave 114	Anion exchange & TFF	N/A	LV-MAX 3-day	5.14E+09
rHIV.H7shN1prPb.hCEF.EGFP (vGM191)	LV-MAX (500mL)	Wave 116	Anion exchange & TFF	N/A	LV-MAX 3-day	1.62E+07
rHIV.H7shN9shPb.hCEF.EGFP (vGM192)	LV-MAX (500mL)	Wave 115	Anion exchange & TFF	N/A	LV-MAX 3-day	7.81E+07
rHIV.V5Vg.CMV.EGFP (DJ001)	N/A	N/A	N/A	N/A	HEK293T 10-day	8.20E+08
rHIV.H7rN1pr.CMV.EGFP Lux	LV-MAX (25mL) + 12.5 Units exogenous neuraminidase	N/A	N/A	N/A	LV-MAX 3-day Luciferase assay	N/A
	LV-MAX (25mL)	N/A	N/A	N/A	LV-MAX 3-day Luciferase assay	N/A
rHIV.H7shN9shPb.CMV.EGFP Lux	LV-MAX (25mL) + 12.5 Units exogenous neuraminidase	N/A	N/A	N/A	LV-MAX 3-day Luciferase assay	N/A
	LV-MAX (25mL)	N/A	N/A	N/A	LV-MAX 3-day Luciferase assay	N/A
rSIV.F/HN.CMV.EGFP (vGM010)	WAVE (5L)	Wave 40	Anion exchange & TFF	Yes	HEK293F 3-day	5.30E+09
	LV-MAX (500mL)	Wave 111	Anion exchange & TFF	Yes	LV-MAX 3-day	7.47E+08
	LV-MAX (500mL)	Wave 197	Anion exchange & TFF	Yes	LV-MAX 3-day	3.13E+08
rSIV.F/HN.CMV.EGFP (vGM216)	LV-MAX (1500mL)	Wave 176	Anion exchange & TFF	Yes	LV-MAX 3-day	3.78E+08
rSIV.F/HN.hCEF.EGFP (vGM107)	WAVE (5L)	Wave 83	Anion exchange & TFF	Yes	HEK293F 3-day	3.02E+09
	WAVE (5L)	Wave 102	Anion exchange & TFF	No	HEK293F 3-day	9.42E+08
	LV-MAX (500mL)	Wave 109	Anion exchange & TFF	Yes	LV-MAX 3-day	4.60E+09
	LV-MAX (500mL)	Wave 110	Anion exchange & TFF	Yes	LV-MAX 3-day	8.78E+08
	LV-MAX (500mL)	Wave 112	Anion exchange & TFF	Yes	LV-MAX 3-day	4.79E+09
	LV-MAX (500mL)	Wave 121	Anion exchange & TFF	Yes	LV-MAX 3-day	4.08E+09
rSIV.F/HN.hCEF.EGFP Lux (vGM141)	WAVE (1L)	Wave 62	Anion exchange & TFF	Yes	HEK293F 3-day	1.75E+10
rSIV.F/HN.hCEF.sogLux (vGM124)	WAVE (5L)	Wave 92	Anion exchange & TFF	Yes	HEK293F 3-day	9.42E+09

2.4.1. LV production using adherent HEK293T cells (Cell Factory system; CF10)

For productions in 10-layer Cell Factories (CF10; surface area 6320cm²; Nunc 170009, Thermo Fisher Scientific), 3.8e8 HEK293T cells were seeded in 1L FreeStyle 293 Expression medium (FreeStyle; Life Technologies) supplemented with 2mM L-Glutamine (Life Technologies). The following day plasmids to a total mass of 770µg per CF10 were transfected using Lipofectamine 2000CD (Invitrogen; 12566014) according to manufacturer's instruction in OptiPRO serum free media (Gibco), and after approximately 7 hours, transfection media was replaced with 1L FreeStyle supplemented with sodium butyrate (Sigma; known to enhance transfection efficiency and transgene expression (Cornelia M. Gorman, Howard, & Reeves, 1983)) to a final concentration of 10mM. 48 hours after transfection, media supernatant was removed, filtered using 0.22µm polyethersulfone membrane (Corning 431098), and treated with Benzonase nuclease (5000U per litre of supernatant; Merk, Darmstadt, Germany) and MgCl₂ (2mM; Sigma) for 1 hour at 37°C to digest nucleic acid contaminants. Vector was then stored at -80°C ahead of activation and purification if not proceeding immediately.

2.4.2. LV production using suspension HEK293T cells (WAVE Cellbag bioreactor)

For productions using serum-free/suspension-adapted HEK293T cells in WAVE bioreactors (1L or 5L scale), the relevant single use Cellbag (GE Healthcare) was filled with FreeStyle media (2L for 5L final production volume in a 10L Cellbag) containing 2.5 mL/L of 10% Pluronic P-68 (Life Technologies), and mounted on a WAVE 20/50 platform (GE Healthcare), rocking at 20 rpm to 8°. Inflated with 5% CO₂ and incubating at 37°C, the Cellbags were seeded with 8e5 HEK293T cells/mL and cultured for 48 hours with the pH of the growth media automatically maintained at 7.2 by CO₂ % adjustment and sodium bicarbonate (Sigma) addition using WAVEPODII controller (GE Healthcare). The cells were then counted, supplemented with 5 mL/L of 45 % glucose (Sigma), and transfected using PEIpro (PolyPlus-transfection) with a total of 0.33µg

plasmid DNA per 1e6 cells at a PEIPRO:DNA volume:mass ratio of 2:1. Approximately 18 hours post-transfection the remaining FreeStyle media to be added (3L for 5L production) was supplemented with 2.5 mL/L of 10% Pluronic P-68 and sodium butyrate (Sigma) sufficient to achieve final concentration 5mM in final volume of media. Approximately 48 hours post-transfection cells were again supplemented with 5 mL/L of 45 % glucose and Benzonase was added (to achieve 5U/mL final concentration). One hour before vector harvest (72 hours post-transfection) Benzonase was again added (same amount as above) along with MgCl_2 to achieve 1mM concentration. Harvest of vector containing supernatant was performed using a GE Ultra 5 μm filter (GE Healthcare) and an AcroPak 0.8/0.45 μm filter (Pall, Portsmouth, UK), and filtrate then stored at -80°C, or immediately activated where necessary (Table 4 B) and then cooled to 4°C ahead of purification.

2.4.3. LV production using suspension LV-MAX cells (LV-MAX Lentiviral Production system)

Productions using LV-MAX cells were carried out following the LV-MAX Lentiviral Production System (Gibco, Thermo Fisher Scientific) according to manufacturer's instruction (all LV-Max reagents obtained from Thermo Fisher Scientific). LV-MAX cells grown in LV-MAX production medium were transfected in the appropriate polycarbonate Erlenmeyer flask (Corning) at a cell density of 4e6 cells/mL as recommended using LV-MAX Supplement (5% of final volume). Plasmid DNA (1250 μg per 500mL production) was complexed with LV-MAX Transfection Reagent (6 μl per mL production volume) in Opti-MEM II Reduced Serum Medium (Thermo Fisher Scientific) made up to 10% of the final volume at transfection for 10 minutes at room temperature (RT; 20-25°C) prior to addition to cells. Approximately 5-6 hours post-transfection, LV-MAX Enhancer was added to achieve final volume of 4%. At 24 hours Benzonase was added to the flasks to achieve 5U/mL final concentration and, at 48 hours, the cultures were centrifuged at 5000g for 10 minutes at RT and the vector containing supernatant was filtered

using an AcroPak 0.8/0.45µm filter (Pall, Portsmouth, UK). The filtrate was then treated with Benzonase (5U/mL) again with MgCl₂ to achieve 1mM, incubating at 37°C for minimum of 30 minutes. The supernatant was then activated if necessary (Table 4 B) and cooled to approximately 4°C ahead of purification or stored at -80°C. Where investigated (Table 4 B; 3.2.3.1), exogenous neuraminidase from *Clostridium perfringens* was obtained from Sigma (N2876) and added to LV-MAX cells the day following transfection in parallel with the first Benzonase treatment.

2.5. LV purification

Crude vector harvests were thawed and activated where necessary (2.4; Table 4 B) immediately prior to purification, and cooled to 4°C.

2.5.1. Anion exchange chromatography of LV

Anion exchange chromatography was performed for LV purification as indicated (Table 4 B) using an AKTA Pure 150L chromatography station (GE Healthcare). Using Mustang Q XT5 quaternary ammonium AEX membranes (Pall), one or three x 5mL, or two x 1mL columns were used for 1500mL LV-MAX, 5L WAVE and 500mL LV-MAX productions respectively. For activated preparations, Defined Trypsin Inhibitor (Gibco; R007100) was added to achieve a 0.1X concentration before subsequent dilution (1:1) in 4°C 50mM Tris (pH7.5). Vector was loaded onto the membranes at a flow rate of 25 mL/min. A low-salt buffer (150mM (WAVE) or 100mM (LV-MAX) NaCl, 25 mM HEPES, pH 7.5) was then used to wash the membranes and the vector was then eluted by running high salt buffer (1 M (WAVE) or 0.5M (LV-MAX) NaCl, 25mM HEPES, pH 7.5). Fractions eluted containing LV were identified by peaks in UV absorbance.

2.5.2. Centrifugation of LV

For LV purifications as indicated in Table 4 B, overnight centrifugation at 6000g was carried out at 4°C for 18 hours using 500mL centrifuge tubes (361690; Beckman Coulter, High Wycombe, UK) and the JLA10.5 rotor (Beckman Coulter). Ultracentrifugation at 68000g was carried out at 20000rpm using a SW 41Ti rotor (Beckman Coulter) and 14x95mm ultracentrifuge tubes (331374; Beckman Coulter) at 4°C for 1.5 hours. Pellets were resuspended in TSSM or dPBS between centrifugation steps where both were carried out (indicated Table 4 B).

2.5.3. Tangential Flow Filtration (TFF)

Crude vector containing production supernatant or eluted fractions following anion exchange were subjected to tangential flow filtration (TFF) to purify and concentrate using a KR1li TFF system (Spectrum/Repligen, CA). Where TFF preceded an ultracentrifugation step (Table 4 B), supernatant was ultra-filtered using a 750kDa pore- 790cm² polyethylsulfone membrane (Spectrum) at sheer force of 3500s⁻¹ (flow rate 600mL/minute). Following anion exchange however, the eluted vector (diluted in TSSM 1-5 volumes) was ultra-filtered to 10mL using a 500kDa pore- 20cm² membrane (Spectrum; 15mL/minute), dia-filtered against 100mL of TSSM and finally concentrated again with ultrafiltration to approximately 5mL, aliquoted in 100µL single-use aliquots in sterile polypropylene 0.6mL microfuge tubes and stored at -80°C prior to use.

2.6. LV titration and testing in cell culture

2.6.1. Integration titre (qPCR)

The number of transducing units (TU) in an LV preparation was determined using quantitative PCR (qPCR) to detect integrated LV genomes after transduction of HEK293 derived cells. Depending on the LV preparation (Table 4 B), TU calculation was determined using either

suspension HEK293F or LV-MAX cells in a 3-day assay in-house (GM), or using adherent HEK293T cells in a 10-day assay at OXB. The number of integrated vector copies was calculated using qPCR of the cellular genomic DNA, targeting either the integrated WPRE or PSI sequences (third-generation LV genome; Figure 3).

In the 3-day assay, cultures of suspension HEK293F or LV-MAX cells were transduced at 5×10^5 cells/mL in FreeStyle or LV-MAX production media, with a five-point dilution series of LV in the presence of $8 \mu\text{g/mL}$ polybrene (Sigma). Approximately 72 hours post transduction, cells were harvested and genomic DNA extracted using QIAamp blood DNA kit (Qiagen). The number of integrated WPRE sequences was quantified in triplicate against a WPRE sequence DNA standard curve using TaqMan FAST Universal Master Mix (Life Technologies). The following primers targeting the WPRE sequence were used: Forward: TGGCGTGGTGTGCACTGT; Reverse: CCCGGAAGGAGCTGACA; Probe: FAM-TTGCTGACGCAACCCCCACTGG-TAMRA. Copies were normalised to the cellular genomic DNA determined either using a Nanodrop spectrophotometer (2.3.1), a Quant-iTTM PicoGreen dsDNA kit (Thermo Fisher Scientific), or duplex qPCR, targeting and quantifying an endogenous control sequence (CFTR gene) in parallel with WPRE. Method for quantification was selected according to sample numbers. Quantification of endogenous CFTR in duplex qPCR was carried out using the following primers: Forward: CTTCCCCCATCTTGGTTGTTC; Reverse: TGACAGTTGACAATGAAGATAAAGATGA; Probe: VIC-TGTCCCCATTCCAGCCATTTGTATCCT-TAMRA. The qPCR was initiated with 2 minutes at 50°C , followed by 10 minutes at 95°C . 40 cycles of 94°C (15 seconds) and 60°C (1 minute) then followed. Analysis to determine TU/mL of LV was performed using Prism 7 software (GraphPad Software, Inc., CA, USA), generating a linear regression curve from the dilution series.

In the 10-day assay used at OXB cultures of adherent HEK293T cells were transduced at $1-3 \times 10^5$ cells per well (12 well; corning 353043) in DMEM media supplemented as described (2.2.1). A three-point dilution series of LV was delivered to the cells in the presence of $8 \mu\text{g/mL}$ polybrene (hexadimethrine bromide; OXB). The transduced cells were passaged three times

following transduction before DNA extraction to quantify integrated LV genome copies, passaging the cells twice 1:5 and once 1:8 into fresh media. On day 10 post-transduction, cellular DNA was extracted using the QIAcube HT/QIAxtractor system (Qiagen). Duplex PCR was then carried out using TaqMan FAST Universal Master Mix (Life Technologies) as before to quantify integrated LV copies, however alternative primers (OXB) were used; targeting integrated LV PSI (packaging signal; rHIV) sequence and endogenous (housekeeping) RPPH1 gene (ribonuclease P RNA component H1) to normalise to cellular genome DNA analysed. qPCR thermal cycling conditions were same as above.

Values obtained using both suspension cell approaches in 3-day assay were statistically indistinguishable (Dr Toby Gamlen, personal communication) and were used interchangeably. However, TU values obtained using 10-day assay in adherent cells were determined as approximately 6-fold lower following repeated titrations of reference LV preparations. For example, the TU/mL of an independent batch of OXB rHIV.VSVg.CMV.EGFP (DJ001; CTRL-015) was determined as 1.4×10^6 TU/mL using 10-day assay at OXB, but a TU value of 1.2×10^7 TU/mL was calculated using the 3-day assay with HEK293F cells in-house. Therefore, to approximate equivalent TU values of LV titrated using the 10-day or 3-day assay, a 6-fold greater TU value of preparations titrated using the 10-day assay was used.

2.6.2. Fluorescence-Activated cell sorting (FACS)

As an alternative measure of functional LV particles, Fluorescence-activated cell sorting (FACS) was used to quantify cells expressing EGFP following transduction. Cells (HEK293T 2.6.4) were collected using recombinant trypsin analog (TrypLEselect; Life Technologies) to detach where necessary, centrifuged (200g, 5 minutes), washed once in PBS, centrifuged again (200g, 5 minutes) and resuspended in PBS prior to measurement on an LSRII cytometer (BD Biosciences, San Jose, USA) using the parameters in Table 5. Percentages were then determined following analysis using FlowJo X software (BD Biosciences). Gating based on forward and side scatter of

particles was used to exclude cell debris and doublets. Untransduced cells were used to set a threshold median fluorescence intensity and facilitate gating for EGFP-positive cells.

Table 5- FACS parameters.

	Dichroic mirror	Bandpass filter	Voltage
Native EGFP	505LP	515/20	350
Forward scatter (FSC)	-	-	400
Side scatter (SSC)	-	-	200

2.6.3. Quantification of EGFP expression by well area

Where not possible to determine a percentage of cells expressing EGFP by FACS, adherent cells were imaged in the clear 96-well plates in which they were seeded and transduced (corning 353916) using the EVOS FL Auto 2 Imaging System; a 10x objective was used to capture images that were automatically stitched to create a complete well image as described (2.8.1). Images were then analysed using Image J software (NIH). Using images from untreated wells the background “threshold” fluorescence was set and subsequently the percentage area of EGFP fluorescence from images of treated wells quantified to determine EGFP expression.

2.6.4. Luciferase assay

Expression of firefly luciferase from cells transduced with Lux-expressing LV was also measured to determine relative amounts of functional LV produced. Using the Luciferase Assay System (Promega) transduced cells were subjected to lysis using Reporter Lysis Buffer (RLB; Promega) and then using a Spectramax i3x (Molecular Devices, San Jose, USA) and Softmax Pro 7 software provided, relative light units (RLU) emitted from the lysed cells were measured after injection of the Luciferase Assay Reagent (Promega). The RLU were then plotted against the dilution of LV delivered to cells to determine relative concentration of functional LV (Figure 17).

2.7. Immunohistochemistry and lectin staining

Immunohistochemistry (IHC) and lectin staining was performed on cells either grown in adherent cultures, or in cryosections generated from tissues, to characterise cell type or presence of receptor. Staining was performed on slides using a humid Staintray staining system (Simport, Quebec City, Canada), or directly on cells maintaining humid conditions using suitable laboratory plasticware. Where fixation was required (fresh samples), cells were treated with 4% PFA (2.1) for at least 20 minutes ahead of, where applicable, antigen retrieval (2.7.1) and subsequent staining (2.7.2 or 2.7.3). Stained samples were mounted using ProLong Gold Antifade medium with DAPI (4',6-diamidino-2-phenylindole; P36935, Invitrogen, Thermo Fisher Scientific) and incubated at RT in the dark overnight prior to immediate imaging or transfer to 4°C for storage.

2.7.1. Antigen retrieval

Where indicated, heat-induced antigen retrieval was performed prior to immunohistochemistry, or lectin staining, using freshly prepared Sodium Citrate buffer (10mM sodium citrate (2.94g/L), 0.0005% Tween 20 (Thermo Fisher Scientific), pH6). Fixed cells or cryosections were washed at RT in Tris Buffered Saline (TBS; Sigma) three times before subjecting to retrieval in Sodium Citrate buffer pre-heated to 98°C in a plastic coplin jar (Thermo Fisher Scientific) using a suitably temperature-controlled water bath. After 15 minutes, the cells or cryosections were transferred immediately into RT TBS and allowed to cool for 20 minutes before staining.

2.7.2. Immunohistochemistry

For immunohistochemistry, a Blocking Buffer (0.1% Triton X-100 (Sigma), 1% Bovine Serum Albumin (BSA; Sigma) and 5% Normal Goat Serum (NGS; Sigma)) was freshly prepared in PBS and used for permeabilization, blocking and for all dilutions of primary and secondary antibodies

used. Cells or sections were washed three times in TBS or transferred from TBS following antigen retrieval and incubated for 1 hour at RT in blocking buffer prepared at RT to permeabilise and block cells. For samples on slides, the sections were then washed in TBS once and allowed to air dry before using a hydrophobic pen (Sigma) to demarcate the tissue/ cell region to be stained. Primary antibodies were then applied (1:100 dilution) and incubated on the slides/cells overnight at 4°C in a dark humidified atmosphere created by a Staintray (Simport) or suitable laboratory plasticware. Slides and cells were then washed three times in Blocking Buffer (RT, 5 minutes) before application of the relevant goat secondary antibody(s) (against the primary IgG species conjugated to Alexa Fluor 594 or 488; Table 6), diluted 1:500 in Blocking Buffer. After 1 hour at RT, cells or slides were washed again (three times in Blocking Buffer, 5 minutes each) and mounted as above (2.7) or incubated in TBS for immediate imaging in wells.

Table 6- Antibodies used.

Cell type	Antibody	Source	Catalog reference number
Ciliated cells	Mouse anti- β -Tubulin	Chemicon Int, Temecula, Ca	MAB3408
Club cells	Mouse anti-CC10	Santa Cruz Biotechnology, Inc Dallas, TX, USA	sc-365992
Goblet cells	Mouse anti-Mucin 5Ac	Abcam plc	Ab212636
Basal cells	Rabbit anti-Cytokeratin 5	Abcam plc	Ab52635
Type II alveolar cells (ATII)	Rabbit anti-Prosurfactant Protein C	Millipore, Temecula, CA, USA	AB3786
GFP	Chicken anti-GFP	Abcam plc	Ab13970
Secondary	Goat anti-mouse Alexa Fluor 594	Life Technologies	A11005
Secondary	Goat anti-rabbit Alexa Fluor 594	Life Technologies	A11012
Secondary	Goat anti-chicken Alexa Fluor 488	Life Technologies	A11039

2.7.3. Lectin staining of sialylated receptors

For staining of sialylated receptors using lectins, a solution of 1% BSA (Sigma) in TBS was freshly prepared and used for blocking and dilution of lectins and conjugates (Lectin Blocking Buffer; LBB). To control for non-specific lectin staining, sample replicates were first pre-treated (following antigen retrieval where necessary) with sialidase from *Arthrobacter ureafaciens* (sialidase A; AdvanceBio GK80040 Agilent, CA, USA), applied at a concentration of 0.5U/mL in

Reaction Buffer B (Agilent); control samples were incubated overnight at 37°C using a HybEZ™ II Oven (ACD, CA, USA) and then washed in TBS. All samples were subsequently washed in TBS (minimum 15 minutes) or subjected to antigen retrieval (2.7.1 where indicated), before blocking using Streptavidin/Biotin Blocking Kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instruction. Samples were washed again in TBS before application of the freshly prepared LBB for at least 20 minutes at RT. Samples were then incubated with Fluorescein-labelled, or biotinylated, lectins (Table 7) prepared to 10µg/mL in LBB, at 4°C in a dark humidified Staintray (Simport) or plasticware overnight. The following day, samples were washed in TBS (3 times, 15 minutes each) and then streptavidin-conjugated Alexa Fluor 594 (Invitrogen; 2mg/mL S11227) was applied at 1:500 dilution in LBB (2 hours, RT). After washing, samples were mounted as above (2.7) or incubated in TBS for immediate imaging in wells.

Table 7- Lectins used for sialylated receptor staining.

Lectin	Source	Catalog reference number
Biotinylated <i>Maackia Amurensis</i> Lectin I (MAI)	Vector Laboratories	B-1315-2
Biotinylated <i>Maackia Amurensis</i> Lectin II (MAII)	Vector Laboratories	B-1265-1
Biotinylated <i>Sambucus Nigra</i> Lectin (SNA)	Vector Laboratories	B-1305-2
Fluorescein labelled <i>Sambucus Nigra</i> Lectin (SNA)	Vector Laboratories	FL-1301-2

2.8. Microscopy

2.8.1. Widefield microscopy

All cells and tissue were imaged for fluorescence or brightfield using an EVOS FL Auto 2 Imaging System (Thermo Fisher Scientific) with 10x or 20x EVOS long working distance fluorite objectives (AMEP4981 and AMEP4624) unless otherwise stated. For capture of stitched images, the sample location was determined using brightfield and a 4x objective (AMEP4680), or was pre-defined using the plate vessel format, and field selected using the EVOS FL Auto 2 software (Thermo Fisher Scientific). The software was then used to automate image capture and stitching of the

selected field using the 10x objective and autofocus every two fields captured. Where fluorescence was to be compared between samples within an experiment, the settings (light intensity, exposure and gain) for image capture were saved and maintained. The EVOS DAPI (AMEP4950), GFP (AMEP4951) and Invitrogen Texas Red (AMEP4955) light cubes were used to detect DAPI, EGFP or fluorescein, and tdTomato or Alexa Fluor 594 fluorescence respectively. Nikon Optiphot-2 microscope was used only for imaging of nasal septum tissue, images were taken using UV fluorescence and GFP-B or Texas Red bandpass filter cubes to detect native EGFP and tdTomato fluorescence respectively.

2.8.2. Confocal microscopy

For confocal imaging, a Zeiss LSM 780 inverted confocal microscope (Zeiss, Jena, Germany) and 25x (LD LCI Plan-Apochromat; Zeiss) or 63x oil immersion objective (Plan-Apochromat; Zeiss) was used, and images captured using the ZEN Black 2011 SP7 LSM software (Zeiss). Alexa Fluor 594 was detected by excitation with a HeNe543 laser and emission filtered by a 595nm pass filter. EGFP was excited using a 488nm laser, filtered with a 505-550 nm band pass filter. DAPI excitation used a 405 nm laser and the detection range was filtered to between 420-490 nm.

2.9. *In vivo* mouse model

All animal experiments were carried out in compliance with the UK Home Office under the approved project and personal licenses. All mouse experiments were performed using BALB/c adult mice (6-8 weeks of age) obtained from Envigo RMS (Loughborough, UK), housed in accordance with the UK Home Office ethical and welfare guidelines and fed on a diet of chow and water *ad libitum*. All mice were allowed to acclimatise following delivery to the Biomedical Services Unit (BMS) (University of Oxford, John Radcliffe Hospital, Oxford, UK) for at least 7 days prior to commencement of any procedures, all of which took place within the BMS.

2.9.1. Intranasal delivery (LV and sialidase)

For nasal instillation, mice were anaesthetised using inhalation of 3-4.5% isoflurane (Abbott, Maidenhead, UK) at induction and 2.5- 3% isoflurane to maintain. Depth of anaesthesia was monitored continuously by observation for breathing rate and reflex response to a foot pad pinch test. After confirming absence of reflex, mice were intranasally dosed as described previously (Xenariou et al., 2007); holding the mouse vertically with their mouth closed, a total volume of up to 100µL was delivered by continuous application of a droplet to the nose. Mice were then allowed to recover from general anaesthesia and regularly observed over the 24 hours following administration. For all mice treated with LV, vector was formulated in TSSM and each mouse received the total TU in 100µL in a single procedure. Mice dosed intranasally with sialidase A (GK80040, Agilent) or sialidase S (from *Streptococcus pneumoniae* AdvanceBio GK80021, Agilent), received 200mU diluted in reaction buffer B provided (5x, 250mM sodium phosphate pH6), or reaction buffer only.

2.9.2. Polidocanol (PDOC)

Mice dosed with polidocanol (2% v/v in PBS, Sigma; P9641; PDOC) received a total of 20µL by intranasal delivery (2.9.1). Immediately following dosing, however, all mice received a dose of 0.1mg Buprenorphine (Vetergesic; BMS)/kg body weight by subcutaneous injection for analgesia and mice were carefully observed for signs of respiratory distress during anaesthesia recovery and over the 48hrs following PDOC administration and euthanised as required.

2.9.3. BLI (IVIS)

Live Bioluminescence Imaging (BLI) of mice dosed with LV expressing Luciferase was performed to measure Luciferase activity. Mice to be imaged were dosed with 100µl of 15 mg/mL D-luciferin (PerkinElmer, Waltham, USA) by intranasal delivery (2.9.1), however, anaesthesia was

maintained (2.5-3% isoflurane) whilst the animals were then visualised using the IVIS Lumina LT Imaging System (PerkinElmer) at 15 minutes after intranasal administration. Using the software programme Living Image v4.5 (PerkinElmer), photon emission was captured and luciferase activity of the nose and lung of each mouse calculated by selecting a region of interest (ROI) and determining the average luminescence as photons/sec/cm².

2.9.4. Mouse tissue processing (cryosections and nasal septum recovery)

All mice were euthanised by cervical dislocation and, where required, nasal septum tissue was recovered immediately post mortem by dissection as described (Antunes et al., 2007); tissue was then laid flat on Superfrost Plus glass slides (VWR, PA, USA) for immediate imaging (2.8.1). All mice were dissected to expose the lungs and trachea. A small incision was then made just below the larynx in the trachea and a cannula of 0.75 mm (Harvard Apparatus, Kent, UK) attached to a blunt 21G needle (Becton Dickinson, Plymouth, UK) was carefully inserted and secured to the trachea with a suture knot (Johnson & Johnson, St-Stevens-Woluwe, Belgium). Using a 5mL syringe (Becton Dickinson) the lungs were then slowly inflated *in situ* with 4% PFA (2.1) before dissection to remove intact trachea and lungs, and submersion in 4% PFA (overnight, RT). The following day the lungs and trachea were re-inflated and submerged in 30% sucrose in PBS (overnight, RT) before final inflation with a 1:1 mixture of 30% sucrose and Optimal Cutting Temperature (OCT) embedding medium (Thermo Fischer Scientific; mixed by vortex and allowed to settle to clear bubbles before use). Immediately following inflation, the lungs were dissected into the lobes required, maintaining the trachea attached to the left lobe where possible. The lobes and trachea were then submerged in plastic embedding moulds (Sigma) pre-filled with sucrose/OCT mixture and transferred to -80°C for rapid freezing ahead of cryosectioning. Cryosections of 7µm were generated using a Thermo Fischer Scientific CryoStar NX50 with the specimen holder set to -30°C. Sections were collected after trimming of the lobes at 30µm increments and where possible the left lung lobe and trachea were cryosectioned to generate

coronal sections exposing the greatest abundance of proximal and distal airways. Consecutive cryosections were collected onto Superfrost Plus glass slides (VWR), allowed to air dry for at least 30 minutes before short term storage at 4°C ahead of mounting or staining (2.7 and 2.9.4.1).

2.9.4.1. Haemotoxylin and Eosin staining

Morphology of lung tissue cryosectioned was assessed using Haemotoxylin and Eosin staining of the cryosections generated. Cryosections were dehydrated by immersion in ethanol (100% and then 70% for 3 minutes each). Sections were then incubated for 5 minutes in water before Haemotoxylin solution (Harris modified; Sigma; 1 minute) and subsequent return to water (5 minutes). Sections were then transferred to 0.5% Eosin Y (MP Biomedicals) prepared in 95% ethanol for 30 seconds before transfer to ethanol (70% and then 100%, 3 minutes each). Sections were then incubated in Xylene for 5 minutes prior to mounting with DPX mountant (Sigma).

2.10. Precision Cut Lung Slices (PCLS)

2.10.1. Generation and culture

Precision Cut Lung Slices (PCLS) were cultured (37°C, 5% CO²) in PCLS culture medium (high glucose DMEM (Sigma; D5796) supplemented with 100 U/mL penicillin plus 100 mg/mL streptomycin (Sigma), 4mM L-Glutamine (Life Technologies), 20µg/mL Gentamicin (Sigma) and 1.25µg/mL Amphotericin B (Sigma)).

For the generation of (PCLS), immediately prior to harvest of fresh unfixed lung tissue, 6% ultra-low melting point (LMP) agarose (Type IX-A; A2576; Sigma) was prepared by dissolving in Hank's Balanced Salt Solution (HBSS; Gibco 14175053) using a microwave and then equilibrating to 42°C (using a temperature-controlled water bath) before addition of penicillin and streptomycin to achieve 100 U/mL and 100 mg/mL respectively (Sigma). Mouse and sheep

lungs were inflated immediately post mortem, maintaining lungs and trachea at, or above RT, for inflation *in situ* with the LMP agarose at 42°C. Human, pig, ferret and macaque lung tissues, however, were transferred immediately following removal into PCLS culture medium at RT due to logistical restrictions for immediate inflation, inflation with LMP agarose was carried out within 4 hours following cadaver removal. Where possible, cannulation of the supplying lung lobe bronchi or trachea was secured before inflation, although for human lung tissue obtained from patient resections, the available airways were injected using a blunt 21G needle (Becton Dickinson). Lung segments were then cooled on ice (≥ 45 minutes) to allow gelling of the agarose before cores of the tissue were taken using a 10mm disposable biopsy punch (VWR) and mounted onto the vibrating microtome (compresstome VF-300-0Z; Precisionary, Boston, USA) for sectioning. Thick (500 μ m) sections were cut using an oscillation and speed setting of 5.

Following cutting, PCLS were collected in the compresstome buffer tray filled with RT HBSS with penicillin and streptomycin (100 U/mL and 100 mg/mL; Sigma) and transferred to Falcon tubes (15mL nominal volume) containing 7mL of PCLS media for washing and subsequent culturing, or into 4% PFA for uncultured PCLS cryosectioning. The tubes were then inverted continuously (37°C, overnight, 18x/minute) using a tube rotator (115 VAC, Cole-Parmer, St Neots, UK). The following day PCLS were transferred to static culture (37°C, 5% CO₂) in PCLS culture medium. One PCLS was cultured in 1mL media per well of a 24 well plate (Corning). Fresh media was exchanged for conditioned media every 2-3 days.

Mouse PCLS were generated using adult BALB/c mice (6-8 weeks) obtained from Envigo RMS and euthanised by cervical dislocation. Fresh human PCLS were generated using normal human lung tissue was obtained from through the Oxford Radcliffe Biobank (ORB, biobank project reference 18/A104), approved by the ORB research bank ethics committee (reference 09/H0606/5+5). Patients undergoing lobectomy were identified in advance of surgery and lobes without any intact bronchioles had to be rejected as they would not feasibly allow for inflation. Ferret PCLS were generated from an adult male (7 months of age) obtained from Marshall

Bioresources/B&K Universal (Hull, UK). Euthanised by a veterinary anaesthetist, the Ferret received a lethal dose of Pentobarbitone under general anaesthesia. With kind assistance from Tamara Ramadan the trachea and lungs were carefully recovered. Fresh pig and rhesus macaque (*Macaca mulatta*) lung were kindly gifted by Tamara Ramadan and Maria Martinez, as excess tissue following terminal procedures at the Department of Biomedical Services (Oxford, UK), in which removal of lung tissue prior to animal perfusion with fixative was feasible. Whole pig lung was removed prior to any perfusion, Macaque lung lobes were detached following PBS perfusion of the cadaver. Fresh ferret trachea explant was cultured without prior agarose inflation or precision cutting, material was transferred directly to static culture in PCLS media without washing.

2.10.2. Assay of PCLS viability

To assess PCLS viability, they were stained using the Live/Dead Viability/Cytotoxicity kit for mammalian cells (L3224; Life Technologies). Untreated PCLS were selected at random and incubated (45 minutes, 37°C) in PCLS media (200µL) containing 4µM calcein-AM (1:1000 dilution) and 4µM ethidium homodimer-1 (1:500 dilution). The PCLS were then imaged immediately in the media/dye applied using the EVOS FL Auto 2 (2.8) with the plate imaging vessel setting, to navigate and capture representative images of each PCLS stained. Green fluorescent calcein indicating live cells (hydrolysis of calcein-AM by intracellular esterases) was detected using the GFP light cube (2.8.1). Fluorescence from ethidium homodimer-1 (upon DNA binding indicating dead cells) was detected using the Invitrogen Texas Red light cube (2.8.1). Untreated PCLS were imaged to determine settings for detection of minimal autofluorescence. A 'dead' PCLS, treated with 70% ethanol for 15 minutes, was stained and imaged in parallel as a control.

2.10.3. Transduction of PCLS

Freshly generated PCLS were transduced within 2-7 days of initiating static culture unless otherwise stated. PCLS were transduced by transfer to wells of a 24 well plate pre-filled with 500µL of PCLS media and 100µL of LV in TSSM. After 3.5hrs media was replaced with fresh PCLS media (1mL) for continued culture ahead of transgene expression detection.

2.10.4. In culture imaging for native EGFP in PCLS

Native EGFP fluorescence from PCLS transduced with LV was detected by imaging 'in culture' using the EVOS FL Auto 2, GFP light cube and 10x objective (2.8). Each PCLS was imaged in 200µL PCLS media in wells of a 24-well plate (Corning). Untreated PCLS were imaged in parallel to determine light settings for detection of minimal autofluorescence and representative images of each PCLS captured.

2.10.5. Cryosections

Cultured and uncultured PCLS were fixed in 4% PFA (2.1) (minimum 20 minutes, RT) before cryoprotection (30% sucrose in PBS overnight at RT) and embedding for cryosectioning. Each PCLS was placed at the bottom of a plastic embedding mould (Sigma), and covered with OCT (Thermo Fischer Scientific) before immediate transfer to -80°C. Cryosections (7µm) were generated as before (2.9.4) however ensuring the mounted PCLS aligned in order to cut coronal sections.

Cryosections of human lung (7µm, fixed in 4% PFA) were gifted from colleagues of the UK Cystic Fibrosis Gene Therapy Consortium (UKCFGTC; Dr Gerry McLachlan, Roslin Institute Edinburgh, UK). All cryosections received were from the untreated right lobe of lung tissue from a single donor (AFFA448), received from the USA (International Institute for the Advancement of Medicine), subjected to *ex-vivo* lung perfusion (EVLPE), processed into PCLS and cryosectioned

before receipt. These cryosections were received, held and used following approval by the Oxford University Medical Sciences Interdivisional Research Ethics Committee (IDREC; R59182/RE001), stored under and in compliance with under Human Tissue Act (HTA) licence 12217. Cryosections used for lectin staining (2.7.3) were consecutive, from a region checked for the presence of airways.

2.10.6. Gaussia luciferase assay

The secretion of Gaussia luciferase (GLux) by PCLS transduced with GLux-expressing LV was assayed by sampling of the PCLS media prior to fresh 3-day media exchange. Samples of 100µL were immediately frozen (-20°C) and thawed once, ahead of assaying for GLux activity. The light produced as a result of the reaction catalysed by GLux in the presence of coelenterazine (BioLux Gaussia Luciferase Assay Kit, NEB) was measured using SpectraMAX i3x microplate reader (Molecular Devices). Fifty µL of GLux assay substrate prepared in the BioLux Gluc assay buffer (NEB) was injected to wells containing 20µL of sample and the light output recorded after 2 seconds in relative light units (RLU).

2.11. Air-liquid interface culture

Air-liquid interface (ALI) culture of cells modelling both the human airway and parenchymal respiratory epithelium were generated and/or maintained. All ALI were cultured on 6.5mm, 0.4µm pore transwell inserts (3470; Corning or Epithelix Sarl, Geneva, Switzerland) in 24 well plates (Corning). Incubated at 37°C and 5% CO₂ (humidity 100%), media in the plates (500-700µL, basal chamber) was replaced with fresh every 48-72 hours (2-3 days).

2.11.1. Human airway ALI

Human airway epithelial cells cultured at an ALI were either generated through differentiation of human bronchial epithelial cells (HBEC) in-house, referred to as Bronchial ALI (B-ALI) or obtained fully differentiated from Epithelix Sarl (Geneva, Switzerland). Differentiated cultures received from Epithelix Sarl were derived from either bronchial (MucilAir) or bronchiolar (SmallAir) epithelial cells and cultured upon receipt according to manufacturer's instruction using the respective MucilAir and SmallAir culture medium provided (Epithelix Sarl). B-ALI were generated using HBEC obtained from Lonza (Basel, Switzerland; CC-2540S), expanded and differentiated using the PneumaCult culture system from Stemcell technologies (Vancouver, Canada) and maintained using complete PneumaCult ALI Maintenance Medium (Stemcell technologies) made up as directed, supplemented with 2.5µg/mL Amphotericin B (Sigma).

Cryovials of HBEC (1mL of $\geq 5 \times 10^5$ cells) were thawed rapidly at 37°C and immediately diluted by dropwise addition of pre-warmed PneumaCult Ex Plus medium (30 minutes at 37°C; Stemcell technologies). Cells were then expanded in submerged culture following manufacturer's instructions with PneumaCult Ex Plus media (Stemcell technologies), exchanging media for fresh every 2 days until 80% confluency was achieved. Cells were dissociated using Animal Component Free Cell Dissociation Kit (Stemcell technologies) and 3.3×10^4 cells were seeded onto 6.5mm, 0.4µm pore transwell inserts (3470; Corning) in 200µL of PneumaCult Ex Plus media according to the PneumaCult ALI protocol. Cells were then cultured submerged in PneumaCult Ex Plus media (200µL apical and 500µL basal) until 100% confluency was reached (approximately 2 days). Cultures were then 'airlifted' to culture in the complete PneumaCult ALI Maintenance Medium (Stemcell technologies) supplied in the basal chamber alone to allow differentiation and subsequent maintenance.

All airway epithelial ALI cultures were subjected to PBS washing once per week, from receipt of MucilAir and SmallAir cultures and day 7 following Airlift of B-ALI cultures. Washing involved application of 200µL pre-warmed dPBS to transwell interior (apical chamber),

incubation (20 minutes, 37°C), with gentle repetitive pipetting of 100µL to dislodge mucus from apical surface, and aspiration of dPBS and mucus.

2.11.2. Surfactant ALI (SALI) cultures

Surfactant ALI (SALI) cultures were generated from H441 cells (HTB-174; ATCC) as described (Munis, Hyde, & Gill, 2020). Briefly, H441 cells were cultured in RPMI-1640 (Gibco; A1049101) supplemented with 10% Fetal Calf Serum (FCS; Sigma), penicillin (50 U/mL) and streptomycin (50 mg/mL). Approximately 5e4 cells were seeded onto 6.5mm, 0.4µm pore transwells (Corning) and cultured submerged (200µL apical and 500µL basal) for 72hrs. Transwells were subsequently airlifted and media in basal chamber exchanged for polarisation medium used to maintain cultures at the ALI (RPMI-1640 supplemented with 4% FCS, 50U/mL penicillin, 50mg/mL streptomycin, 1uM Dexamethasone (Sigma), 1% insulin-transferrin-selenium (Gibco) and 2mM L-Glutamine (Life Technologies)).

2.11.3. Trans Epithelial Electrical Resistance (TEER)

The TEER of ALI cultures was measured using a Millicell 392 ERS-2 Voltohmmeter (MERS00002; Milipore, Merk) according to manufacturer's instructions. Cultures were submerged (200µL apical and 500µL basal) in dPBS (RT) and equilibrated for 15 minutes prior to measurement using the 'chopstick' electrodes. Resistance values were calculated by subtraction of values measured using transwells without cells and then normalised to the culture surface area of the transwells (0.33cm²) for presenting as Ω.cm².

2.11.4. Sialidase, lectin treatments and transductions of ALI cultures

ALI cultures were transduced by apical administration of the appropriate dose (TU) of vector made up to 100µl of TSSM buffer (3.5hr, 37°C). Airway epithelial cell ALI cultures were treated

or transduced a minimum of 8 weeks post airlift, and 24hrs following washing. SALI cultures were transduced at 14 days post-airlift. For sialidase treatment, ALI transwell inserts were apically treated with 25mU sialidase A (GK80040, Agilent) or sialidase S (GK80021, Agilent) diluted in reaction buffer provided (buffer B) to 50µL. Sialidase was incubated on the ALI culture (1hr, 37°C), aspirated, and washed briefly in dPBS (RT) before immediate transduction or fixation to process for cryosections (2.11.7). SALI cultures were pre-treated where indicated (6.2.8) with unconjugated lectins. MAI, MAII or SNA (Vector Laboratories; L-1310, L-1260 and L-1300 respectively) were prepared to the appropriate concentration in 100µL dPBS, applied apically and incubated on the SALI for 1hr at 37°C, aspirated, and washed briefly in dPBS (RT) before immediate transduction.

2.11.5. In culture imaging of ALI cultures

ALI cultures were visualised throughout maintenance to confirm ciliated cell motility (airway ALI) and to detect native EGFP fluorescence using the EVOS FL Auto 2 (2.8). Following transduction of ALI cultures with EGFP expressing LV, the cultures were imaged (using the GFP light cube and 10x objective) to detect EGFP fluorescence by automated scanning and stitching of the entire cultured surface area under identical settings within a single experiment (2.8).

2.11.6. Quantification of ALI EGFP fluorescence.

The percentage area of EGFP fluorescence above threshold was quantified using the stitched images captured of the entire transwell (2.11.5). A circular ROI was generated using Image J (NIH) to exclude analysis of transwell edges. Background 'threshold' fluorescence was then determined using images captured from untreated ALI cultures and the area EGFP fluorescence above threshold was subsequently calculated for each transwell.

2.11.7. Cryosections of ALI culture transwells

ALI cultures were fixed by submersion of transwells in 4% PFA (2.1) (20 minutes, RT) before cryoprotection in 30% sucrose in PBS (at least 1 hour, RT). The transwell membranes (and cultured cells attached) of each ALI culture were then extracted using a scalpel, taking care to avoid disturbance/detachment. Membranes were then embedded in OCT using a plastic embedding mould and transferred to -80°C. Cryosections (7µm) were generated as before (2.9.4), though ensuring where possible that the membrane was perpendicular (approximately 90°) to the blade to allow representative capture of the stratified cell layers in a cross-section of the culture.

2.12. Investigating receptor binding using non-pulmonary cells

2.12.1. Glycans

All glycans used in Chapter 6 were obtained from either Dextra (Reading, UK; specifically 3'-Sialyl-LacNAc (SLN302), 6'-Sialyl-LacNAc (SLN306) and LSTc (SLN506)), or from Elicityl (Crolles, France; specifically LSTd (GLY083), Galacto-N-Neopentaose (GLY024), LSTa (GLY081), GM3 (GLY090) and GM1a (GLY096)). All were reconstituted in RT dPBS (maximum concentration 6.4µmol/mL), stored at +4°C (<4 weeks) prior to use.

2.12.2. Sialyltransferase and sialidase treatment of MDCK-Siat1 cells

α2,3-sialyltransferase from *Pasteurella multocida* (S1951), α2,6-sialyltransferase from *Photobacterium damsela* (S2076) and substrate CMP-Sia (cytidine-5'-monophosphate-sialic acid, C8271) were all obtained from Sigma and reconstituted in nuclease-free water. Where MDCK-Siat1 cells were treated with sialyltransferase in 96-well plates (6.2.5.3), 20,000 cells/well were incubated with 31.25mU plus 62.5µmol CMP-Sia, in 50µL dPBS at 37°C. Where MDCK-Siat1 cells were treated with sialidase (6.2.5.2), 20,000 cells/well were incubated with 50µL of

sialidase A (GK80040, Agilent) at 50mU/mL, or sialidase S (GK80021, Agilent) at 200mU/mL, prepared in reaction buffer provided (buffer B).

2.12.3. Hemagglutination assay.

Chicken red blood cells (RBC) preserved in Alsever's solution were purchased from TCS Biosciences (FB010AP; Buckingham, UK) and prepared in dPBS to 1% (RBC volume) immediately prior to use in each assay, following three washes (repeated centrifugation at 1400g for 5 minutes and resuspension in dPBS). Prior to each Hemagglutination Inhibition assay (6.2.3), the minimum concentration of each LV preparation required to hemagglutinate the RBC used (known as the HA unit; (Hirst, 1941) was determined by titration. Serial dilutions of LV in 50 μ L TSSM were prepared in 96 well U-bottom plates (CLS3367; Corning). Subsequently, 50 μ L of the 1% RBC preparation was added to each well to create a final RBC concentration of 0.5%; after incubation (45 min, RT), hemagglutination was determined by visual inspection, indicated by absence of an RBC pellet in the plates.

2.13. Statistical Analysis

All statistical analyses were performed using Prism 7, (GraphPad, La Jolla, CA, USA). A calculated p value of > 0.05 was deemed non-significant (indicated with 'ns' on figures). Calculated p values of < 0.05 were deemed a significant difference and indicated with one star (*) on figures. More statistically significant p values of < 0.01 , < 0.001 and < 0.0001 were indicated with two (**), three (***), or four stars (****), respectively.

3. Chapter 3- Pseudotyped lentiviral vectors to target murine lung *in vivo*

3.1. Introduction

3.1.1. Pseudotypes

Both LV.F/HN (1.4.2.4) and LV.Rostock (1.4.2.5) pseudotypes can efficiently transduce the murine airway epithelium *in vivo* (Alton et al., 2017; Patel et al., 2013), but a direct comparison of efficiency and specific lung cell type tropism has not been made. The WT viruses from which these pseudotypes are derived appear to share a preference for α 2,3 sialylated receptors (as described in 1.5.1.1 and 1.5.2.1). In contrast, the hemagglutinin (HA) from avian influenza H7N9 (A/Shanghai/2/2013), displays enhanced α 2,6 binding (Yang et al., 2013). To allow for a comparison, two novel lentiviral vector pseudotypes based on the H7N9 Shanghai strain were investigated (gift of Oxford Biomedica). Both new pseudotypes include the H7 hemagglutinin protein from H7N9 Shanghai and the M2 protein from Rostock, but they differ in the use of the accompanying neuraminidase protein – one incorporates the N9 neuraminidase from the H7N9 Shanghai strain, while the other uses the N1 neuraminidase from the A/PR/8/34 H1N1 strain of influenza used in the Rostock pseudotype (Figure 10). In both cases, the H7 hemagglutinin protein was engineered to include a polybasic cleavage site (2.3), which eliminates the need for activation of these pseudotypes with a trypsin-like protease during production and/or purification.

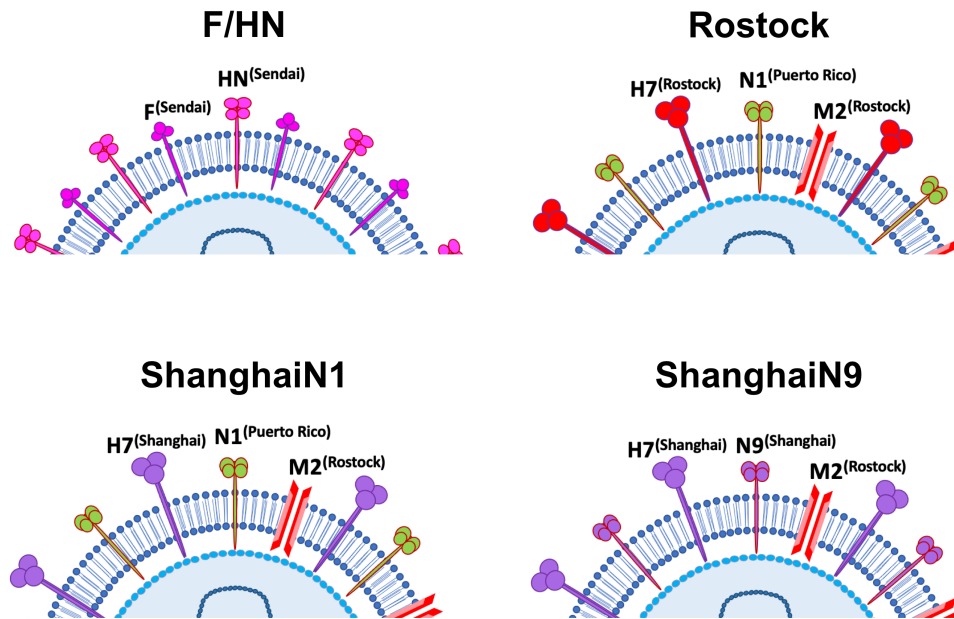


Figure 10- Schematic to show LV pseudotypes investigated. Illustration of glycoproteins expressed in production of each pseudotyped LV. F/HN: fusion (F) and hemagglutinin-neuraminidase (HN) glycoproteins derived from Sendai virus and modified as described (1.4.2.4, Kobayashi et al., 2003). Rostock: H7 hemagglutinin and M2 proton channel from H7N1 A/Fowl Plague Virus/Rostock/8/1934 with N1 neuraminidase from H1N1 A/Puerto Rico/8/1934 as pseudotyped by McKay et al., 2006. ShanghaiN1: same M2 and N1 glycoproteins as used in Rostock pseudotype, plus H7 from H7N9 A/Shanghai/2/2013 modified as described (2.3) to incorporate polybasic cleavage site. ShanghaiN9: same M2 and H7 glycoproteins as ShanghaiN1, plus N9 from H7N9 A/Shanghai/2/2013.

3.1.2. *In vivo* mouse lung model

Intranasal delivery to the lungs of mice under anaesthesia is a widely used model of lung gene delivery, recapitulating many of the barriers faced in targeting human lung *in vivo* by inhalation (apical) delivery (1.4.1). The mouse lung presents many of the diverse cell types found in the human lung, with similar relative abundances and distributions between proximal airway, distal airway and the parenchyma (reviewed Rock, Randell, & Hogan, 2010). Additionally, it has been confirmed using a non-absorptive radioactive tracer that up to 56% of a 50µl volume delivered via intranasal inhalation is retained within the lungs (lower respiratory tract)(Southam, Dolovich,

O'Byrne, & Inman, 2002). Although the life span of the mouse is limited to approximately 2 years, it is useful for modelling duration of expression. In particular, lineage tracing demonstrates pulmonary cell turn over and epithelium regeneration to probe the longevity of transgene expression. (Rock et al., 2009). An alternative method to investigate targeting of progenitor cells, and persistence of vector/transgene expression, is by forced regeneration of the epithelium through administration of a detergent (e.g. polidocanol) to strip away the airway epithelium. This approach has previously been used to strip the murine tracheal airway epithelium, such that only patches of epithelial remnants remain visible at 24hrs post-delivery; importantly epithelium regeneration follows, with partial effects noted by day 3, and full regeneration by day 7 (Borthwick, Shahbazian, Krantz, Dorin, & Randell, 2001). This approach, is however, limited to modelling turnover of cells in those parts of the lung accessible to the polidocanol volume administered, which in turn is restricted in order to minimise toxicity and damage to the (more distal) functional parenchyma. This powerful tool has also been investigated for disruption of the airway epithelium ahead of stem cell engraftment, which can be observed from as little as 2hrs following epithelial ablation (Farrow et al., 2018).

Although the mouse lung displays a range of sialylated receptor subtypes that differs from the human lung, with an absence of $\alpha 2,6$ and an abundance of $\alpha 2,3$ sialylated receptors stained by MAII in the proximal airway (described (1.5.3) (Figure 9), the small volume of the mouse lung offers a huge advantage as an experimental model and facilitates *in vivo* studies, with delivery volumes significantly lower than would be required for larger animals or humans.

3.1.3. Detection reporter transgene activity

In order to investigate LV transduction, a reporter transgene is typically employed for simple detection. Fluorescent reporter transgenes such as enhanced Green Fluorescent Protein (EGFP) allows identification of transduced cells through accumulation of EGFP, although detection *in vivo* can suffer due to limited sensitivity as a result of background cellular auto-fluorescence.

Alternatively, enzyme-based reporter systems can offer greater sensitivity. For example, reporter genes with luciferase activity may either be secreted (Gaussia luciferase; GLux) or localised within the cells targeted (Firefly luciferase; Lux); both emit light following reaction with the substrate luciferin and can be used to monitor gene expression across a wider dynamic range than reporters such as EGFP. Also, as a reporter transgene, bacterial β -galactosidase (lacZ) is commonly used, whereby expression is determined using a colorogenic substrate (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside; X-gal), which when hydrolysed by lacZ generates a blue stain within expressing cells. These approaches, although offering good sensitivity and minimal background compared with fluorescent reporters, are limited by a dependency on exogenous substrate delivery. The ideal reporter gene is therefore selected based on the specific detection needs within a given study.

As a refinement, where characterisation of transgene expression using a single transgene is insufficient, a protein-fusion or multicistronic vector can be used to express two reporter genes (or one alongside the gene of interest) from a single mRNA transgene cassette. Two commonly used approaches to achieve this are (i) the use of a second (internal) ribosome entry site (IRES) (Pelletier & Sonenberg, 1988) placed between the transgenes, and (ii) the use of a sequence encoding a short amino acid sequence (for example the 2A peptide (Ahier & Jarriault, 2014)) that causes ribosome skipping – omitting the formation of a peptide bond whilst continuing to translate the two proteins. Drawbacks of these strategies include a reduced protein expression from the downstream transgenes when using IRES, variable efficiency in ribosome skipping using 2A peptides (H. Y. Chan et al., 2011), and the additional packaging burden of including additional nucleic acid sequence (particularly with the IRES approach as it requires inclusion of ~430 base pairs (Bochkov & Palmenberg, 2006)).

The challenges of EGFP detection (photobleaching, high background autofluorescence in some situations and sensitivity to denaturation in the processing of cells/tissues required for cell type identification) are often overcome using immunohistochemistry targeting EGFP as an

antigen. This approach, however, is labour intensive, squanders the dynamic range of fluorescence, thus reducing the information available on expression levels, and also increases the potential for both false positive and negative staining. Use of native fluorescence such as that resulting from EGFP expression can, however, offer the advantage of allowing both the characterisation of the cell type(s) expressing the EGFP transgene, as well as the relative expression level achieved, whilst mitigating potential impact of inconsistent immunohistochemistry against the EGFP. Although EGFP is by far the most widely used fluorescent reporter, proteins that fluoresce at longer wavelengths (e.g. red or far-red), offer an advantage for detection in eukaryotic cells and tissues, where there is lower background autofluorescence at longer wavelengths (Miyawaki, Sawano, & Kogure, 2003). TdTomato is one such reporter protein, reported to be approximately three times brighter than EGFP, but similarly photostable (Shaner, Steinbach, & Tsien, 2005). Although this reporter has been investigated in several mouse organs (Morris, Klanke, Lang, Lim, & Crombleholme, 2010), it is not known how this reporter compares with EGFP for detection in the lung.

3.1.4. Aims

Both the F/HN (1.4.2.4) and Rostock (1.4.2.5) surface glycoproteins have been shown to successfully pseudotype rHIV-based LV (Gélinas et al., 2017; McKay et al., 2006). Furthermore, efficient gene transfer to the mouse lung epithelium following intranasal delivery of Rostock pseudotyped rHIV (rHIV.Rostock) to mice has been confirmed (Patel et al., 2013). Thus, rHIV was selected as a platform for pseudotype comparison. The first experimental aim was to develop a reliable method to quantify native fluorescent reporter expression in both the mouse airway and parenchyma in order to facilitate evaluation of the transduction efficiency and distribution of cells targeted by these rHIV pseudotypes. Using such a method, the next aim was to select the optimal fluorescent reporter transgene for incorporation into pseudotyped rHIV vectors and

to produce them in sufficient quantities to compare transduction efficiency and cell-type targeting *in vivo*.

3.2. Results

3.2.1. Development of a method to quantify native reporter transgene fluorescence in the mouse lung following intranasal LV delivery

Delivery of F/HN pseudotyped rSIV (rSIV.F/HN) results in quantifiable EGFP expression in the mouse airways and parenchyma (Alton et al., 2017). Unfortunately, using paraffin-embedded tissue to best preserve tissue morphology, the traditional method used involved exposing tissue to fluorophore-destroying (or quenching) treatments and chemicals (e.g. high temperatures and ethyl alcohols); this results in the need for immunohistochemistry against the fluorophore (Jiang et al., 2005; Swenson, Price, Brazelton, & Krause, 2007). To eliminate the requirement for immunohistochemistry whilst still preserving sufficient morphology to characterise expressing cells, strategies to detect and quantify native EGFP fluorescence in mouse lung were investigated, using the rSIV.F/HN vector as a positive control.

3.2.1.1. Determination of dose and timepoint for native EGFP detection in the mouse lung using rSIV.F/HN

A dose of either 1e8 or 3e8 TU of rSIV.F/HN.hCEF.EGFP (See Table 4 A) was delivered to mice intranasally, in a total volume of 100µL TSSM buffer, under inhalation anaesthesia (2.9.1). Mice were sacrificed 7 or 28 days after delivery and the lungs processed using freshly prepared 4% PFA (2.1; v/v), (in the absence of methanol which is a common additive to stabilise non-polymerised formaldehyde but is known to destroy native fluorescence) to achieve chemical fixation of the mouse lung prior to dehydrating and embedding the tissue for cryosections (described 2.9.4). Importantly, using this process, 7µM cryosections were generated capturing

the expected lung morphology, and EGFP was readily detected from all mice treated with rSIV.F/HN at both time points and doses. High levels of EGFP-dependent fluorescence could be observed throughout the airways as well as in regions of the lung parenchyma. Although diffuse background autofluorescence was detected in cryosections generated from naïve untreated mice, cell-associated fluorescence in the tissue of treated mice was clearly greater (example shown Figure 11 A versus B).

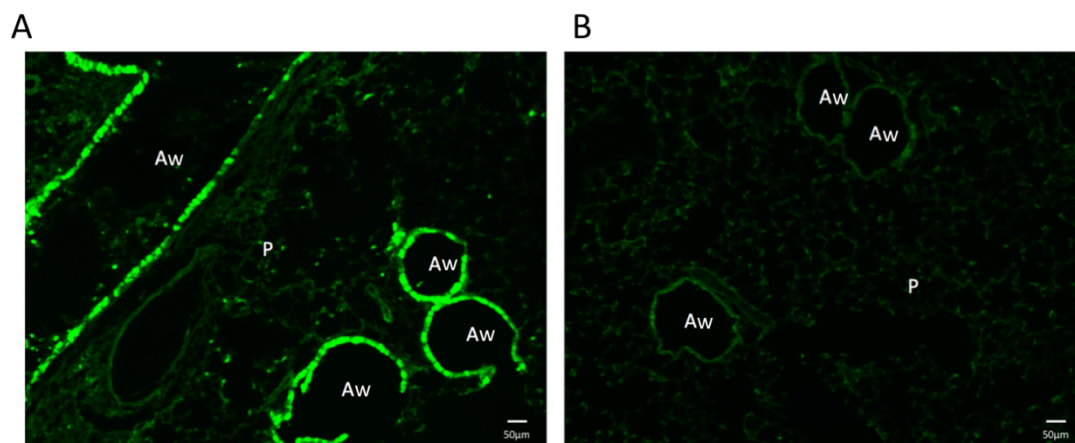


Figure 11- Native EGFP detection following rSIV.F/HN.hCEF.EGFP intranasal delivery to mouse lung. Mice were treated with 3×10^8 TU rSIV.F/HN.hCEF.EGFP (100µL intranasal delivery) or left untreated (naïve) and culled 7 days later. Cryosections of mouse lung were generated (2.9.4) and images captured for detection of EGFP using the EVOS Auto 2 using the same brightness settings (exposure and gain, 2.8.1). (A) Treated mouse. (B) Naïve mouse lung cryosection demonstrating representative autofluorescence levels. Parenchymal tissue (P) and the airways (Aw) are identified. Scale bar represents 50µM.

3.2.1.2. Develop method for quantifying EGFP expression and distribution throughout the mouse lung

In order to use the native fluorescence from EGFP to evaluate the efficiency of transduction by LV, a method to quantify both the fluorescence observed and the relative distribution of transduced cells was developed. A single coronal plane left lung lobe cryosection from each mouse was selected for quantitative analysis. Selection was based on morphology and the

presentation of the greatest abundance of airways, without knowledge of fluorescence intensity. Each section was scanned and a wide-field image created by stitching multiple (typically tens to hundreds) smaller images using the EVOS FL Auto 2 imaging system (Thermo Scientific; see 2.8.1). To ensure comparable images were generated, consistent exposure and brightness settings were used across all sections. These wide-field stitched images were analysed using area selection tools available in Image J software (NIH). Regions of interest (ROI) for quantification were selected systematically: (i) up to six ROI capturing small airways, (ii) a single ROI for each secondary bronchi visible, and (iii) two ROI capturing the primary bronchi were used for quantification. Using brightness thresholding criteria in Image J software, and selection tools to identify and distinguish airway epithelia and parenchymal tissue area from background fluorescence, a % image area exceeding a threshold fluorescence (minimising detection of autofluorescence using untreated tissue) was calculated for each ROI (Figure 12).

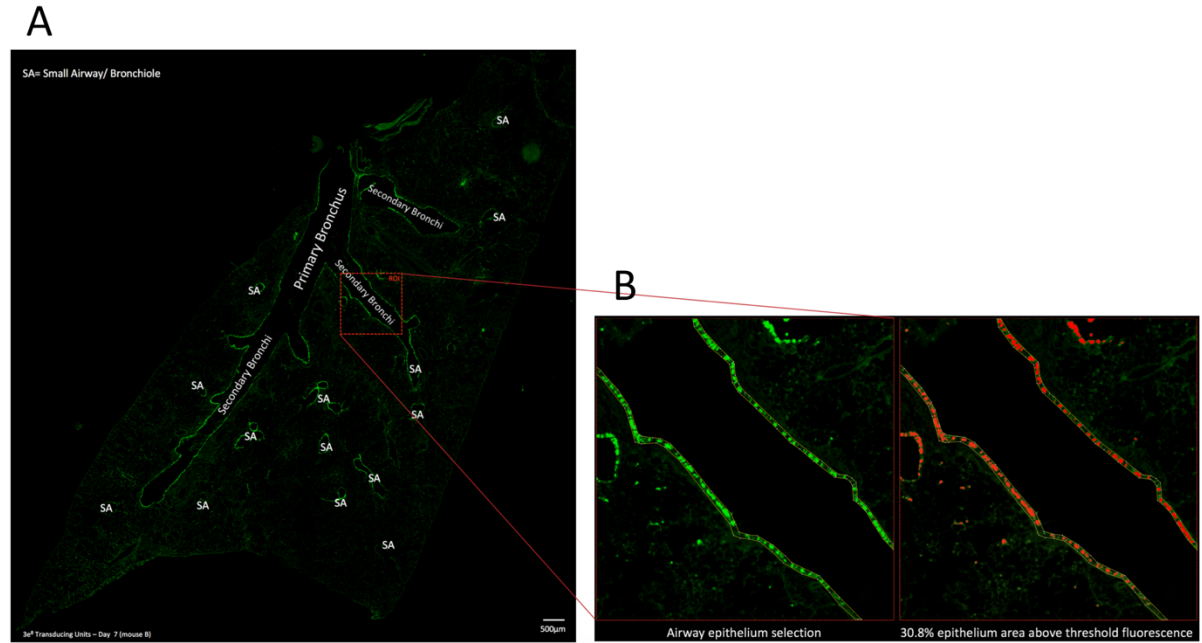


Figure 12- Anatomy and airway categorisation of left mouse lung lobe cryosections used for ROI selection and example of thresholding to quantify percentage area above autofluorescence for an airway within ROI. Mice were treated with 3e8 TU rSIV.F/HN.hCEF.EGFP (100µL intranasal delivery) and culled at day 7. Cryosections of mouse lung were generated (2.9.4) and stitched images capturing EGFP fluorescence generated (Section 2.8.1) using the EVOS Auto 2 (A). These images were subsequently used for systematic selection of regions of interest (ROI, B), using detected airway anatomy to orientate. EGFP quantification of percentage area (airway epithelium or parenchymal tissue) above autofluorescence threshold, set using cryosections from naïve mice, was performed for each ROI. (A) Stitched image of left lung lobe coronal cryosection. Scale bar represents 50µM. (B) ROI selected from stitched image. Image J selection tools used to demarcate airway epithelium (yellow borders) and fluorescence above threshold (red) indicated. SA: Small airway or bronchiole.

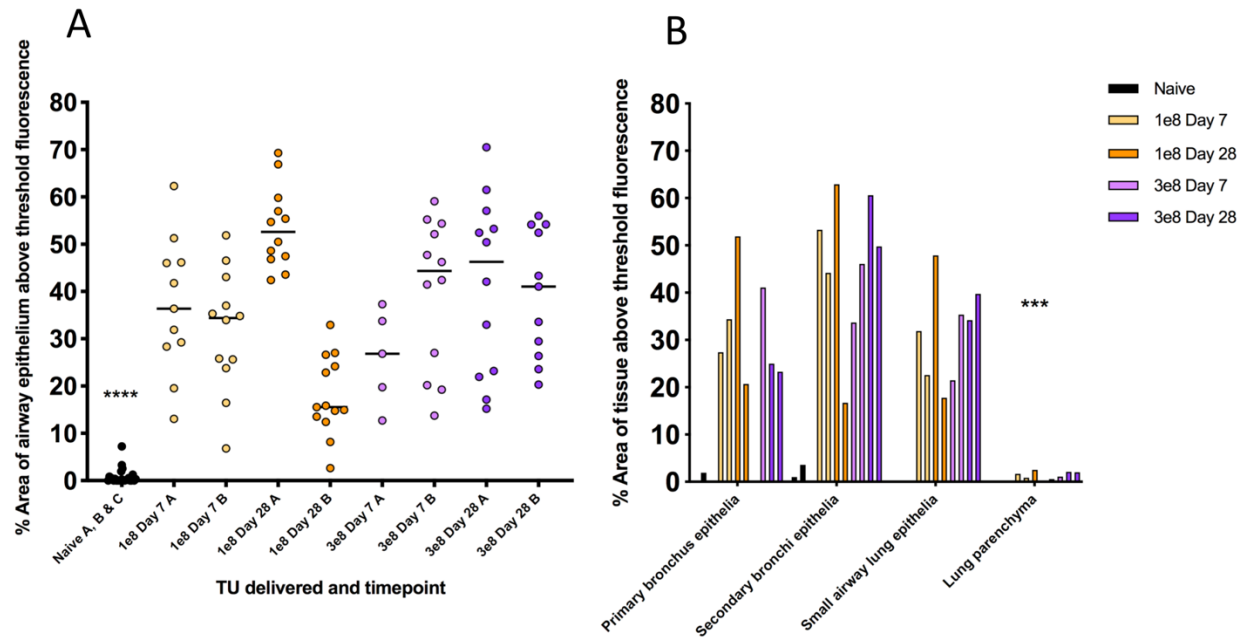


Figure 13- EGFP detection by percentage area of airway epithelium above threshold fluorescence in mice transduced with rSIV.F/HN and distribution of fluorescence detected between proximal and distal airways and parenchyma. Mice treated with 1e8 or 3e8 TU rSIV.F/HN.hCEF.EGFP (Wave 83 see Table 4) were sacrificed on day 7 or 28 following delivery and native EGFP detected in left lung lobe cryosections. The percentage of airway area within regions of interest (ROI) above fluorescence threshold were determined using Image J software (NIH). A, B and C refer to individual mice. (A) Each data point represents an airway measurement from a single ROI. Fewer data points for a specific mouse correspond to fewer airways captured (and therefore ROI assessed) on the selected section (minimum airways n=5 for a single mouse). Median represented by solid line. All treated mouse airway values were compared with naïve mouse airway values (****, $p < 0.0001$; Mann-Whitney U test). (B) Mean percentage area of tissue above threshold fluorescence for each anatomical ROI category for each mouse. Each bar represents an individual mouse. Parenchymal values versus all airway values (***, $p = 0.0001$; Mann-Whitney U test).

The median area (%) of airway epithelium above threshold fluorescence quantified for all treated mice was approximately 40%, significantly greater than naïve (median 0.132%) ($p < 0.0001$, Mann-Whitney U Test), validating use of this method (Figure 13 A). Whilst some variation was observed between treated animals (median percentages ranging from 15% to 53%), there was no significant difference detected between levels of airway expression

measured at day 7 and day 28 post-dosing ($p>0.9999$), and no significant difference between the $1e8$ and the $3e8$ TU vector doses ($p>0.9999$, Kruskal-Wallis test with Dunn's multiple comparison post-hoc test for both).

In addition to airway epithelium, area (%) values were also determined for the parenchymal tissue captured within the ROI used for small airway quantification, by demarcating all tissue excluding that used for the small airway calculation. Values were subsequently grouped according to tissue and airway type/proximity, and used to characterise the distribution of transduction (Figure 13 B). Significantly lower levels of fluorescence were recorded for the parenchymal tissue area compared with airway epithelia values, however no significant difference between each category of treated airways was detected ($p=0.1522-0.9999$, Kruskal-Wallis followed by Dunn's Multiple Comparison test) despite a trend observed towards greater percentages recorded in secondary bronchi versus primary and small airways. In order to confirm that these 'software-calculated' values of percentage area above threshold fluorescence for each airway reflected the actual percentage of airway epithelial cells transduced, a comparative study was performed where EGFP positive and negative cells were counted manually. For this, three mice were dosed with the same LV ($1e8$ TU rSIV.F/HN.hCEF.EGFP), sacrificed 7 days following delivery, and the percentage area quantification for airway epithelium was analysed both using the automated Image J area approach as described above and a manual count of EGFP positive cells. Analysing the same airways in ROI; one from primary bronchus, three of secondary bronchi and three of small, a total of 7 values were determined per mouse using both methods. For manual counting, DAPI staining of nuclei was captured in parallel with EGFP detection (merged images) and used to identify individual cells. The EGFP visualised and quantified in the airways of these mice was unexpectedly reduced (median 7.5%, Figure 14) versus that observed previously using this LV (median 35.4%, Figure 13 A), despite equivalent TU delivered and timepoint assessed. This likely reflects variability between different batches of LV produced (Wave 121 versus Wave 83, Table

4 B) and highlights the importance of using equivalent production and titration methods for comparison of LV preparations. The percentage of airway epithelium cells manually counted as EGFP positive ranged between 5-18% with a median of 13.6%, approximately twice that determined using the automated method ($p=0.0014$; Wilcoxon matched-pairs signed rank test). Overall, this suggested that the percentage determined by area represented a conservative estimate to the number of EGFP-expressing cells in the airway.

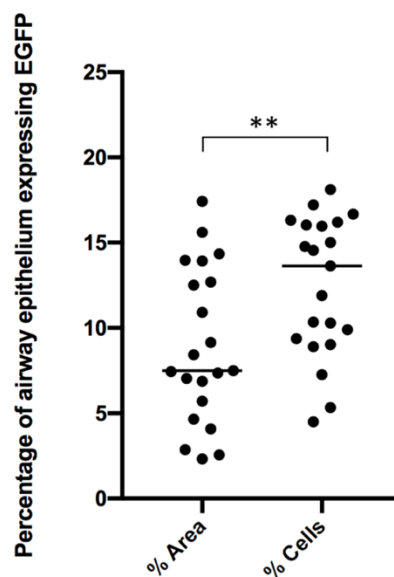


Figure 14 - Airway epithelium EGFP quantification by percentage area versus percentage EGFP positive cells. Quantification of EGFP in airway epithelium ROI from three mice after intranasal delivery of $1e8$ TU rSIV.F/HN.hCEF.EGFP (Wave 121) and sacrificed on day 7, determined by either percentage area above threshold fluorescence or by counting of cells. Each data point represents an airway measurement from a single ROI. Median represented by solid line. All % area values were compared with % cell values (**, $p=0.0014$; Wilcoxon matched-pairs signed rank test).

3.2.1.3. Selection of fluorescent reporter transgene for maximal detection

Having established a method to quantify transgene expression using a fluorescent reporter without the need for manual cell counting, the next aim was to determine whether tdTomato

could offer greater sensitivity for detection, given the reported 3-fold enhanced brightness (Shaner et al., 2005) versus EGFP, and the lower levels of autofluorescence from tissue at this wavelength. It was anticipated that tdTomato might allow greater sensitivity, allowing the background threshold to be lowered.

Two rHIV.F/HN vectors (vGM182 and vGM181, Table 4) were produced and titrated in parallel, expressing either EGFP or tdTomato for direct transgene comparison. Production of sufficient LV for *in vivo* comparison was challenging with low yields of concentrated TU obtained. Production of these vectors using Cell Factories (2.4.1), purified using two centrifugation steps (OBM335 and OBM336), or exchanging the initial overnight centrifugation for tangential flow filtration (OBM337 and OBM338) achieved only a maximum concentration of 1.74×10^8 TU/mL (Table 4 B). Production using a Wave Cellbag bioreactor approach at the 5L scale (5L; 2.4.2, Wave 95 & 96) however, generated sufficient concentrated LV such that doses of 7.98×10^7 TU (EGFP) or 6.15×10^8 TU (tdTomato) could be delivered to mice (100 μ L intranasal dose; Wave 96 and Wave 95 respectively, see Table 4 B). Although expression of both tdTomato and EGFP were visualised in wet mounts of the nasal septum from all treated mice (punctate signal; Figure 15 A), there was no evidence that the tdTomato transgene offered increased detection of native fluorescence in the mouse lung since only EGFP vector treatment generated expression in the airways which was greater than naive animals (Figure 15 B), despite the greater TU dose of tdTomato expressing rHIV.F/HN. Consequently, despite the theoretical advantages of tdTomato, EGFP was selected as the transgene reporter for subsequent studies.

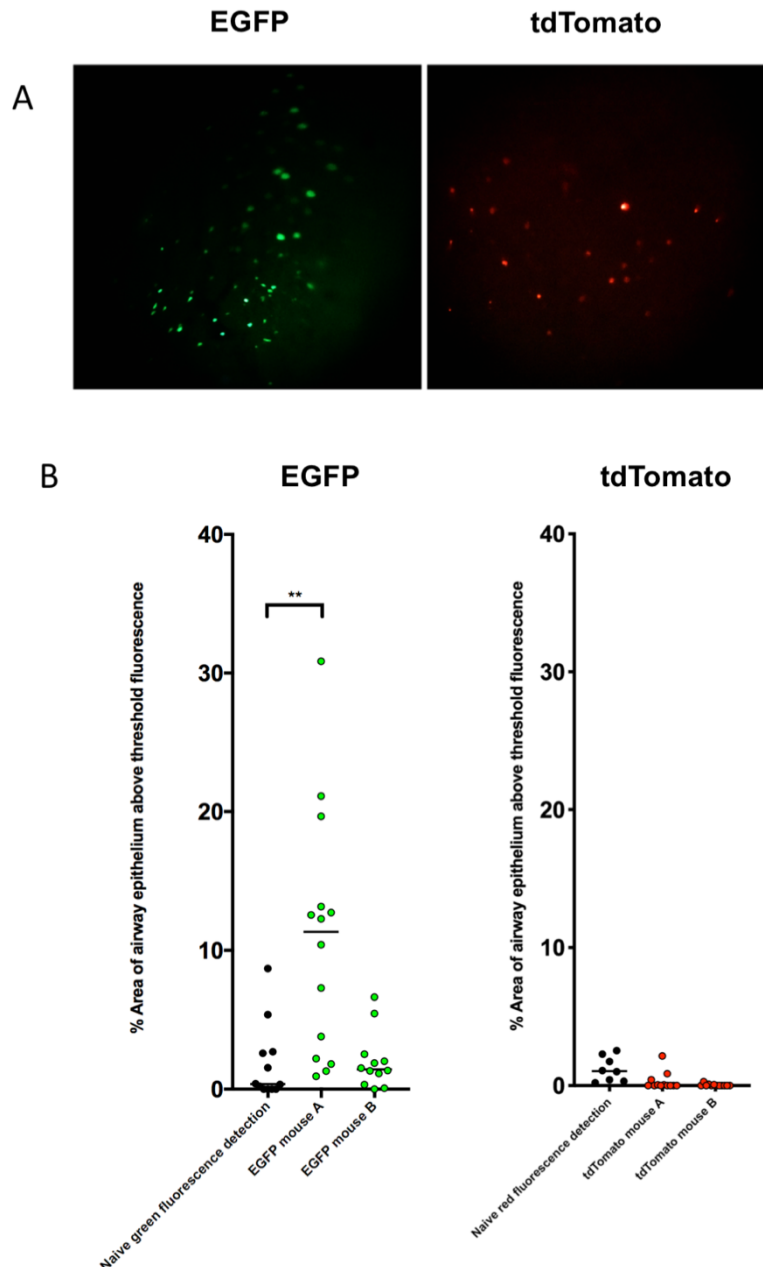


Figure 15- Detection of native EGFP or tdTomato fluorescence following delivery of rHIV.F/HN. Mice (n=2) were dosed with either 7.98e7 TU (Wave 96) rHIV.F/HN.CMV.EGFP or 6.15e8 TU (Wave 95) rHIV.F/HN.CMV.tdTomato and sacrificed on day 7, with images captured using Nikon Optiphot-2 microscope (10x) and GFP filter (EGFP) or Texas red filter (tdTomato). (A) Representative detection of transgene fluorescence in nasal septum, (B) Quantified percentage area airway above fluorescence threshold. A and B refer to individual mice. Each data point represents an airway measurement from a single ROI. Median represented by solid line. Significance detected between mouse A expressing EGFP versus naïve (**, p=0.0013; Kruskal–Wallis followed by Dunn’s Multiple Comparison test).

3.2.2. Pseudotyped rHIV for *in vivo* comparison

Levels of EGFP detected from *in vivo* delivery of rHIV.F/HN (Figure 15, 7.98e7 TU) were limited versus rSIV.F/HN (Figure 13 & 14, minimum TU 1e8), despite similar TUs delivered. Additionally, production of rHIV.F/HN using cell factories had proved challenging (3.2.2). However, with pseudotyping of rHIV by Rostock pseudotype established previously (McKay et al., 2006), production of rHIV using adherent cell factories as well as suspension LV-MAX production was trialled aiming to produce sufficient concentrated rHIV of all pseudotypes for direct comparison *in vivo* at an equivalent TU doses. To confirm that the limited detection of EGFP with rHIV.F/HN versus rSIV.F/HN *in vivo* was not a result of the alternative plasmid used to supply the F/HN pseudotype (OXB versus GM plasmids, see section 2.3), an alternative rHIV.F/HN.CMV.EGFP vector (vGM188) was produced which used the GM plasmids for F and HN. Using the Wave (5L) suspension process (Wave 105), a very similar titre of concentrated vector was achieved (5.45e8 TU/mL for vGM188 using GM F/HN plasmids, versus the 7.98e8 TU/mL for vGM182 using OXB F/HN plasmids). Disappointingly, the yield of concentrated rHIV obtained by these production methods was modest and dependent on the pseudotype manufactured (Table 4 B). Although 1e8 TU/mL concentrations could be obtained for F/HN and Rostock pseudotypes (vGM182/vGM188 and vGM222), these titres are significantly lower than those achieved for rSIV.F/HN (typically >1e9 TU/mL). Additionally, the ShanghaiN9 pseudotype was concentrated to a maximum of only 4.65e7TU/mL (vGM220) and even lower titres were achieved for ShanghaiN1 vectors (maximum 4.27e6TU/mL vGM221).

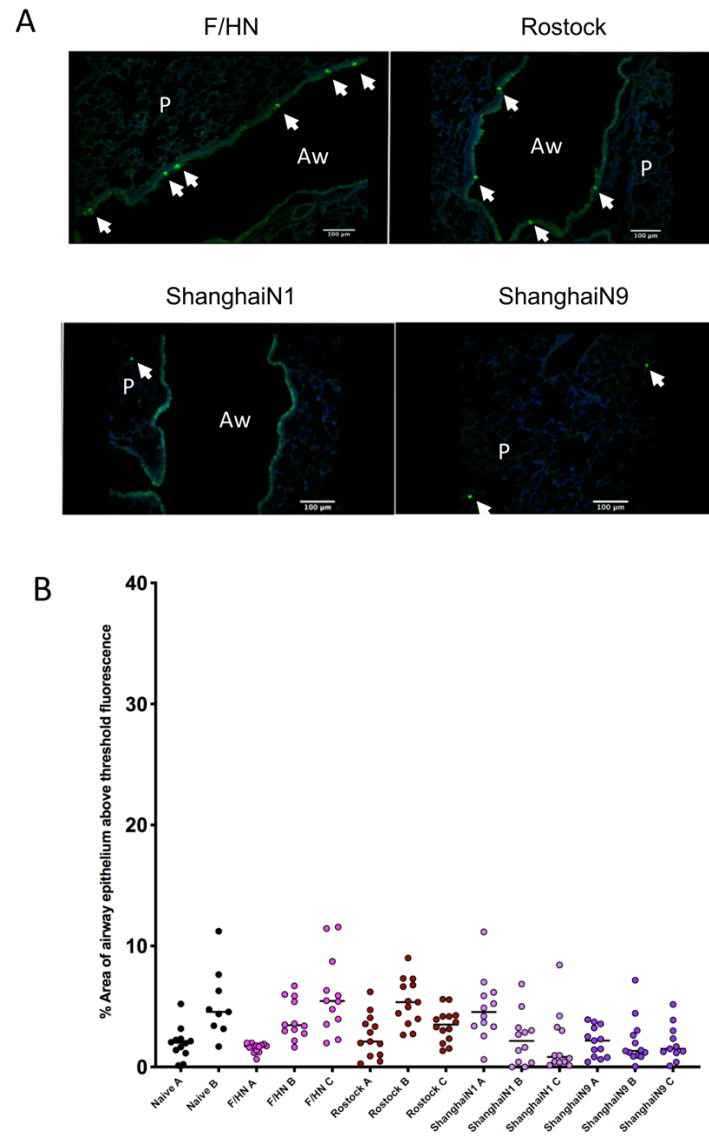


Figure 16- Detection of EGFP fluorescence following delivery of influenza-based pseudotypes of rHIV.CMV.EGFP to the mouse lung. Mice were delivered the maximum feasible dose (MFD) of rHIV.CMV.EGFP pseudotyped with F/HN (vGM188, Wave 105; 5.45e7 TU), Rostock (vGM222 OBM368 1.33e7 TU), ShanghaiN1 (vGM221 OBM369 1.51e5 TU), or ShanghaiN9 (vGM220 OBM370 1.13e6 TU) and sacrificed day 7 for cryosection analysis. (A) Example of EGFP detection (white arrows) in airway and parenchyma from mice treated with each pseudotype. Parenchymal tissue (P) and the airways (Aw) are identified. Merged Images taken using the EVOS Auto 2 using the same EGFP brightness settings (exposure and gain). Nuclei counterstained with DAPI (blue), scale bar 100μM. (B) Quantified percentage area airway above fluorescence threshold. A, B and C refer to individual mice. Each data point represents an airway measurement from a single ROI. Median represented by solid line. No significance detected between naive and treated groups (Kruskal–Wallis followed by Dunn’s Multiple Comparison test).

Mice treated with the maximum feasible dose (MFD comprising maximum TU in 100 μ L) of these vectors, generated EGFP-expressing cells that were visualised (Figure 16 A), but, these studies did not result in sufficient EGFP fluorescence in the airways to quantify expression above naïve levels using the automated method developed (Figure 16 B). Ultimately, this meant, disappointingly, that these rHIV LV could not be used to compare pseudotypes for transduction efficiency in this *in vivo* model, although qualitative observations were still possible. The minimal EGFP detected from vGM188 treated mice was similar to that detected from vGM182 treated mice that received a similar TU dose (Figure 15), suggesting that there was little to no impact of the source of the F/HN pseudotype supplying plasmids used. The few EGFP positive cells visualised from F/HN and Rostock pseudotypes were confined to the airway epithelium, whereas those from mice administered either ShanghaiN1 or ShanghaiN9 pseudotypes were identified in the parenchyma (Figure 16 A); this indicated an alternative cell type tropism of the rHIV pseudotyped with Shanghai H7 versus F/HN and Rostock.

3.2.2.1. Investigating production using exogenous neuraminidase

One hypothesis to explain the low yield of concentrated-influenza based pseudotypes could be insufficient supply of neuraminidase to facilitate particle release from LV producer cells; a known challenge for production of hemagglutinin-based LV pseudotypes. One way to address such a limitation, and increase production yields, would be to supply neuraminidase, either by expression within the host cell and packaging into LV particles or, delivered exogenously to the LV producer cells. To test this hypothesis, we investigated production of rHIV pseudotyped with Rostock or ShanghaiN9 with and without supplementation of exogenous neuraminidase. Using the LV-MAX suspension process (2.4.3) and the pHIV.CMV.EGFP_{Lux} genome (pGM290; Table 3), exogenous neuraminidase was supplied to the producer cells 24 hours post-transfection. Based on an approach previously shown to be effective (S. Scott et al., 2012; S. D. Scott, Kinsley, Temperton, & Daly, 2016) cells were treated with 0.5 U (units) per mL to achieve an excess

supply of exogenous neuraminidase per cell. The impact on yield of functional LV was determined by serial dilution of the crude LV containing supernatant on LV-MAX cells and measuring the expression of Lux transgene expression as described (2.6.2.4). As expected, Figure 17 shows a greater yield of Rostock pseudotyped rHIV versus ShanghaiN9 consistent with previous findings (3.2.3), however no increase in yield was detected with supplementation of exogenous neuraminidase.

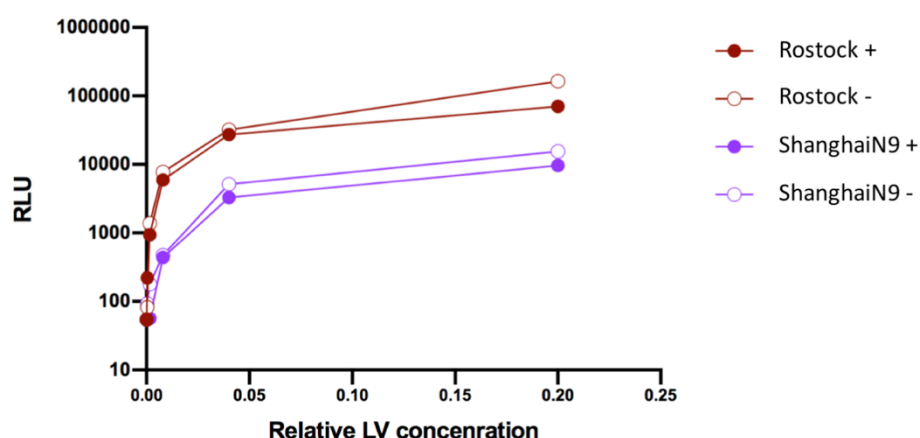


Figure 17- Yield of rHIV pseudotyped with Rostock or ShanghaiN9 in the presence or absence of exogenously supplied neuraminidase. rHIV pseudotyped with Rostock or ShanghaiN9 were produced with (+) or without (-) exogenous neuraminidase (Table 4, rHIV.H7rN1pr.CMV.EGFPLux and rHIV.H7shN9sh.CMV.EGFPLux; 2.4.3). Following titration of crude vector on LV-MAX cells, luciferase (Lux) activity was determined as described 2.6.2.3 to quantify relative yield Relative light units (RLU).

3.2.2.2. Investigating an alternative constitutive transgene promoter (hCEF)

Another method to enhance detection of transduced cells from the various LV pseudotypes under investigation is the use of a stronger constitutive transgene promoter, although this approach merely improves detection rather than vector yield or titre. As an alternative to the commonly used CMV promoter, the hCEF enhancer/promoter element is a combination of a CpG-free human CMV enhancer and a CpG-free human elongation factor 1 α promoter (Hyde et

al., 2008), and has previously been shown to direct greater and more sustained transgene expression than the CMV promoter following delivery to the mouse lung. Consequently, rHIV.hCEF.EGFP vectors were manufactured in parallel using the LV-MAX process for all four pseudotypes (vGM191-4, Table 4 B). Despite titres of up to 5.14×10^9 TU/mL achieved for Rostock pseudotype, 6.76×10^8 TU/mL for F/HN, and modest improvement to yield of ShanghaiN1 and ShanghaiN9 pseudotyped rHIV (1.62 – 7.81×10^7 TU/mL), EGFP detection in mouse lung was absent following MFD (in $100 \mu\text{L}$) delivery of all vectors (Figure 18). Taken together, these data suggest that the limitation in EGFP detection in mouse lung following treatment with the various rHIV LV pseudotypes was unlikely to be as a result of insufficient promoter activity given the robust detection of EGFP expression from rSIV.F/HN.hCEF vectors.

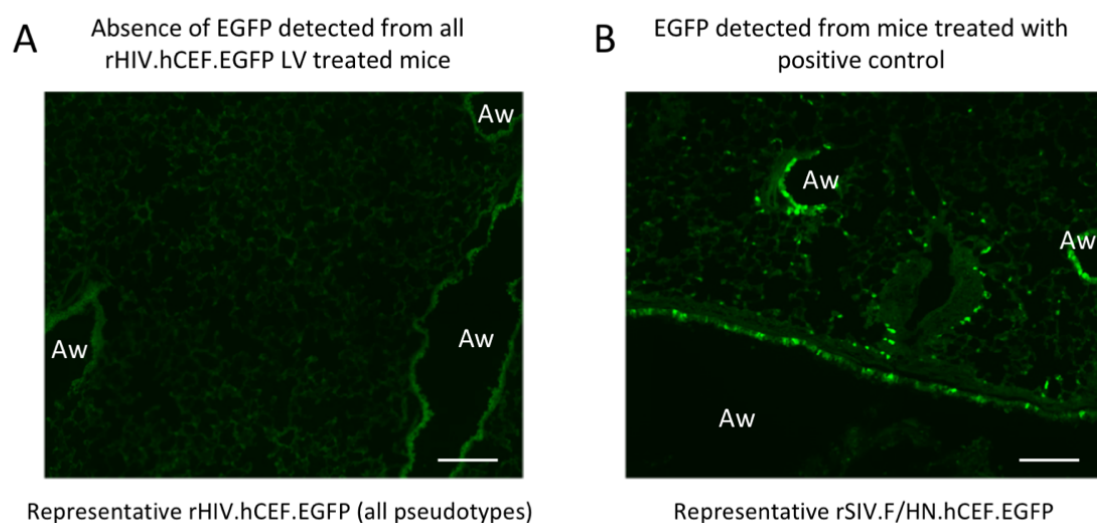


Figure 18- Absence of native EGFP detection in mouse lung following delivery of alternatively pseudotyped rHIV.hCEF.EGFP. Mice ($n=2$) were administered the MFD of rHIV.hCEF.EGFP pseudotyped with F/HN (vGM193 Wave 113; 6.76×10^7 TU), Rostock (vGM194 Wave 114; 5.14×10^8 TU), ShanghaiN1 (vGM191 Wave116; 1.62×10^6 TU), or ShanghaiN9 (vGM192 Wave115; 7.81×10^6 TU) and sacrificed on day 7 for cryosection analysis. Mice ($n=2$) were also administered the MFD of rSIV.F/HN.hCEF.EGFP (vGM107, Wave 109; 4.60×10^8 TU) in parallel as a positive control. (A) and (B) Exemplar images of EGFP detection in representative ROI. Parenchymal tissue (P) and the airways (Aw) are identified. Scale bar $100 \mu\text{M}$.

3.2.3. Characterising cells transduced by F/HN pseudotyped LV *in vivo* using high titre rSIV.F/HN

Despite these efforts to improve the yield or detection of transduction by rHIV LV *in vivo*, insufficient native EGFP was detected to allow comparison of pseudotypes in this model. With sufficient expression detected following delivery of rSIV.F/HN however, such that transduction could be quantified, and cellular distribution between airways and parenchyma assessed (Figure 13), the transduction achieved by this pseudotype *in vivo* was investigated further.

3.2.3.1. Cell types transduced

Although native EGFP detection from cryosections allows for quantification and the distribution of transduced cells between airways and parenchyma, the specific cell-type of transduced cells requires immunohistochemistry (IHC) to identify cell-type specific markers. By combining native EGFP detection with cell-type IHC we anticipated that transduced cell-types could be determined without the IHC compromising EGFP detection of EGFP. Cryosections from mice dosed with rSIV.F/HN.hCEF.EGFP, after quantification of native EGFP fluorescence (Figure 13), were subjected to cell-type specific IHC (without heat-based antigen retrieval that would destroy native EGFP fluorescence). Visualising stained cryosections using confocal microscopy, colocalization of native EGFP with ciliated, club, goblet and alveolar type-II (ATII) cell specific markers was observed, confirming transduction of these cell types (Figure 19). Although the frequency of colocalization was not quantified, infrequent transduction of ciliated cells was apparent given the abundance of both markers in the airways (Figure 19 A). Conversely transduced ATII cells were readily detected despite the less frequent detection of EGFP positive cells in the parenchyma (Figure 19 D).

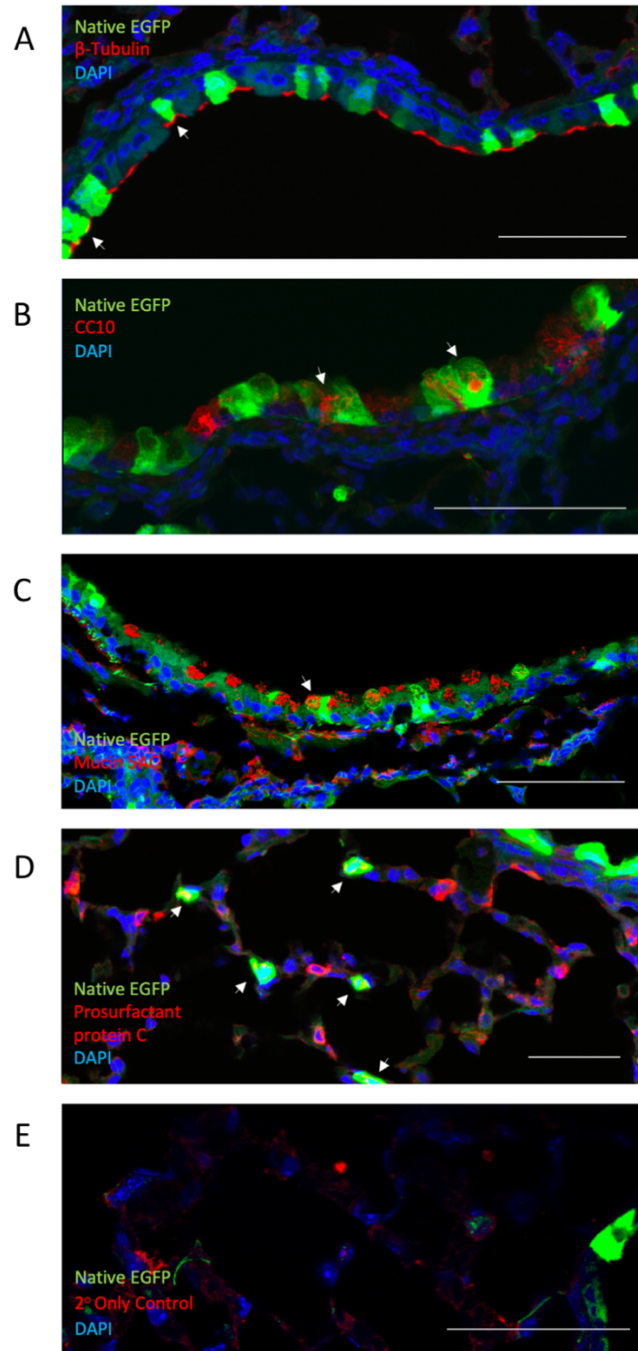


Figure 19- Confocal images of native EGFP colocalization with cell-type specific immunohistochemistry. Mice (n=2) were treated with 3e8 TU rSIV.F/HN.hCEF.EGFP, sacrificed 7 days later and lung cryosections immunostained. Cell type-specific antibodies against (A) ciliated (β -Tubulin), (B) club (CC10; Club cell secretory protein), (C) Goblet (Mucin 5AC) and (D) alveolar type-II cell (ATII; Prosurfactant protein C) markers, all shown in red. (E) Secondary antibody only control. Examples of native EGFP colocalization are indicated (white arrows) and nuclei are stained blue (DAPI); scale bar represents 50μM. Images captured using Zeiss LSM 780 inverted confocal microscope.

Additional imaging of stained cryosections using widefield microscope (EVOS FL Auto 2) to scan and stitch images was performed to allow further assessment of the distribution of specific cell types transduced. Stitched images from sections stained for ciliated cells (β -Tubulin) revealed an apical airway staining pattern (Figure 20), similar to that detected using confocal microscopy (Figure 19), validating the widefield microscopy approach. The distribution of ciliated cells appeared to be enriched in the primary bronchi, and inversely distributed to native EGFP-expressing cells, visualised in the secondary and smaller airways (Figure 20). This could indicate limited tropism of the F/HN pseudotype for this cell type in the mouse airway, despite transduction confirmed by colocalization with confocal microscopy (Figure 19). Regrettably, in cryosections stained for club, goblet and ATII cells, cell-type specific signal observed using confocal microscopy could not be detected at the light and exposure settings required to eliminate autofluorescence detection (high in airways and blood vessels; example shown ATII cells Figure 20; see also airway autofluorescence Figure 11).

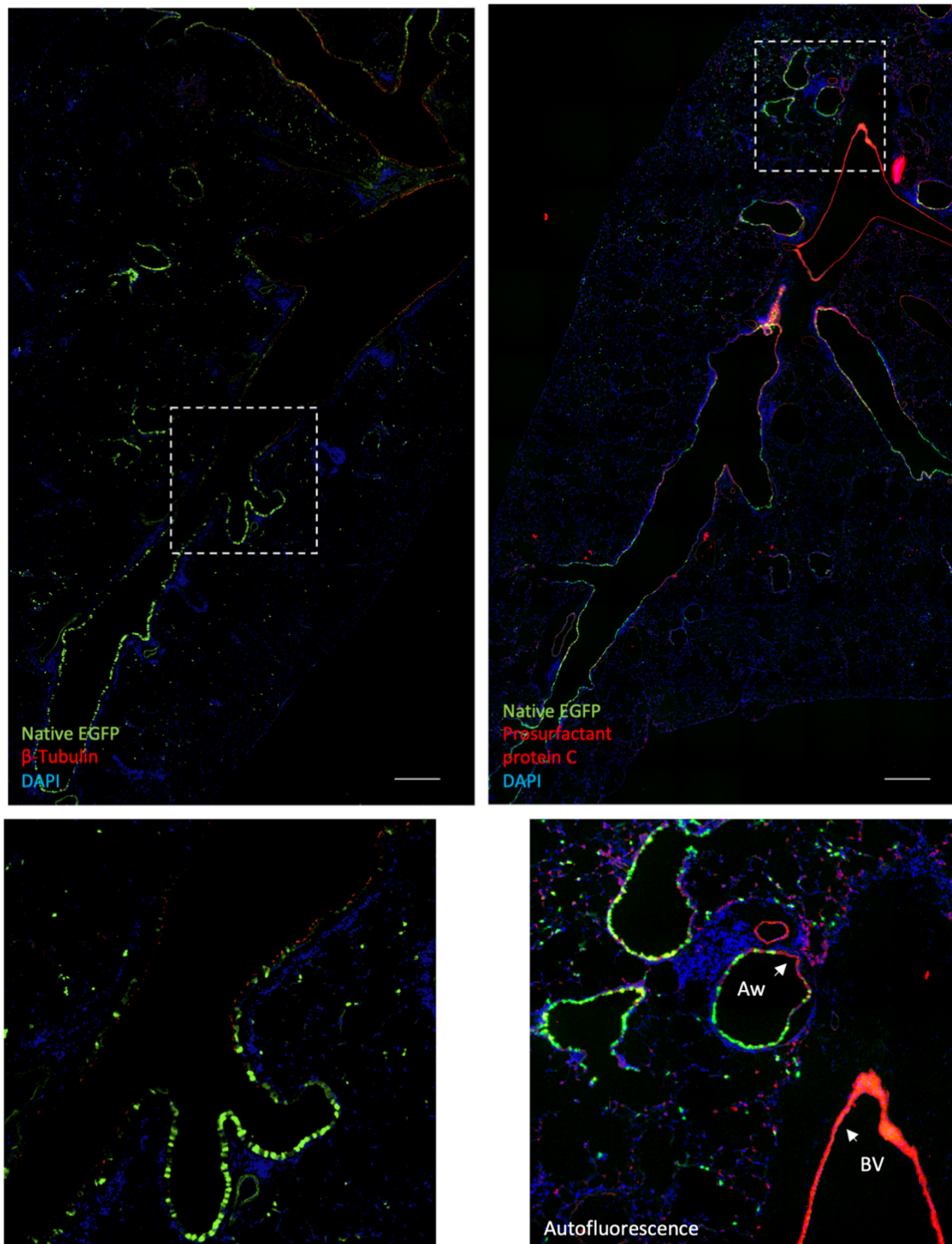


Figure 20- Widefield stitched images of native EGFP and cell- type specific immunohistochemistry. Stitched images of whole left lung lobe cryosections generated from mice treated with 3e8 TU rSIV.F/HN.hCEF.EGFP and sacrificed on day 7. Sections were stained for ciliated cells (β -Tubulin; Left) and alveolar type-II cells (ATII; Prosurfactant protein C; Right). White box indicates region expanded below. Representative example of autofluorescence signal in airways and blood vessels indicated (white arrows), scale bar represents 500 μ M. Nuclei are stained blue (DAPI), images captured and stitched using EVOS FL Auto 2.

3.2.3.2. Progenitor cells transduced

One cell type most opportune to target with integrating LV is the progenitor basal cell, with the potential to facilitate transgene expression in daughter cells after division of a transduced cell. Basal cells are reportedly rare/absent in mouse airways and largely confined to the trachea (Rock et al., 2009). In order to determine basal cell transduction by rSIV.F/HN, cryosections of treated mouse lung and trachea were immune-stained for cytokeratin 5, preserving native EGFP fluorescence, but no staining was observed in the mouse lung or tracheal sections. However, using heat-based antigen retrieval, and dual IHC against both cytokeratin 5 and GFP, basal cells could be identified in cryosections of tracheal epithelium (Figure 21). No colocalization of staining and EGFP expression was detected however and so transduction of basal cells by rSIV.F/HN remains at best an infrequent event.

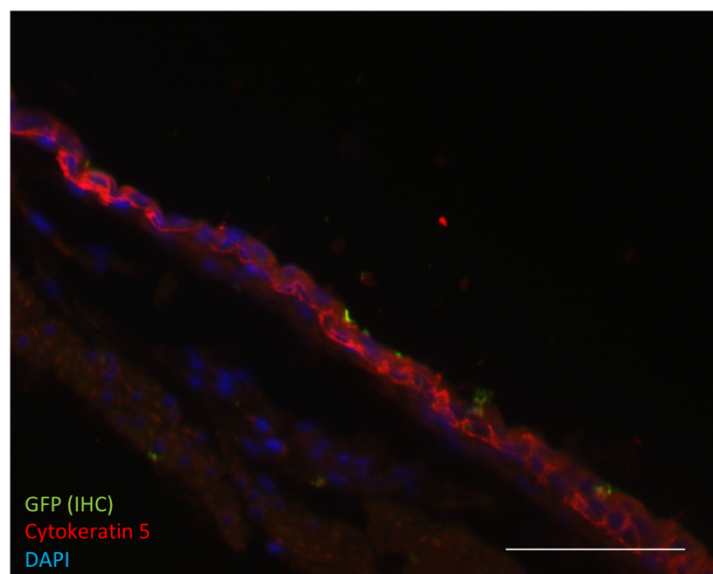


Figure 21- Basal cell and GFP staining in mouse trachea cryosections. Example of mouse trachea cryosection generated from mouse treated with 3e8 TU rSIV.F/HN.hCEF.EGFP and sacrificed on day 7. Immunostaining of basal cells (Cytokeratin 5; red) and GFP (green) are counterstained with DAPI (blue); scale bar represents 50µM. Images captured using EVOS FL Auto 2.

3.2.3.2.1. Long term expression *in vivo*

As an alternative to immunohistochemistry to detect progenitor cell transduction, mice treated with 3e8 TU of SIV.F/HN.hCEF.EGFP were sacrificed 1 year following delivery and native EGFP fluorescence evaluated using the method described above. Although EGFP quantification was lower (~25% area) in airways at 1 year (Figure 22 A) compared with mice treated in parallel and sacrificed at day 7 and 28 (Figure 13 A; ~40%), native EGFP expression was distributed similarly between the airways and parenchyma (Figure 13 versus 22 B).

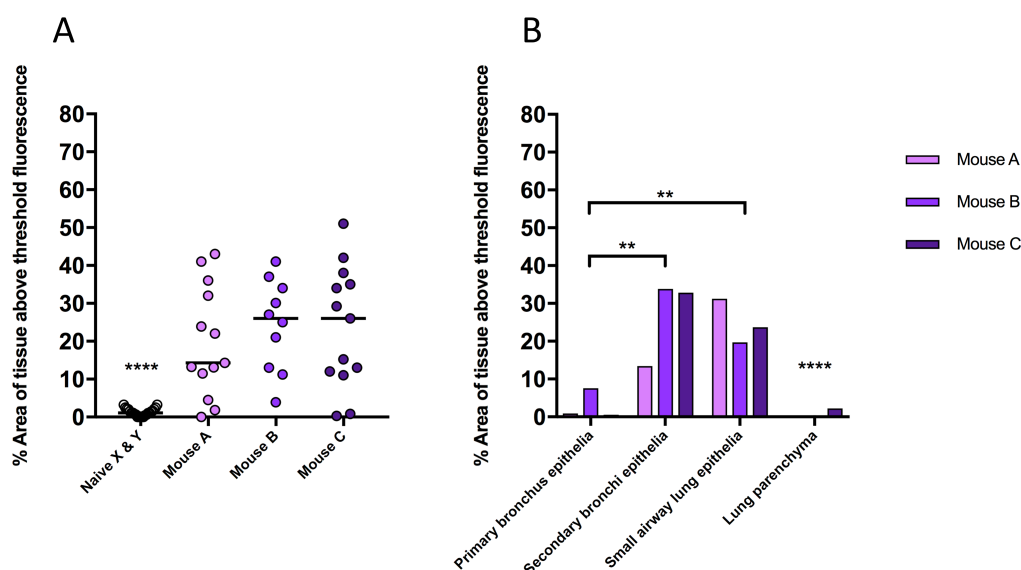


Figure 22- EGFP quantified in mice treated with rSIV.F/HN and analysed after 1 year. Mice (n=3) were treated with 3e8 TU of rSIV.F/HN.hCEF.EGFP or left untreated (n=2 naïve), and were sacrificed 1 year later and lung cryosections prepared. The percentage area of airway within ROI above fluorescence threshold were determined using Image J software (NIH). A, B and C refer to individual treated mice. (A) Each data point represents an airway measurement from a single ROI. Median represented by solid line. X and Y refer to individual naïve mice. All treated mouse airway values were compared with naïve mouse airway values (****, $p < 0.0001$; Mann-Whitney U test). (B) Mean percentage area of tissue above threshold fluorescence for each anatomical ROI category for each mouse. Each bar represents an individual mouse. Data from naïve mice not shown: mean % area from these animals was always $< 2\%$. Parenchymal values versus all airway values. (****, $p < 0.0001$; Mann-Whitney U test). Significance also detected between values from the different types of airways from treated mice (primary bronchus versus secondary bronchi, **, $p = 0.0016$; primary bronchus versus small airway, **, $p = 0.0017$ Kruskal–Wallis followed by Dunn’s Multiple Comparison test).

Although no significant difference was detected between airway values from anatomical categories at day 7 and 28 (3.2.1.2), the trend observed (greater values from secondary bronchi versus primary and small airways) is upheld in the values from mice sacrificed at 1 year. However, primary bronchi airway EGFP expression was significantly lower in secondary and small airways at 1 year (Figure 22 B). This could suggest proportions of EGFP expressing cells in the proximal airways are more greatly reduced between the 28 day and 1-year time point relative to secondary and more distal airways. Interestingly, upon wide-field cryosection imaging of mouse lungs at 1 year after treatment, a series of clustered EGFP-positive cells were observed in secondary bronchi in two of the three treated mice (mouse A and C Figure 23). To further determine the enrichment of EGFP-positive cells in these bronchi, consecutive cryosections were imaged and a similar pattern of enrichment in the corresponding regions of secondary bronchi was observed (Figure 23 purple boxes). This supports the notion that these EGFP-expressing cells are clustered within the same region of airway epithelium. Such clusters may arise as a result of transduced cell mitosis and are suggestive of stem/progenitor cell transduction by F/HN in the secondary bronchi.

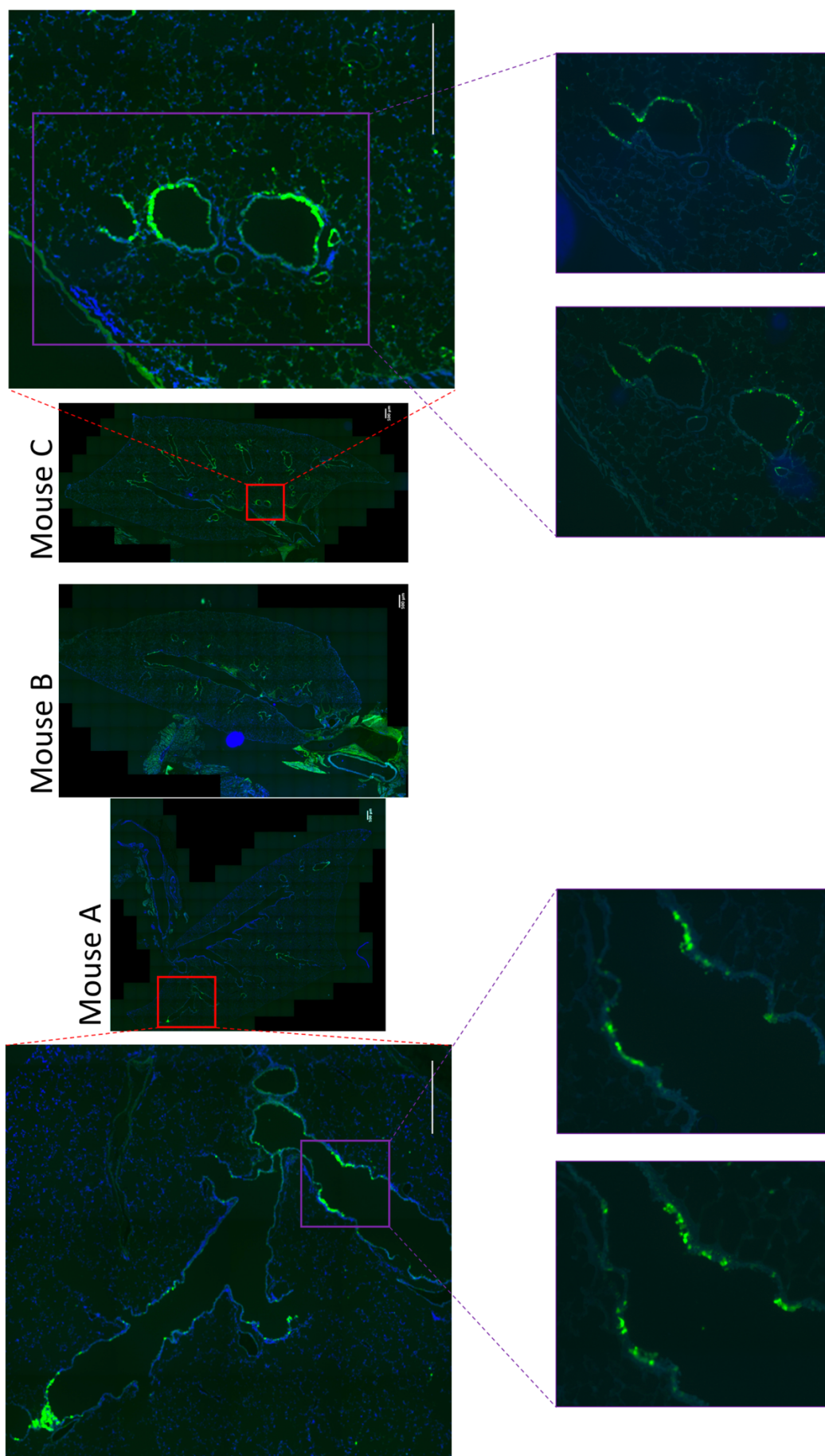


Figure 23- EGFP visualised in mice treated with rSIV.F/HN and analysed after 1 year. Stitched images of whole left lung lobe cryosections generated from mice treated with 3e8 TU rSIV.F/HN.hCEF.EGFP (n=3; mouse A, B and C) sacrificed at 1 year. Examples of native EGFP detected in airways identified as secondary bronchi indicated by selection in red, enlarged. In purple, ROI across consecutive cryosections exemplifying corresponding regions of enriched native EGFP detection are shown. Scale bar represents 500µM. Nuclei are stained blue (DAPI), images captured and stitched using EVOS FL Auto 2.

3.2.3.2.2. Polidocanol to model regeneration of the epithelium

Expression detected at 1 year following LV delivery could indicate transduction of cells *in vivo* that persist and express EGFP throughout their lifetime (>1 year), or that the initial transduced cells had progenitor/stem properties and the observed signal is from daughter cells expressing the integrated transgene at 1 year. To further investigate progenitor/stem cell transduction by F/HN in this model, regeneration of the epithelium was forced following LV delivery. The detergent polidocanol has been used to model regeneration of both the nasal epithelium delivering volumes of up to 10µL (Mitomo et al., 2010), and the tracheobronchial epithelium by intranasal (Farrow et al., 2018) or direct intratracheal instillation of volumes up to 25µL (Leblond et al., 2009). Aiming to model regeneration of airway epithelium within the lung (bronchi) however, it was first determined whether an intranasal dose of 20µL would be tolerated by mice, as well as the extent of airway epithelium stripping (24 hours), and regeneration (7 days) following polidocanol administration. Cryosections were generated from mice following Polidocanol treatment and stained for morphology using haematoxylin and eosin to visualise nuclei and extracellular matrix/cytoplasm respectively (Figure 24). Naïve mice were included as controls.

Evidence of thinning and loss of epithelium integrity was observed throughout the primary bronchi of polidocanol-treated mice sacrificed at 24 hours (Figure 24 B & E); which contrasted with the bronchi of untreated naïve mice where a continuous, linear epithelium was

observed (Figure 24 A & D). In contrast, the primary bronchi from mice treated with polidocanol sacrificed after 7 days showed a thickened epithelium with a dark continuous and linear staining pattern, consistent with a regeneration of an integral epithelium (Figure 24 C & F). These results indicate that polidocanol, when delivered as a 20 μ L bolus via intranasal delivery, can be used to force regeneration of the primary bronchi epithelium in mouse lung. Using this model, native EGFP detected from mice treated with rSIV.F/HN.hCEF.EGFP was investigated $\pm$ polidocanol induced primary bronchi epithelium regeneration. Following delivery of 1e8 TU, mice were sacrificed (n=2; LV only (day 7)) or treated with polidocanol. 24 hours following polidocanol treatment (n=2) dosed mice were sacrificed to confirm epithelium stripping (LV + polidocanol (day8)) and at 7 days following polidocanol treatment (day 14 post LV delivery) the remaining mice were sacrificed to determine native EGFP expression (n=6; LV + polidocanol (day 14)).

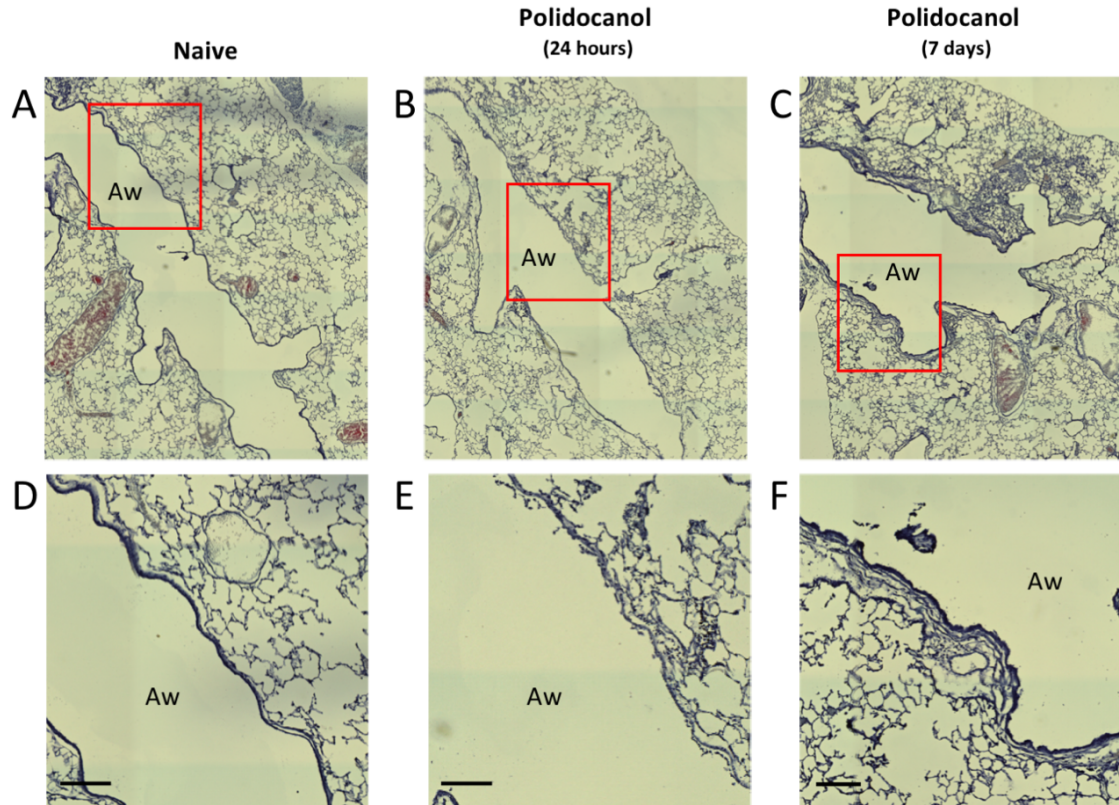
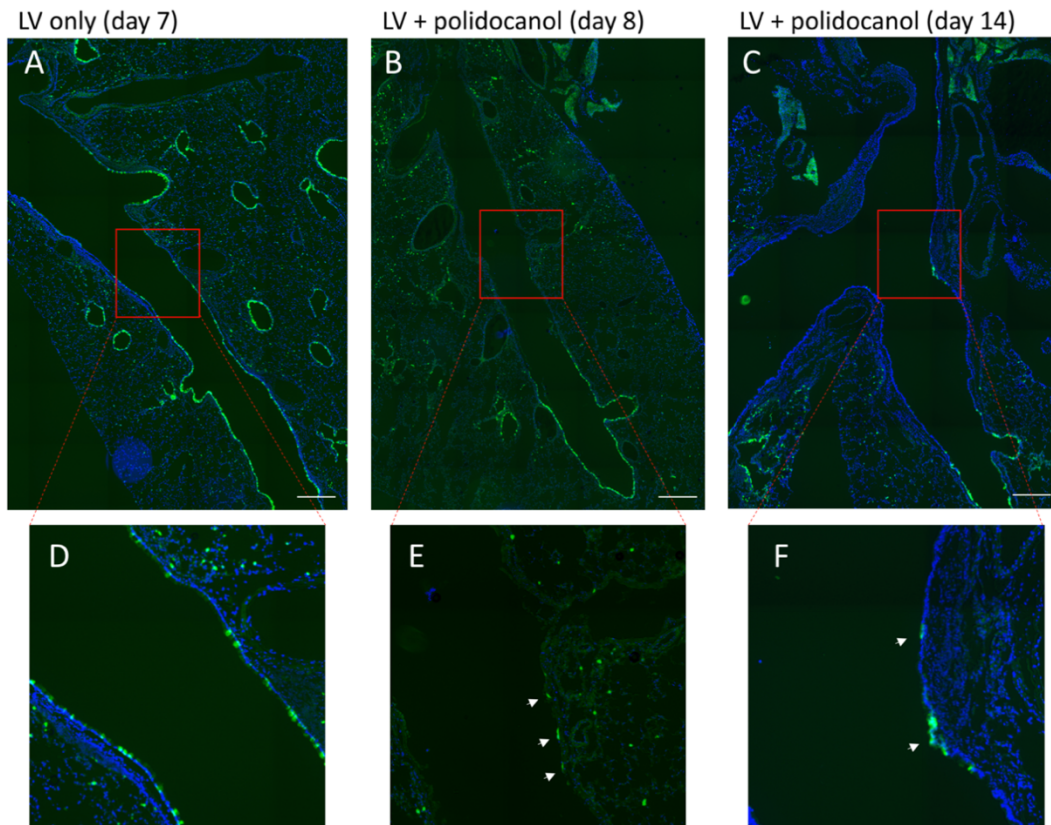


Figure 24- Effect of intranasal polidocanol on primary bronchi epithelium integrity. Representative stitched images of primary bronchi captured using whole left lung lobe cryosections generated from naïve (n=1, A & D), or polidocanol (20 μ L; 2%) treated mice, and sacrificed 24 hours (n=2, B & E) or 7 days (n=2, C & F) after treatment. Sections stained with haematoxylin (dark blue; nuclei) and eosin (pink; cytoplasm and extracellular matrix). Scale bar represents 100 μ M, Aw: airway, images captured and stitched using EVOS FL Auto 2. Red box (A-C) indicating enlarged portion of epithelium illustrating epithelium integrity (D-F).



LV: LV delivery
PDOC: Polidocanol delivery
*: Sacrifice

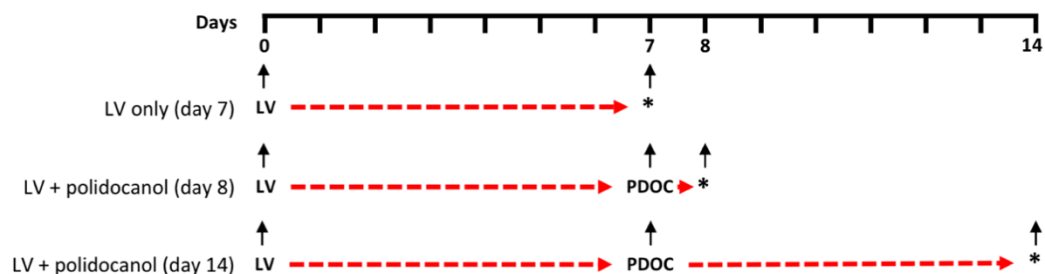


Figure 25- Use of polidocanol to model regeneration of primary bronchi epithelium following transduction with rSIV.F/HN. Representative stitched images of primary bronchi captured using whole lung lobe cryosections from mice transduced with 1e8 TU of rSIV.F/HN.hCEF.EGFP (Wave 121). Mice were sacrificed (LV only (day 7); n=2, A & D), or further treated with polidocanol to model stripping (LV + polidocanol (day 8); n=2, B & E) and regeneration (LV + polidocanol (day 14); n=6, C & F) of the epithelium. Red box (A-C) indicating enlarged portion of primary bronchi epithelium (D-F). White arrows (E & F) indicating examples of native EGFP expression in primary bronchi following polidocanol treatment. Scale bar represents 500μm. Nuclei are stained blue (DAPI), images captured and stitched using EVOS FL Auto 2. Timeline of experimental procedures shown below (LV: LV delivery, PDOC: polidocanol delivery, *: sacrifice).

As observed previously (Figure 13), native EGFP was detected along the primary bronchi of mice transduced with 1e8 TU of LV only (Figure 25 A & D). In contrast, very little native EGFP was detected in cryosections from primary bronchi from mice subsequently treated with polidocanol and sacrificed after 24 hours (day 8, Figure 25 B & E), consistent with the stripping of the transduced epithelium (Figure 24 B & E). The few EGFP-expressing cells observed on primary bronchi epithelium were squamous in morphology (Figure 25 E, white arrows), in stark contrast to those found in the epithelium in the absence of polidocanol treatment (Figure 25 A & D). Of the six mice processed for cryosections of primary bronchi following LV delivery and subsequent forced regeneration of the epithelium (LV + polidocanol (day 14)), native EGFP expression in the primary bronchi was detected in three. In contrast to the dispersed pattern of EGFP detected in mice in the absence of polidocanol treatment (Figure 25 A & D), EGFP-expressing cells detected following forced epithelium regeneration tended to appear in clusters (Figure 25 C & F).

3.2.3.2.3. Polidocanol to enhance LV access to repopulating cells

The model of forced primary bronchi epithelium regeneration was used to investigate the potential of rSIV.F/HN to target cells responsible for epithelium regeneration, by delivering the LV following stripping of the epithelium (24 hours after Polidocanol treatment). All six mice pre-treated with polidocanol and subsequently transduced with rSIV.F/HN had patchy, but markedly enhanced, levels of native EGFP detected in the primary bronchi as well as in the adjacent surrounding parenchyma (Figure 26 B). In contrast to the dispersed pattern of EGFP expressing cells observed in mice without pre-treatment (Figure 26 A), EGFP expression was contiguous in many portions of the epithelium (Figure 26 B). These data suggest that rSIV.F/HN can transduce stem/progenitor cells under the right circumstances (likely regarding physical access) with levels and pattern of EGFP expression in regenerated airways indicative of transduced cell proliferation.

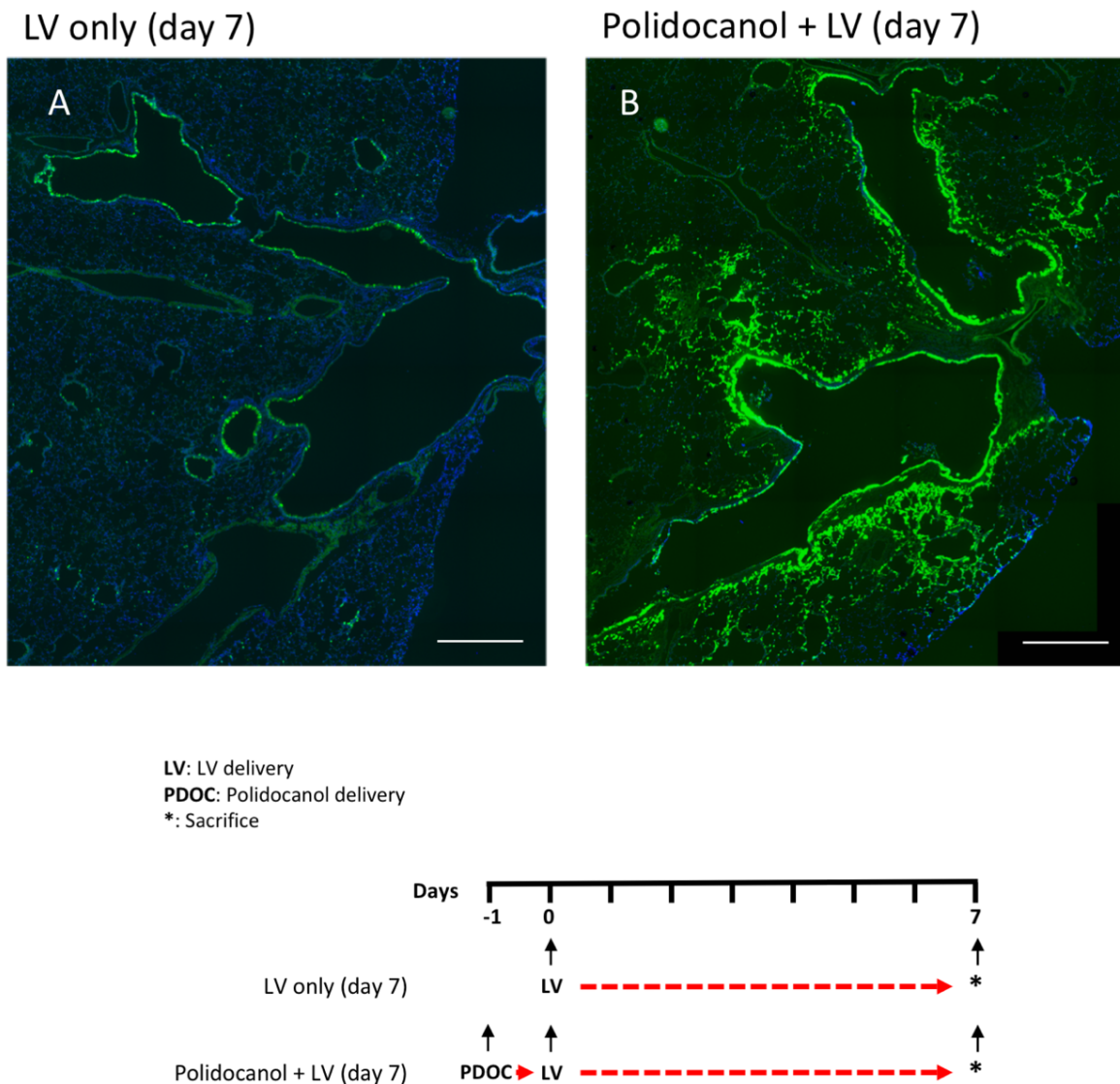


Figure 26- Use of polidocanol to enhance rSIV.F/HN access to repopulating cells of the primary bronchi airway epithelium. Representative stitched images of primary bronchi captured using whole lung lobe cryosections generated from mice transduced with 1e8 TU of rSIV.F/HN.hCEF.EGFP (Wave 121). Mice were sacrificed after 7 days, with (n=6, B) or without (n=3, A) pre-treatment of polidocanol 24 hours prior to LV delivery. Scale bar represents 500µM. Nuclei are counterstained blue (DAPI), images captured and stitched using EVOS FL Auto 2. Timeline of experimental procedures shown below (LV: LV delivery, PDOC: polidocanol delivery, *: sacrifice).

3.3. Discussion

3.3.1. Detection of native EGFP in the mouse lung

The direct detection of a transgene product offers significant advantages over methods dependent on substrate, or immunostaining, mainly by reducing the risk of false positive and negative results. By investigating transduction of a range of LV pseudotypes *in vivo* using native EGFP expression, it was possible to determine the relative efficiency of transduction in the murine airways. All mice treated intranasally with rSIV.F/HN.hCEF.EGFP resulted in high levels of native EGFP visualised in the airway and parenchyma (Figure 11); an observation consistent with the expression pattern and efficiency determined for this vector previously using EGFP immunostaining (Alton et al., 2017). Quantification of native EGFP by area (%) above threshold fluorescence was developed as an automated method to measure EGFP expression after LV delivery to the mouse lungs, and showed levels significantly greater than untreated/naïve mice. By direct comparison with traditional counting approaches, the automated quantification, was shown to modestly underestimate the percentage of transduced epithelial cells; importantly this meant that the median EGFP detection in airway epithelium (7.5% for automatic versus 13.63% by manual cell counting; Figure 14) was a conservative estimate. Importantly, quantification using percentage area facilitated a higher throughput. Further, by exploiting this method to capture native EGFP expression from many airways throughout the mouse lung using ‘whole lobe’ cryosection images, the distribution of EGFP-expressing cells could also be characterised and ROI used for quantification selected using an unbiased, systematic approach; something which is difficult to achieve with manual counting.

Having produced a range of rHIV.F/HN.CMV vectors for comparison of fluorescent reporter transgenes for detection *in vivo*, using expensive and time-consuming cell-factory methods, it was disappointing to observe only one treated mouse resulting in quantifiable EGFP detection in the airways. Interestingly, evidence for expression of both EGFP and tdTomato in treated mice using nasal septum wet mount imaging (Figure 15 A) highlighted the fluorescence

limitations of cryosection preparation. Disappointingly, there was a lack of tdTomato expression detected in the airways of treated mice, despite a greater TU dose delivered compared with rHIV expressing EGFP; this could be as a result of either insufficient expression of the dimer tdTomato protein in murine lung cells, or a greater sensitivity of this fluorophore to the tissue processing and fixation steps required in the generation of cryosections suitable for fluorescence imaging.

3.3.2. Insufficient yield of concentrated rHIV for *in vivo* for pseudotype characterisation

Production of sufficient rHIV for comparison of pseudotypes *in vivo* was very challenging. Vector concentrations of F/HN and Rostock pseudotyped rHIV achieved similar (F/HN; 7.98e8 TU/mL Wave 96) or greater (Rostock; 5.14e9 TU/mL Wave 114) titres than rSIV.F/HN (1-3e9 TU/mL, Figure 13), with very few EGFP-positive cells detected with any of the rHIV vectors produced for comparison of pseudotype (or transgene, Figure 15), and despite delivery of the MFD (Figure 15, 16 & 18). This is in contrast to robust expression observed with the rSIV.F/HN vector (Figure 13). It is therefore difficult to attribute any significance to the observation that the few EGFP-expressing cells identified in mice treated with rHIV vectors were confined to the airway (F/HN and Rostock), or parenchyma (ShanghaiN1 and ShanghaiN9) (Figure 16), given their infrequency. The significantly greater percentage EGFP area in the airway epithelium versus parenchymal tissue using rSIV.F/HN, however, is at least consistent with the epithelial distribution of the few cells transduced with rHIV/F/HN. An increased affinity of Shanghai H7 for the α 2,6 subtype of receptors (Yang et al., 2013) is also in line with the reported availability of this receptor subtype in the mouse lung parenchyma (Ning et al., 2009)(Figure 9).

Although yields of functional particles pseudotyped with F/HN may be similar for rHIV.F/HN and rSIV.F/HN vectors, the lower efficiency of rHIV.F/HN could be due to a number of reasons. For example, the F/HN pseudotype, comprising the addition of the cytoplasmic tail

from SIVagm envelope glycoprotein to the N-terminus of the HN protein (Kobayashi et al., 2003), might be sub-optimal for rHIV vectors. It is possible that the difference could be due to the alternative pseudotyping plasmids used, but this was deemed unlikely given the low EGFP detection from rHIV.F/HN vectors prepared with alternate versions of the pseudotyping plasmids (vGM182 Figure 15 and vGM188 Figure 16). Additionally, disadvantaged detection of EGFP through use of CMV versus hCEF to mediate expression was discounted as there was no improvement in EGFP detection from any of the pseudotyped rHIV.hCEF vectors versus rHIV.CMV despite delivery of greater TU (Figure 16, Figure 18). The many potential restrictions on the HIV-1 capsid (described in sections 1.2.3.1, 1.2.3.2) could limit the efficiency of transgene delivery and EGFP detection, but is unlikely given reports of high transgene expression in mouse lung with other rHIV-based pseudotypes (Buckley et al., 2008; Stocker et al., 2009). The inefficiency of rHIV.Rostock observed here contrasts with the reported efficiency of this pseudotype for rHIV in mouse lung, which was demonstrated following delivery of 2×10^7 TU via intranasal delivery of a 20 μ l volume (Patel et al., 2013). Reconciling TU values determined using different functional titration methods (Table 4 B, 2.6.1) is always a challenge and may explain some discrepancies. In the studies reported in this chapter a method based on enumerating DNA integration events was employed, while the studies of Patel *et al* utilised a method based on enumerating LacZ positive cells. It is possible that these methods have diverged to the point where comparison of titres is unhelpful. This is not unreasonable given that even DNA-based titration methods can vary by 10-50 fold between laboratories and 2-4 fold with a laboratory (historical laboratory observations) depending on cell types used in the determination, the precise transduction conditions, the duration of the cell culture phase and the use of alternate DNA standards (particularly circular supercoiled DNA versus linear DNA molecules).

A further confounding issue was that the yield of concentrated rHIV pseudotyped with ShanghaiN1 or ShanghaiN9 was low (maximum of 7.81×10^7 TU/mL vGM192). Production of this novel hemagglutinin pseudotype was a greater challenge than even the Rostock HA, which is

perhaps uniquely advantaged for pseudotyping LV efficiently. It has been previously demonstrated the Rostock HA can be pseudotyped in the absence of NA to HIV particles, in contrast to a human IAV HA (H3N2- Aichi/2/68)(V. Bosch et al., 2001). As discussed (see 7.3.1), production of LV pseudotyped with Shanghai HA may be of greater dependence on sufficient incorporation of neuraminidase glycoprotein compared with the Rostock pseudotype, adding to the challenge in manufacturing. Further, in contrast to the natural polybasic cleavage site found in Rostock HA facilitating cleavage by furin or furin-like proteases within the trans-Golgi network, the Shanghai HA incorporates a synthetic polybasic site. Although removing the need for an activation step following LV harvest, this polybasic site renders the particles infective immediately upon release, potentially promoting transduction of producer cells which might ultimately limit productivity. Additionally, although M2 incorporation has been shown to enhance yield of infectious Rostock HA pseudotyped particles (McKay et al., 2006), the impact of M2 incorporation on transduction by rHIV remains unclear (see 7.2.2), and although no detriment to infectivity of Rostock pseudotyped particles was detected by McKay et al., pseudotyping with M2 could negatively impact the transduction by Shanghai HA pseudotyped rHIV.

In contrast to influenza based pseudotypes, F/HN facilitates fusion, with no F glycoprotein dependency/sensitivity to pH, at the cell membrane. This could be advantageous to production of functional particles- a more stable pseudotype, invulnerable to an irreversible and inactivating pH driven conformational change during intracellular processing, explaining greater yields of TU with this pseudotype. Further, this pseudotype benefits from use of a single glycoprotein to mediate both binding (hemagglutinin) and cleavage (neuraminidase) of sialylated residues, which embodies a balance of these competing activities in functional particles (Benton et al., 2015). To further investigate whether insufficient neuraminidase activity was limiting release of functional vectors with influenza-based pseudotypes, exogenous neuraminidase was added, but there was no evidence of benefit to yield (Figure 17). The

importance of this balance may contribute to the greater yield of ShanghaiN9 versus ShanghaiN1, as the N9 neuraminidase from the WT strain could be a better match for achieving the right balance of effective binding and cleavage of non-functional sialylated residues, versus an alternative source of neuraminidase (from the H1N1 A/Puerto Rico/8/1934 strain), as used in ShanghaiN1 pseudotype.

3.3.3. rSIV.F/HN in the mouse lung

High, quantifiable levels of native EGFP fluorescence were reliably detected following delivery of 1e8 TU of rSIV.F/HN.hCEF.EGFP, across production batches (vGM107; Wave 83, Figure 13; Wave 121, Figure 25 and Figure 26), each individually titrated. Transduction of ciliated, club and goblet cells in the airway, and ATII cells in the parenchyma, were confirmed using confocal microscopy and immunohistochemistry, and these observations agreed with previous colocalization studies using immunohistochemistry for both EGFP and cell-specific markers (Alton et al., 2017). However, imaging using widefield fluorescence microscopy and image stitching was confounded by high levels of autofluorescence – particularly in blood vessels and airways - which tended to mask the cell-type specific immunohistochemistry signal. Staining for ciliated cells using β -tubulin antibody was an exception, however, with signal sufficient to detect both EGFP and cell type-specific staining. This could be a result of either the highly concentrated apical localisation of the β -tubulin antigen, or greater affinity and staining with this antibody combination. The rSIV.F/HN.hCEF.EGFP vector tended to show greater transduction of secondary bronchi than primary airway epithelium (Figure 13 B), perhaps explained by a greater F/HN pseudotype tropism for non-ciliated cells (more frequent in the distal airways) over ciliated cells (more frequent in the proximal airways) (Rock et al., 2010). This result is in stark contrast to the targeting of ciliated cells in cultures of mouse tracheal epithelial cells by with Sendai virus (Ibricevic et al., 2006) upon which this pseudotype is based and could indicate that the F/HN pseudotype cell tropism is different from Sendai virus.

3.3.4. F/HN evidence of progenitor cell targeting

Although basal cells expressing cytokeratin 5 (marker of progenitor cells) were identified in the mouse trachea using immunohistochemistry (Figure 21), assessment of vector transduction using native EGFP fluorescence was not possible, as the required antigen retrieval processing destroyed native EGFP fluorescence. Furthermore, no evidence for colocalization was obtained when dual immunohistochemistry was attempted (Figure 21). However, support for rSIV.F/HN transduction of lung stem/progenitor cells was obtained by investigating EGFP expression in mice 1 year after rSIV.F/HN delivery. Interestingly, the area of EGFP airway fluorescence, quantified by the automated method, was somewhat lower at 1 year (approximately 25% of airway epithelium area) than in mice sacrificed at day 7 or 28 (~40% airway area, Figure 13, all mice treated at same time with same vector lot); suggesting either a reduction in transgene expression over time, or the turnover of terminally differentiated transduced cells. As significantly lower levels of EGFP were found in the proximal versus distal airways, it could be speculated that this difference in expression (early versus 1 year timepoint) is a result of a greater epithelial cell turn over in the proximal airway potentially depleting transduced cells; this would be consistent with reports of increased ciliated cell turnover in the proximal airways in lineage tracing studies (Rawlins & Hogan, 2008).

The infrequent proliferation of epithelial cells in the more 'steady-state' secondary bronchial epithelium has been attributed to a subset of club cells, which in the absence of basal cells (Figure 4) play a role in the maintenance of the bronchial/bronchiolar epithelium in human and mouse (Hong et al., 2004; Montoro et al., 2018; Rawlins et al., 2009). The clusters of EGFP-positive cells observed in regions of the secondary bronchi epithelium at 1 year after rSIV.F/HN delivery could be due to transduction of club cells that subsequently underwent proliferation/differentiation. Polidocanol induced regeneration of the primary bronchi epithelium resulted in clusters of EGFP-expressing cells similar to those observed in the secondary bronchi 1 year after vector administration (Figure 23 versus 25). Interestingly, while

cytokeratin 5 positive (basal) cells were not observed in the murine primary bronchi of mice, the regeneration of the airway epithelium after polidocanol treatment requires cells with functional stem/progenitor properties to be available. At least some of these stem/progenitor cells were presumably local survivors of the polidocanol treatment as this is the simplest explanation for their EGFP-positive phenotype. Thus, these observations support the notion that F/HN vectors are able to transduce stem or progenitor cells, with the important caveat that without characterising the clonality of the cells in these clusters, stem/progenitor transduction can only be speculated. Further, although modelling regeneration of airway epithelium induced by polidocanol could prove to be a useful tool, differences may exist in the proliferative roles played by individual cell types involved in provoked injury repair as opposed to general ongoing maintenance of the epithelia. Nevertheless, the studies presented support the notion that F/HN vectors are capable of transducing cells that are not terminally differentiated. The conspicuous pattern of EGFP-expressing cells from rSIV.F/HN following polidocanol induced injury and subsequent regeneration (Figure 26) seems most simply explained as resulting from proliferation of transduced cells.

3.3.5. Conclusion

A method to allow rapid quantification of murine lung cell transduction using native EGFP detection facilitated assessment of both efficiency and distribution of transduction without compromising detection of transgene expression and allowed enumeration of specific cell types. Although it was not possible to make a direct comparison of transduction by the novel influenza-based pseudotypes using the *in vivo* mouse model, efficient transduction of mouse airway and parenchyma by F/HN pseudotyped rSIV was readily characterised. The broad murine lung cell type tropism observed with rSIV.F/HN vectors was in agreement with previous observations (Alton et al., 2017), although studies described in this Chapter showed a greater transduction efficiency for airway epithelial cells versus cells of the parenchyma, as well as a preference for

transduction of non-ciliated cells in the distal airway epithelium. This finding is somewhat in contrast to reports of Sendai virus targeting $\alpha 2,3$ sialylated residues on ciliated cells (Ibricevic et al., 2006), and requires further investigation. Intriguingly, results in mice transduced 1 year previously, or after undergoing forced regeneration of the airway epithelium, supports the notion that rSIV.F/HN vectors can transduce stem/progenitor cells in the lung.

4. Chapter 4- Precision Cut Lung Slices (PCLS) as a model for lentiviral vector transduction

4.1. Introduction

4.1.1. PCLS as an *ex vivo* model of the lung

Lung explant tissue, prepared as precision cut lung slices (PCLS), have been widely used as a model of the lung (reviewed (Sanderson, 2011)). The use of low melting-point agarose to inflate the lung tissue allows preservation of cut tissue structure and facilitates the investigation of aspects of respiratory physiology, such as airway contractility (Koziol-White et al., 2016) and lung cell populations such as alveolar macrophages (W. Wu et al., 2010). The PCLS model has also been used to investigate respiratory infections such as influenza (Van Poucke, Nicholls, Nauwynck, & Van Reeth, 2010), as well as transduction with viral vectors, e.g. Vaccina (Diallo, Roy, Abdelbary, De Silva, & Bell, 2011). In addition to lung tissue, tracheal explants cultured *ex vivo* have also been used to demonstrate apical tropism of LV pseudotyped with Ebola glycoproteins (1.4.2.1) compared with basolateral targeting using the VSVg pseudotype (Kobinger et al., 2001); this study suggests there is potential to model delivery to polarised epithelium *ex vivo*, although this has not been confirmed using airways captured within PCLS.

The PCLS model has significant advantages over *in vivo* animal models with regards to practical, ethical and financial considerations and can be generated from any source of fresh lung tissue including human. Another specific advantage for gene therapy investigations is the ability to more precisely control the delivery of limited and/or precious gene transfer agents to target tissues. The uniformly sized lung slices may also provide an opportunity for reproducible and higher-throughput screening than can be performed *in vivo*.

4.1.2. PCLS of lung tissue from multiple species

The ability to generate PLCS from any source of fresh lung tissue extends the use of this model to multiple animal species, which may differ in the distribution of sialic acid receptor subtypes. As discussed (1.5.3), such putative differences in sialylated receptors between species could ultimately affect subsequent cell targeting and efficiency of LV transduction, thus understanding the species and cell tropisms for a pseudotyped virus could be very useful in selecting the appropriate animal species for testing. In studies of respiratory viruses that utilise sialic acid as a receptor for infection, ferrets and pigs are widely used to model and predict human infection, as there is an abundance of the 'human' $\alpha 2,6$ receptor subtype in the upper respiratory tract and airways of these two species (Figure 27) (Nelli et al., 2010; Q. Xu, Wang, Cheng, Zengel, & Jin, 2010). In particular, both ferrets and pigs have been used to study influenza transmission, infectivity and pathogenesis (Belser, Katz, & Tumpey, 2011; Rajao & Vincent, 2015). Non-human primates (NHP) are less frequently used to model pulmonary infections with such hemagglutinating viruses, due to limited knowledge of sialylated receptor distribution (Davis, Taubenberger, & Bray, 2015). However, NHPs are still valuable for evaluating therapeutics with respect to potentially toxic or inflammatory responses (Miller, Royer, Pinkerton, & Schelegle, 2017).

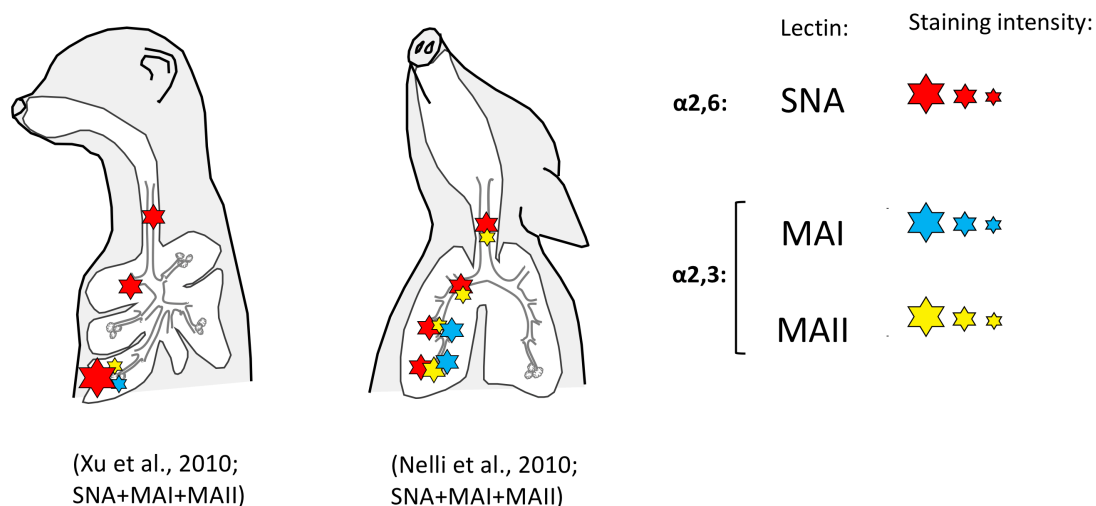


Figure 27- Distribution of sialylated receptor subtypes in the respiratory tract of ferret and pig.

The distribution of sialylated receptors, as determined using lectins *Sambucus nigra* agglutinin (SNA; red stars) to detect α 2,6 sialylated linkages and *Maackia amurensis* (MAI; blue stars) or (MAII; yellow stars) to detect α 2,3 sialylated linkages, in the airway and respiratory epithelium of ferret (Q. Xu et al., 2010) and pig (Nelli et al., 2010). Size of the symbol corresponds to the relative lectin staining intensity.

4.1.3. Aims

The potential for PCLS to model *in vivo* LV transduction was determined using rSIV.F/HN transduction of murine PCLS, in comparison with *in vivo* mouse lung delivery (3.2.1 & 3.2.3). In addition, transduction using F/HN and the alternative, influenza-based pseudotypes was investigated in PCLS from different species to assess the impact of species and pseudotype on the transduction profile in lung.

4.2. Results

4.2.1. Establishing PCLS as a tool to investigate LV transduction

To evaluate transduction by pseudotyped LV in PCLS, lung tissue from multiple species was obtained either as a whole lung, with potential for perfusion and preservation of tissue *in situ* (preferred option), or by procurement of tissue samples following lobectomies. In order to

control for any impact of the less preferred procurement process on PCLS in culture, the viability of PCLS from each source was determined at the point of transduction, using the live/dead staining assay as described (2.10.2).

4.2.1.1. Detection of murine PCLS transduction by rSIV.F/HN

In Chapter 3, robust detection of native EGFP fluorescence was observed following *in vivo* delivery of rSIV.F/HN.hCEF.EGFP to the murine lung (vGM107, Table 4, 3.2.1 and 3.2.3), therefore, murine PCLS were prepared for *ex vivo* delivery of rSIV.F/HN. Naïve BALB/c mice were sacrificed and PCLS generated immediately as described (2.10.1). Prior to LV transduction, several PCLS were selected at random and assayed for viability using the live/dead stain (2.10.2).

Figure 28 A shows a representative image from (n=2) PCLS stained with both calcein-AM and ethidium homodimer-1 indicating live and dead cells respectively, showing a mixed population of mostly live cells, with a small proportion of dead cells at the PCLS edge. In contrast, those PCLS pre-treated with ethanol, to serve as a positive control for cell death, did not stain with Calcein-AM. The secreted reporter Gaussia luciferase (gLux) was selected as the transgene since it is sufficiently sensitive to quantitatively detect transgene expression over a wide dynamic range, with the opportunity to monitor transduction over time via repeated media sampling. Although levels of activity were variable between individual PCLS, statistically significant gLux activity was detected in media from all murine PCLS treated with rSIV.F/HN.hCEF.sogLux (vGM124, Table 4), persisting from day 10 to at least day 45 (Figure 28 B). Following transduction with rSIV.F/HN.hCEF.EGFP (vGM107, Table 4), native EGFP was detected in murine PCLS (n=3) both in culture and in cryosections (Figure 28 C). Expression was localised mostly to the airway epithelium, similar to that observed and quantified in cryosections following *in vivo* delivery to the murine lung (Figure 11, Figure 13 B) using the same LV (vGM107). This similarity in distribution of EGFP expressing-cells between the two models demonstrates the potential of PCLS to reflect LV transduction *in vivo*.

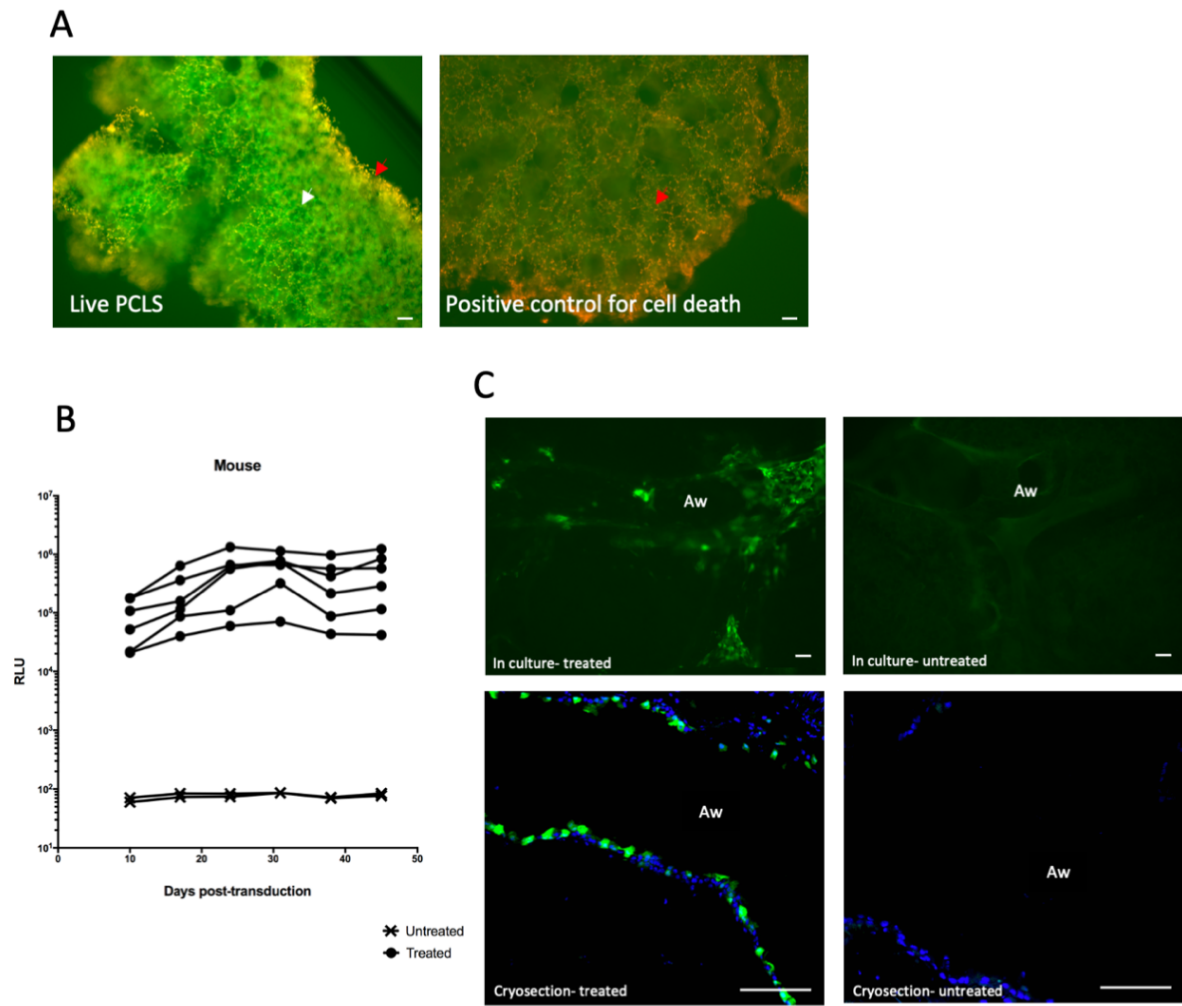


Figure 28- Transduction of murine PCLS with rSIV.F/HN. (A) Prior to LV transduction, the viability of murine PCLS was assayed. (LEFT) Representative image of murine PCLS (n=2) stained and imaged to determine viability in parallel with a positive control for cell death in PCLS (RIGHT) (green cells/ white arrow= live, red cells/arrows= dead). (B) PCLS (n=6) were treated with rSIV.F/HN.hCEF.sogLux (vGM124; 3e8 TU) or left untreated (n=2). GLux activity in media was sampled post- transduction (RLU; relative light units). The Log of area under the curve (AUC) for treated versus untreated was significantly different (****, $P < 0.0001$, Unpaired two-tailed t-test. (C) Representative images captured to detect native EGFP from PCLS (n=3) treated with rSIV.F/HN.hCEF.eGFP (vGM107; 3e8 TU) or left untreated (n=3), 7 days post-transduction in culture, or from cryosections of the PCLS (processed on day 7 and counterstained using DAPI; blue). All scale bars represent 100 μ M, Aw: Airway.

4.2.2. Investigating transduction by F/HN pseudotyped LV in PCLS from different species

PCLS were generated from ferret, pig and rhesus macaque lung tissue, as well as from resected lung tissue from two human donors. The viability of PCLS generated from each species was variable (Figure 29) with a mix of live and dead cells observed, similar to that observed in murine PCLS (Figure 28). Ferret PCLS appeared to be the most viable tissue, with the fewest (red/orange-stained) dead cells, whereas PCLS from macaque and human donors appeared to have a greater proportion, possibly a result of less controlled/prompt methods of tissue procurement. No airways were identified within the PCLS used to determine viability from human and ferret, due to their infrequent capture in the PCLS generated.

When PCLS (n=6-8) were taken from each species and transduced with 3e8 TU rSIV.F/HN.hCEF.sogLux, secreted gLux activity was observed in the media confirming transduction of all species. This result was statistically significant versus untreated controls following exclusion of outlier PCLS replicates in human donor 2 and pig (shown in red in Figure 30 A) when, following a lack of gLux activity, the treated PCLS were confirmed as non-viable, showing no evidence of live cells. Expression of gLux reporter appeared stable in treated PCLS from all species for at least 45 days and was detectable for >100 days in human, ferret and pig PCLS. When PCLS were transduced with 3e8 TU rSIV.F/HN.hCEF.EGFP, native EGFP expression was detected in culture and cryosections from all treated PCLS from all species compared with untreated (Figure 30 B). Although no airways were identified in human PCLS, likely a result of restricted availability within resected (distal) lung tissue, native EGFP was clearly observed in the parenchyma. In Ferret PCLS, native EGFP was concentrated to the airway as seen in mouse PCLS (Figure 28). In contrast, EGFP detected in pig and macaque PCLS was observed in both airways and parenchyma (Figure 30 B).

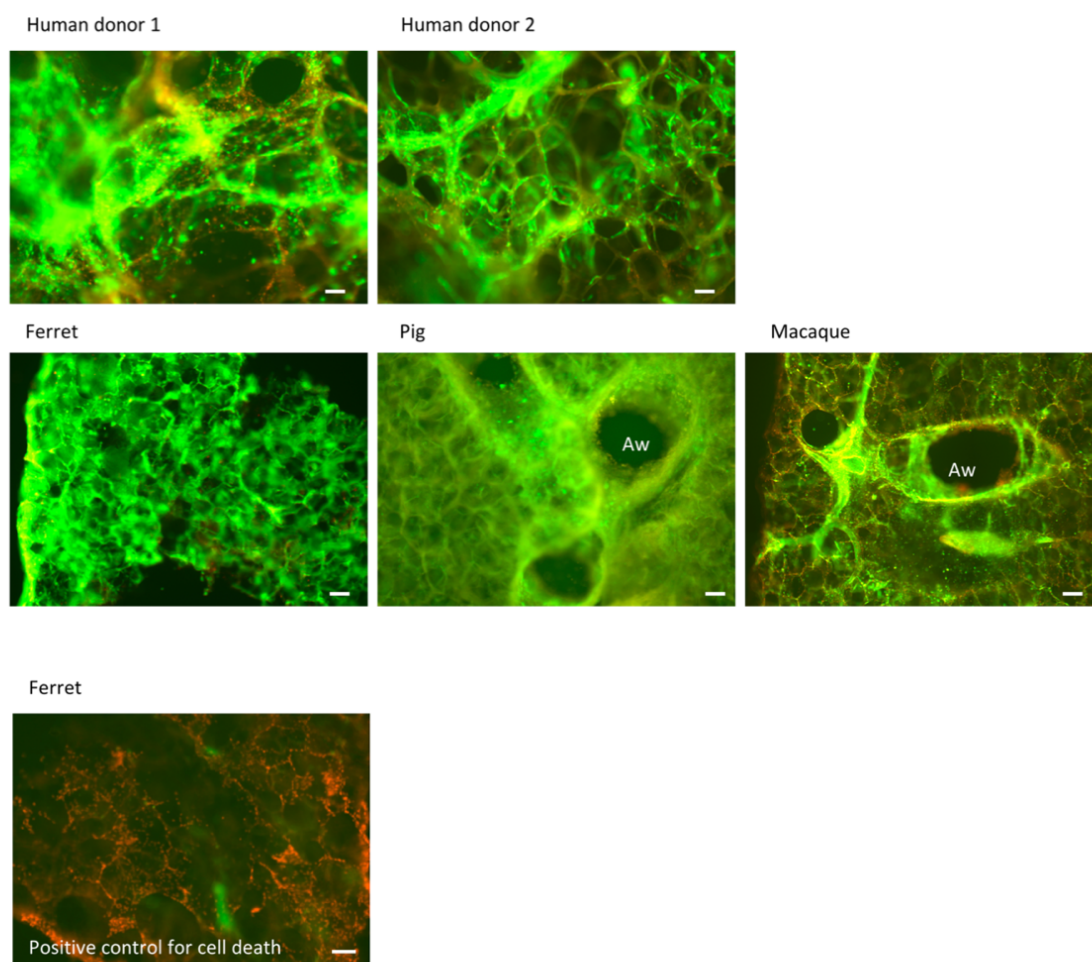


Figure 29- Viability of PCLS from human, ferret, pig and macaque lung. Representative images of PCLS (n=2 per species and donor) stained to determine viability. Positive control for cell death prepared using ferret PCLS also shown (2.10.3; green: live cells, red: dead cells). Scale bars represent 100 μ M.

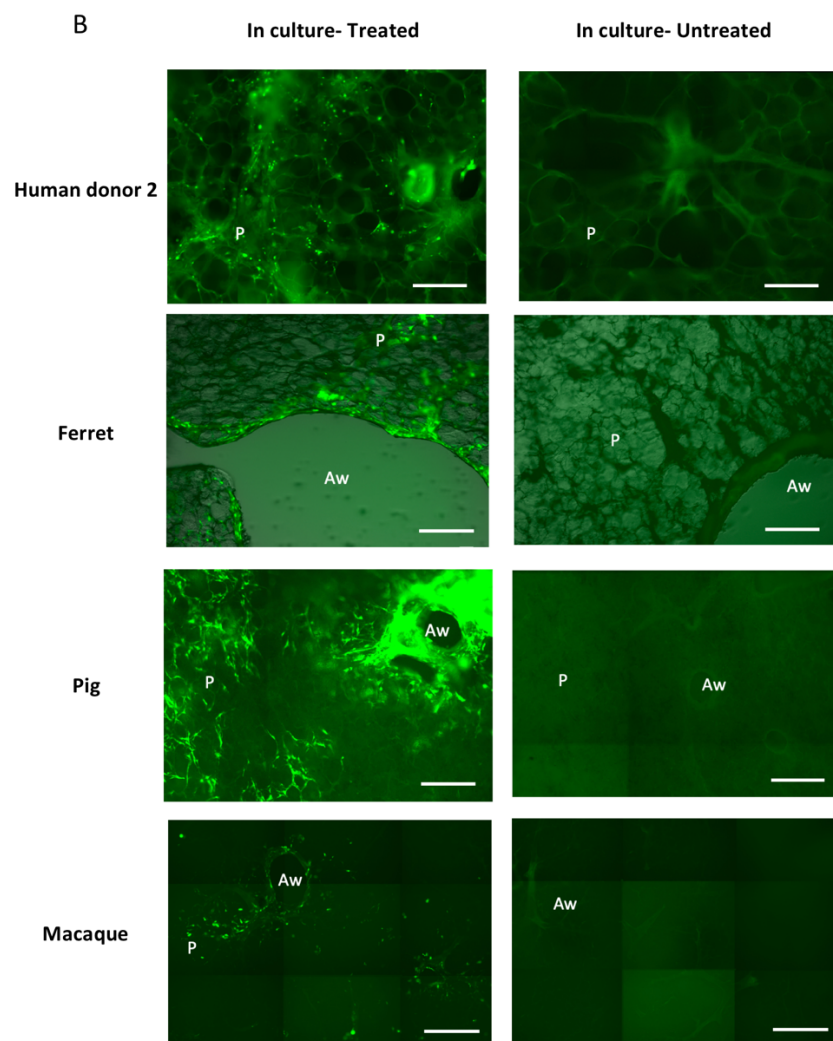
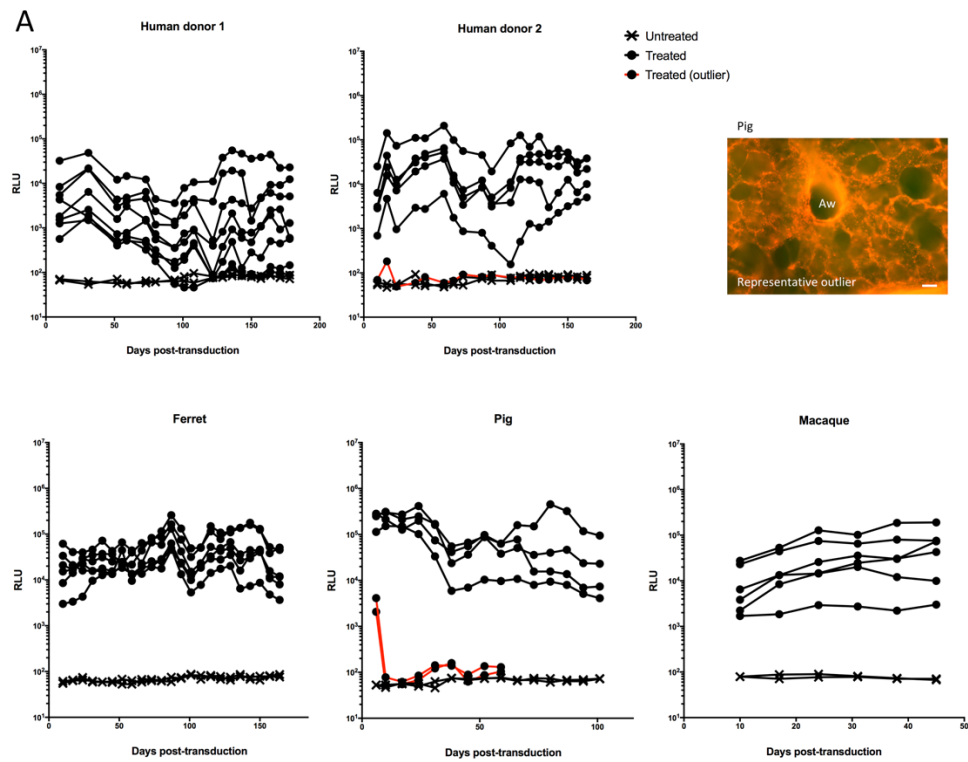


Figure 30- Transduction of PCLS from different species with rSIV.F/HN. (A) GLux activity detected in media sampled from PCLS treated with 3e8 TU rSIV.F/HN.hCEF.sogLux (vGM124; n=6-8) or left untreated (n=2) over time. Activity determined as described (2.10.7; RLU; relative light units). Log (Area under the curve) for treated versus untreated was significantly different for all species following removal of outlier replicates indicated (red lines): Human 1: P=0.0139 (*); Human 2: P=0.0031 (**); Ferret: P<0.0001 (****); Pig: P<0.0001 (****); Macaque: P=0.0013 (**). Significance determined using an unpaired two-tailed t- test. Representative image of outlier viability stain on pig PCLS also shown, red: dead cells, scale bar represents 100µM. (B) Representative images captured in culture to detect native eGFP from PCLS (n=3-6 PCLS per species) 7-28 days following treatment with 3e8 TU rSIV.F/HN.hCEF.eGFP (vGM107; Wave 110) or left untreated. Imaged; ferret PCLS images were captured with brightfield merge due to low autofluorescence of tissue for visualisation. Scale bars represent 500µM. Aw: Airway, P: Parenchyma.

4.2.2.1. Transduction of ferret trachea explant

The efficient transduction of the airway observed in ferret PCLS (Figure 30 B) could contradict the preference of Sendai virus, from which the F/HN pseudotype is derived, for binding α 2,3 sialylated receptors (1.5.1.1). Ferret airways are widely considered to model the human epithelium with a rich abundance of α 2,6 receptors, yet lack of α 2,3 characterised in bronchial and tracheal epithelium (Figure 27; Xu et al 2010). To investigate this further, cultured ferret trachea was transduced with rSIV.F/HN.hCEF.EGFP and cryosectioned. EGFP-expressing cells were identified along the epithelium indicating the transduction of ferret airways cells by F/HN is not limited to more distal airway epithelium such as that captured in PCLS. Further, transduction of airway resident ciliated cells was confirmed by native EGFP colocalization with β -tubulin immunostaining (example shown Figure 31).

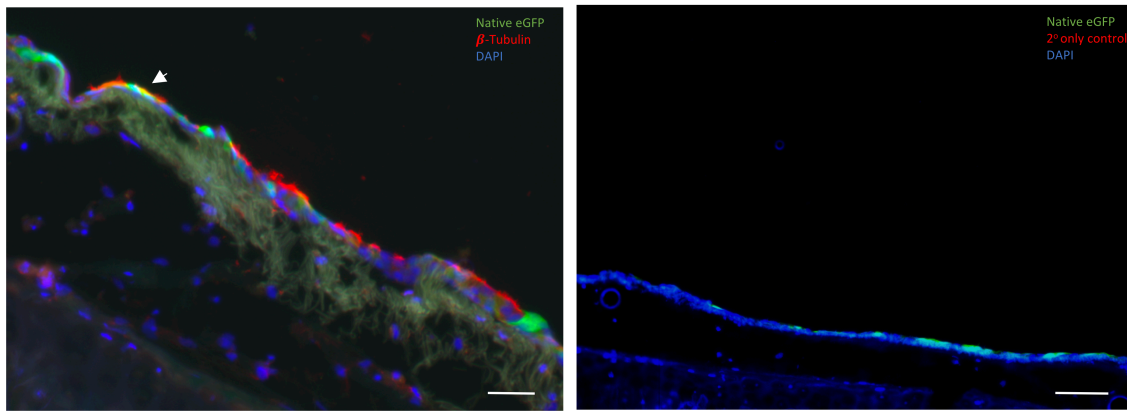


Figure 31- Transduction of ciliated cells in ferret trachea by rSIV.F/HN. Ferret tracheal tissue (n=2 explants from one trachea) was treated with 3e8 TU rSIV.F/HN.hCEF.eGFP and cryosectioned 7 days later. Example image shown of colocalization (white arrow) of native eGFP (green) with immunostaining for ciliated cells (β -Tubulin; red). Secondary antibody-only control shows native EGFP only. Cryosections were counterstained with DAPI (blue); scale bar represents 100 μ M. Images captured using EVOS FL Auto 2.

4.2.3. Transduction of PCLS with alternative pseudotypes

Having demonstrated transduction of all species of PCLS with rSIV.F/HN, the influenza-based pseudotyped vectors were investigated. In Chapter 3, rHIV vectors were generated to compare F/HN, Rostock, ShanghaiN1 and ShanghaiN9 pseudotypes *in vivo* (3.2.2), and the limited quantities available were used to treat PCLS from each species at a maximum feasible dose (MFD). Following treatment with rHIV.F/HN, native EGFP was detected in culture for all species of PCLS, confirming that rHIV could transduce all species investigated similar to rSIV (4.2.2), with the exception of human (Table 8, Human donor 2). The absence in human could be anticipated however given the relatively low (++, Table 8) levels of EGFP detected using rSIV and the ~6-fold lower TU of rHIV treated. Where detected, the distribution of EGFP (airway, parenchymal or both) for each species was also similar to that observed using the greater TU dose of rSIV.F/HN.eGFP (3e8 TU; Figure 30 B, also summarised in Table 8), supporting a possible species-specific cell type targeting by the F/HN pseudotype. With the lower TU doses of rHIV.F/HN (CMV or hCEF), fewer EGFP-expressing cells were observed as expected. Despite treatment with the

MFD of each vector, only minimal native EGFP was detected from rHIV pseudotyped with Rostock, ShanghaiN1 or ShanghaiN9 (in addition to F/HN) in murine PLCS, corresponding to the very poor efficiency achieved *in vivo* (Figure 16), with only a few cells detected in cryosections of PLCS also (Table 8, Figure 32). Additionally, no native EGFP was detected from these vectors in PLCS generated from human, ferret or pig lung and only a few cells detected in culture of Macaque PLCS in the parenchyma following ShanghaiN9 treatment, indicating no enhanced transduction by these pseudotypes for detection using alternative species PLCS.

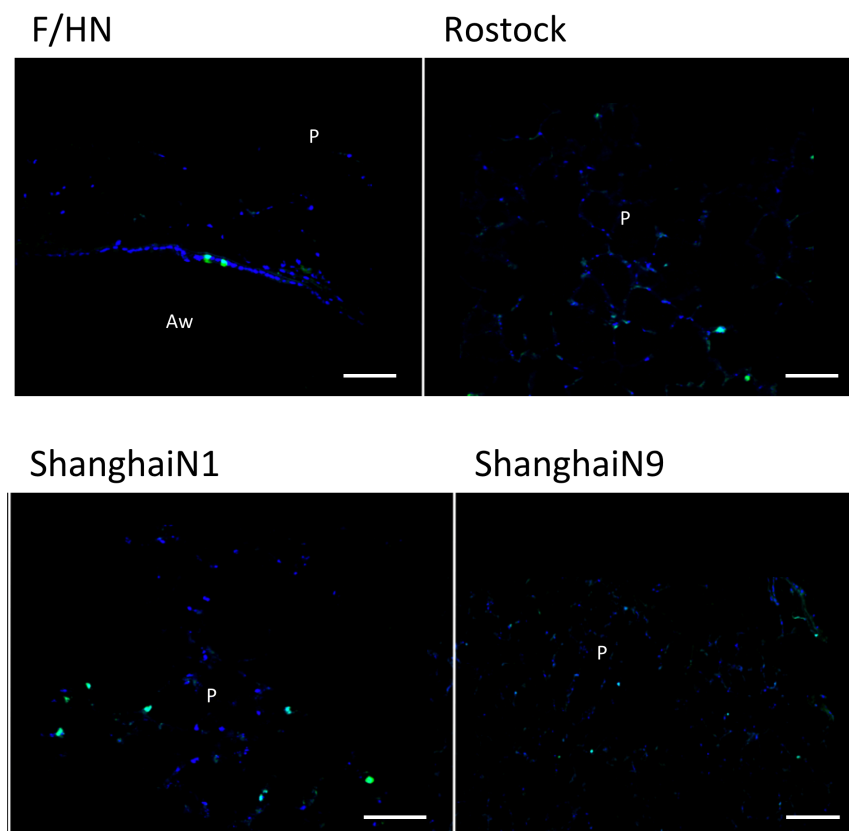


Figure 32- Native EGFP detection in murine PLCS cryosections following treatment with alternative pseudotyped rHIV.CMV. Murine PCLS treated with rHIV.CMV.EGFP vectors pseudotyped with F/HN (Wave 105; 5.45e7 TU), Rostock (OBM368 1.33e7 TU), ShanghaiN1 (OBM369 1.51e5 TU), or ShanghaiN9 (OBM370 1.13e6 TU) were processed for cryosections at day 7. Aw: Airway, P: Parenchyma. Native eGFP (green) counterstained using DAPI (blue). Scale bars represent 100µM.

Table 8- Summary of native EGFP detected in PCLS from different species following LV transduction. Native EGFP detected in PLCS from all species at day 7 post-LV treatment in Airway (Aw), Parenchyma (P) or not detected (-) using in-culture or cryosection imaging.

Relative abundance of eGFP expressing cells:

+, few cells detected in culture, none in cryosections;

++, cells detected in culture and few in cryosections;

+++, cells detected in culture and many in cryosections.

PLCS were treated with 3e8 TU of rSIV.F/HN.hCEF.EGFP vGM107 (Wave 110; n=3 mouse, human donor 2, ferret, macaque; n=6 pig).

For HIV vectors, a maximum feasible dose (MFD; 100ul) was applied to all species (n=3): TU of rHIV.CMV for each PCLS: F/HN, 5.45e7 (vGM188; Wave 105); Rostock, 1.33e7 (vGM222; OBM368); ShanghaiN1, 1.51e5 (vGM221; OBM369); ShanghaiN9, 1.13e6 (vGM220; OBM370). TU of rHIV.hCEF for each PCLS: F/HN, 6.76e7 (vGM193); Rostock, 5.14e8 (vGM194); ShanghaiN1, 1.62e6 (vGM191); ShanghaiN9, 7.81e6 (vGM192). Representative images of in culture native eGFP detection from PLCS treated with rSIV.F/HN shown in previously (Figure 30). *denotes example of few eGFP expressing cells detected in mouse PCLS cryosections shown (Figure 32).

	rSIV hCEF: 3e8 TU	rHIV				
	F/HN	Promoter	F/HN CMV: 5.45e7 TU hCEF: 6.76e7 TU	Rostock CMV: 1.33e7 TU hCEF: 5.14e8 TU	ShanghaiN1 CMV: 1.51e5 TU hCEF: 1.62e6 TU	ShanghaiN9 CMV: 1.13e6 TU hCEF: 7.81e6 TU
Mouse	Aw +++	CMV	Aw* ++	p* ++	p* ++	p* ++
		hCEF	Aw ++	-	-	-
Human donor 2	P (no Aw present) ++	CMV	-	-	-	-
Ferret	Aw +++	CMV	Aw +++	-	-	-
Pig	Aw & P +++	hCEF	Aw & P +++	-	-	-
Macaque	Aw & P ++	hCEF	Aw & P +	-	-	P +

4.3. Discussion

4.3.1. Use of PCLS to compare transduction by pseudotyped LV

Studies in Chapter 3 showed that *in vivo* delivery of rSIV.F/HN to the murine lung resulted in EGFP-positive cells mainly in the murine airways (Figures 11 & 13), indicating a preference for the F/HN pseudotype to target airway epithelial cells. It could be argued, however, that the minimal parenchymal transduction was a result of inadequate delivery to the distal mouse lung. The intranasal delivery technique (insufflation of small volumes of liquid) is a widely used lung delivery method, which can be affected by dosing volume and depth of anaesthesia (Southam et al., 2002). With expertise, however, it is highly reproducible, with delivery to the murine parenchyma that is consistent enough to demonstrate a preferential targeting for parenchyma with recombinant AAV5 and AAV9, preferentially transducing murine ATII (Sumner-Jones et al., 2007) and ATI cells (Limberis & Wilson, 2006), respectively. Together, these observations argue against poor parenchymal delivery being the reason for rSIV.F/HN transducing mainly airway epithelium in the murine lung.

The PCLS model was selected to further investigate LV transduction as delivery to parenchymal tissue is unlikely to be a problem for the *ex vivo* application of vector in this model. Using the same rSIV.F/HN vector and dose, *ex vivo* delivery to murine PCLS resulted in a similarly high efficiency, persistence and airway cell tropism to that observed *in vivo* (Figure 28 & Figure 13), suggesting that the PCLS model may be useful for predicting *in vivo* lung targeting. These data also reinforce the observation that rSIV.F/HN transduces murine airway epithelium more efficiently than parenchymal cells. This is also consistent with the reported preference for Sendai virus (from which the F/HN pseudotype is derived) to bind α 2,3 sialylated receptors (1.5.1.1, (T. Suzuki et al., 2001)), with a rich distribution of these receptors reported in these tissues (J. P. Guo et al., 2011; Ibricevic et al., 2006; Ning et al., 2009) (Figure 9).

It was hoped that the more controlled *ex vivo* delivery offered by the PCLS model might allow for a comparison of F/HN with influenza-based pseudotypes, using the limited supply of

rHIV vectors (3.2.2). Unfortunately, although transduction by rHIV.CMV vectors could be observed in murine PCLS (Figure 32), it was sparse, with no enhancement compared with *in vivo* (Figure 16), despite using the maximum feasible dose. These results suggest that poor vector distribution and exposure to lung tissue *in vivo* is not the cause of the low transduction efficiency observed.

Interestingly, compared with the relatively low level rHIV.F/HN transduction in murine PCLS, greater levels were observed in ferret and pig PLCS (Table 8). This is somewhat surprising, given the reported lack of $\alpha 2,3$ receptors in the ferret airways (Figure 27, (Q. Xu et al., 2010)), receptors preferentially used by Sendai virus from which the F/HN pseudotype is derived. This result is however supported by reports of efficient gene transfer by a Sendai virus vector to ferret airway *in vivo* (Yonemitsu et al., 2000). Conversely, there was no increase in EGFP detection with influenza-based pseudotypes in any of these species of PCLS compared with mouse PCLS. This suggests that the receptors available in the mouse lung are not a species-specific limitation to transduction by the influenza-based pseudotypes *in vivo* (Figure 16). Instead, the greater transduction efficiency of F/HN in ferret airway versus influenza pseudotypes could suggest that there is an even greater availability of the receptor(s) used *only* by the F/HN pseudotype in this model compared with mouse. Additionally, this result indicates that the (poor) transduction achieved by the influenza-based pseudotyped rHIV across species is not limited by species-specific rHIV restriction.

4.3.2. Impact of species on transduction by F/HN pseudotyped LV

Transduction with F/HN pseudotyped LV was investigated in different species of PCLS using the secreted gLux reporter and, although somewhat variable, gLux activity was detected in the media of PCLS from all species (Figure 30). Secreted gLux expression was stable (from 10-45 days) in treated murine PLCS in line with the similar levels of EGFP detected in mice *in vivo* at day 7 and 28 (Figure 13). Comparison of transduction efficiency between species, however, was

not possible with the observed variability in gLux measurements between PCLS and between samples; for this, a larger number of PLCS replicates, a better understanding of the factors contributing to the proportion of live and dead cells, as well as control of the tissue structures captured in each PCLS would be required.

Table 8 provides a summary of multiple studies in which native EGFP was detected in PCLS species following transduction of various LV configurations. For the F/HN pseudotype, native EGFP detected in PCLS from different species was slightly increased with rSIV.F/HN compared with rHIV.F/HN, although this is likely due to the lower (~10-fold) dose used. For each species, the distribution of transduced cells (localised to airway or parenchyma) was consistent for both rHIV and rSIV vectors, suggesting a potential species-specific transduction profile for F/HN. The F/HN preference for transduction of airways in both mouse and ferret (including trachea), is unexpected, however, with a reported prevalence of $\alpha 2,3$ receptors in mouse and $\alpha 2,6$ receptors in ferret (Figure 9, Figure 27). This could imply the use of both $\alpha 2,3$ receptors in mouse and $\alpha 2,6$ receptor subtypes by F/HN, or possibly, a common receptor prevalent across both species.

4.3.3. Modelling transduction of human lung

A key driver for all these studies is to understand which species might be the best ‘predictor’ of successful transduction in the human lung. Transduction of human lung PLCS was confirmed using rSIV.F/HN expressing gLux and EGFP, however, no EGFP was detected following rHIV.F/HN treatment (donor 2; Table 8), likely to be a result of the lower TU (versus rSIV.F/HN) used. The transduction observed in rSIV.F/HN treated PLCS was parenchymal with no airways identified in the human PLCS generated. Parenchymal targeting of human lung with F/HN is not unexpected given the mixed population of all $\alpha 2,3$ and $\alpha 2,6$ sialylated receptor subtypes in human parenchyma that can be characterised with lectins (Figure 9). Despite the inability to capture and model human airway transduction using human PLCS obtained from patient lobectomies,

the transduction of airways in ferret, pig and macaque (rhesus) is promising for F/HN transduction of human airway. In particular, transduction of ferret airway and trachea epithelium in PLCS implies potential for F/HN transduction of human respiratory tract, with similar sialylated receptors (Q. Xu et al., 2010) and viral attachment patterns (Van Riel et al., 2007) reported. Although extensive literature supports use of ferret airways as a model to predict human airway infection and transduction with hemagglutinating viruses (Buhnerkempe et al., 2015), some studies suggest the ferret respiratory tract contains sialylated residues that could serve as receptors, which are not present in human epithelium (Jia et al., 2014). Further, some studies noted differences in viral attachment between human and ferret trachea, indicating reduced receptor availability in ferret trachea versus human, such as with an isolate of the H7N9 2013 LPAIV outbreak (A/Anhui/1/2013)- demonstrating less efficient attachment to ferret trachea compared with human (Siegers et al., 2014). The inefficiency of this isolate for infection of ferret trachea was additionally confirmed (relative to human influenza H3N2) using *ex vivo* explants. This indicates translation of this difference detected in virus attachment/binding between models to their relative infectivity, reinforcing this possible flaw in use of ferret trachea/airway to model transduction of human airway.

Although the respiratory tracts of pigs are also often used to model human airway transduction, the airways captured in PLCS from such a large animal are likely to represent small airway/ bronchiolar epithelium. In contrast to proximal, these airways present a mixed population of all $\alpha 2,3$ and $\alpha 2,6$ sialylated receptor subtypes as detected by the MAI, MAII and SNA lectins (Nelli et al., 2010), implicating less restricted subtype availability and so potentiating transduction beyond that possible using receptors available in human airway.

The implications of F/HN transduction of rhesus macaque airway are unknown. Some studies have characterised the distribution of receptors in NHPs, including in African green monkey which shows similar distribution to human, rich in $\alpha 2,6$ receptors (Matsuoka et al., 2014), but this is not observed in the chimpanzee (N. M. Varki, Strobert, Dick, Benirschke, &

Varki, 2011), and a lack of $\alpha 2,6$ receptors has been inferred in cynomolgus macaque (Van Riel et al., 2007). No characterisation of the distribution of sialylated receptors in rhesus macaque have been reported to date, which makes any interpretation of the data difficult, but studies described in Chapter 6 attempt to address this.

4.3.4. Conclusion

The use of murine PLCS to investigate LV transduction *ex vivo* showed many similarities to results obtained with the *in vivo* mouse lung with regards to transduction efficiency and distribution. The usefulness of PLCS to characterise transduction by rHIV with influenza-based pseudotypes was limited due to the very low frequency of EGFP-expressing cells, although again, this was similar to that seen *in vivo*, irrespective of the species of PCLS used. Transduction of human lung parenchyma by the F/HN pseudotype was confirmed in PCLS, but transduction of human airway epithelium was not possible in this model due a lack of airways. Transduction mediated by F/HN was detected in PCLS from all species however, with some evidence for species-specific transduction profiles. In particular, transduction of ferret and pig airway epithelia could suggest efficient F/HN transduction using receptors found in human airway.

5. Chapter 5- Air-Liquid Interface (ALI) cultures as a model for lentiviral vector transduction

5.1. Introduction

Studies presented in Chapter 4 showed that PCLS from a variety of species could be used to model transduction of the lung. Human PCLS generated from donor lobectomies, however, lacked discernible airway structures and therefore, human Air-Liquid Interface (ALI) cultures were selected as a model in which to investigate F/HN transduction of airway epithelium in proximal airways. ALI cultures also have potential for greater inter-replicate reproducibility compared with PCLS, ensuring controlled vector delivery to the apical surface of epithelial cells, and facilitating the comparison of transduction efficiency between vectors.

5.1.1. ALI cultures can model the human airway epithelium

ALI cultures have been widely used to model the airway epithelium in health and disease, recapitulating many of the characteristics and cell types observed in polarised epithelium *in vivo* (Whitcutt, Adler, & Wu, 1988). Most commonly, ALI cultures are derived from human bronchial epithelial cells (HBEC), which can be isolated from patients undergoing surgery or post-mortem (De Jong, Van Sterkenburg, Kempenaar, Dijkman, & Ponec, 1993). In contrast to submerged cultures of HBEC, which do not differentiate in a polarised manner (Wu, Sato, & Whitcutt, 1986), HBEC cultured at an air-liquid interface on inserts with 0.4 µm pores to control nutrient delivery, simulate *in vivo* epithelial conditions. These cells differentiate to form a pseudostratified epithelium with a mucociliary phenotype, comprising at least three of the major cell types represented in the proximal human airway *in vivo*: basal, goblet and ciliated cells (Prytherch et al., 2011). Single-cell RNA sequencing of cells differentiated from HBEC at an ALI confirmed the expression of many different types, subsets and lineages of airway epithelial cells (Plasschaert et al., 2018), that express transcript profiles consistent with those observed *in vivo*.

Consequently, ALI cultures are well accepted for use in the study of the pseudostratified airway epithelium.

A key cell type found in the airway is the club (CC-10 expressing) cell, abundant in the smaller airways of human lung but infrequent in the proximal airways (Figure 4, (Boers et al., 1999). ALI cultures derived from bronchial cells (HBEC) typically possess club cells, but abundance varies; for example, club cells are reportedly absent in the commercial MucilAir™ (HBEC-derived ALI cultures from Epithelix Sarl) (Huang et al., 2017), but present in other ALI cultures from alternate sources of bronchial HBEC (Prytherch et al., 2011). This variability may result from differences in the approach to culture and differentiation of HBEC, but the responsible factors remain unclear. This variation in abundance between cultures could also reflect the intermediate role club cells play in the reported cell type lineage hierarchy (Montoro et al., 2018); interestingly, HBEC cultures are also observed to express more rare cell types such as pulmonary neuroendocrine, tuft and ionocyte cells.

5.1.2. ALI cultures to model human airway epithelium transduction

Differentiated ALI cultures are routinely used to model infectivity by respiratory pathogens as they have a polarised distribution of cellular receptors thought to recapitulate the native epithelium. For example, ALI cultures are regularly used to model infection of the human airway by influenza virus, as they express sialylated glycan receptors on their surface that are comparable to human lung bronchi (Ibricevic et al., 2006). The specific distribution of receptors within ALI cultures is unclear, however, with differing reports on the receptor subtypes present, as well as a lack of clarity as to which cells express which receptors.

The confusion over receptor distribution may be the result of different lectins used for staining, with important differences in binding specificity often overlooked (Geisler & Jarvis, 2011), as well as use of different detection protocols (Nicholls et al., 2007). There is some agreement that α 2,3 sialylated receptors (section 1.5) are found selectively on ciliated human

airway epithelial cells, identified both in ALI culture (Mikhail N. Matrosovich, Matrosovich, Gray, Roberts, & Klenk, 2004) and also in human bronchus (Ibricevic et al., 2006), using an indiscriminate mixture of the MAI/MAII lectins. However, this was identified only as a 'predominant pattern' in airway ALI cultures, with infrequent detection of non-ciliated cells also observed (L. Zhang et al., 2005). The distribution of $\alpha 2,6$ sialylated receptors on ciliated versus non-ciliated cells is further debated. Using SNA lectin, $\alpha 2,6$ receptors were identified on non-ciliated human airway cells in ALI cultures (Mikhail N. Matrosovich et al., 2004; L. Zhang et al., 2005) and also in bronchus (Ibricevic et al., 2006), but expression on ciliated cells was less often observed (Ibricevic et al., 2006; L. Zhang et al., 2005). Together, these studies indicate some common patterns in receptors present on specific cell types, but also highlight difficulties in interpretation.

When polarised ALI cultures were used to model LV transduction, it was shown that the VSVg pseudotype did not permit transduction from the apical surface, presumably due to a lack of available VSVg receptors; transduction was only evident in damaged areas of ALI cultures (Jonsdottir et al., 2019). Since apical access to the VSVg receptor located predominantly on the basolateral membrane is restricted *in vivo* (Kremer, Dunning, Parsons, & Anson, 2007), these observations support the premise that the ALI culture can model the polarised airway epithelium. Importantly, while it has been previously reported that ALI cultures can be efficiently transduced by rSIV.F/HN vectors (Griesenbach et al., 2012), the precise cell types remain unresolved. Furthermore, the transduction of human airway ALI cultures by alternative (Rostock or Shanghai) pseudotyped LV has not been investigated.

5.1.3. ALI culture types

ALI cultures are most often prepared using HBEC isolated from the bronchial (proximal) human airway, but cells isolated from more proximal (e.g. nasal) or distal (bronchiolar) airway epithelium have also been used. Often, only minimal attention is paid to the impact of epithelial cell isolation protocols or culture conditions. Such differences could contribute to the variable reports in cell type abundance and receptor profiles (5.1.1, 5.1.2); for instance, supplemental growth factors can influence differentiation at an ALI (Luengen et al., 2020). The commercially available SmallAir™ (Epithelix Sarl) cultures are derived from epithelial cells isolated by enzymatic digestion of distal (bronchiolar) airway and are reported to comprise a greater proportion of club (CC-10 expressing) cells and, conversely, a lower proportion of goblet (Mucin 5AC expressing) cells compared with equivalent MucilAir (bronchial) cultures (Huang et al., 2017) available from the same manufacturer. The use of both ALI culture types has the potential to model the difference in cell type abundance, and their corresponding cellular receptor distribution, in the proximal and distal regions of the human airway epithelium.

Importantly, disease states can also be modelled by preparing ALIs using HBEC from patients with conditions such as CF. ALI cultures of CF airway recapitulate aspects of the CF disease phenotype, such as impaired chloride conductance with poor mucociliary clearance and diminished airway surface liquid (ASL) volume (Harvey, Tarran, Garoff, & Myerburg, 2011). Using CF cultures, CFTR modulator drugs have been tested in Ussing chamber studies and showed rescue of the CF ALI culture electrophysiology (Gentzsch et al., 2017). The opportunity to investigate the impact of disease phenotype on LV transduction could help inform the translation to a clinical setting.

ALI cultures can also be prepared from immortalised cell lines such as human and mouse lung adenocarcinoma cell lines grown under ALI conditions. Surfactant ALI (SALI) cultures have been developed to model the human distal lung epithelia and have also been demonstrated to be transduced with rSIV.F/HN (Munis et al., 2020). Culture of the human lung adenocarcinoma cell line (H441) resulted in expression of both alveolar cell-specific surfactant proteins and tight

junction proteins, as well as epithelial barrier properties in line with previous reports (Salomon et al., 2014). This type of model, as with airway ALI cultures, offers potential to investigate transduction efficiency, with controlled delivery of vector to the epithelia surface

5.1.4. Aim

The aim of the studies in this Chapter was to explore the usefulness of human ALI cultures in modelling LV transduction of the human lung. Three types of human airway ALI culture were characterised and then assessed for transduction with a variety of LV pseudotyped vectors.

5.2. Results

5.2.1. Characterisation of airway ALI cultures as a model of human airway epithelium

Human ALI cultures used to model the bronchial airway epithelium were either HBEC (bronchial, Lonza) differentiated in-house to generate Bronchial-ALI cultures (B-ALI, Section 2.11.1), or were obtained commercially in a fully differentiated state from Epithelix Sarl (MucilAir). To investigate ALI cultures derived from epithelial cells from distal airway epithelia, SmallAir cultures were also obtained from Epithelix Sarl that had been generated from bronchiolar epithelial cells sourced from the same donors as the bronchial epithelial cells used to generate MucilAir cultures. Table 9 describes the airway ALI cultures derived from donors varying in age and gender, along with the respective TEER values and mucous phenotype. In addition, MucilAir cultures were obtained which had been generated from the bronchial epithelia of two CF patients with the most common, loss-of-function, CFTR mutation (deletion of Phenylalanine 508; >90% of CF cases) (Thibodeau et al., 2010).

Table 9- Airway ALI cultures generated and used. TEER measurement were performed as described (Section 2.11.3; Mean $\pm$ SEM; n=5) or by manufacturer (Epithelix Sarl; Mean $\pm$ SEM;

n=12). Mucus production scored as: +, mucus detected in wash procedure only; ++, mucus visible prior to wash (<7days after removal); +++, mucus visible 48hrs after removal; +++, mucus visible on ALI 24hrs after removal. Mucus mobility reported as: mobile (regular globule formation between 7 day washes) or cohesive (little evidence for globule formation and mucus slower to dislodge during wash).

Donor	Age	Gender	Airway epithelial cell source	Type	TEER (Ω .cm ²)		Mucus production	Mucus mobility
					In house measurement	Commercial measurement		
1	3	Female	Bronchial	B-ALI	245±12	N/A	++++	Cohesive
2	19	Female	Bronchial	B-ALI	309±19	N/A	++++	Mobile
3	16	Female	Bronchial	B-ALI	290±12	N/A	++++	Mobile
4	56	Male	Bronchial	B-ALI	460±25	N/A	++++	Cohesive
5	61	Female	Bronchial	MucilAir	535±14	544±9	+	Mobile
			Bronchiolar	SmallAir	939±19	941±9	+	Mobile
6	56	Female	Bronchial	MucilAir	304±7	299±19	++	Mobile
			Bronchiolar	SmallAir	822±12	815±22	++	Mobile
7 (CF)	30	Female	Bronchial	MucilAir	535±11	449±22	++	Cohesive
8 (CF)	24	Male	Bronchial	MucilAir	659±13	626±17	++	Cohesive

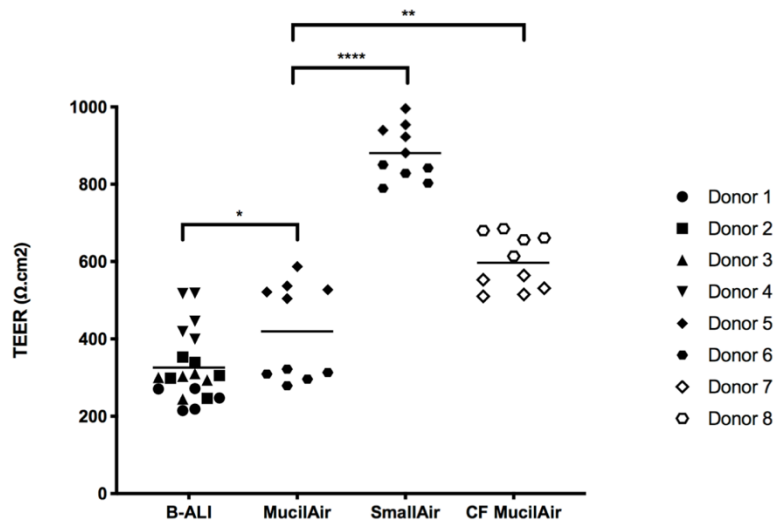


Figure 33- Trans Epithelial Electrical Resistance (TEER) in different types of human ALI culture from different donors. Trans-Epithelial Electrical Resistance (TEER) was measured as described (Section 2.11.3; n=5) for each airway ALI culture type. Each data point represents a biological replicate; the bar represents the grand mean. *, P=0.0244 (B-Ali versus MucilAir), ****, P<0.0001 (MucilAir versus SmallAir), **, P=0.0029 (MucilAir versus CF MucilAir), as determined using Mann- Whitney U test.

The TEER values were measured (Section 2.11.3) and indicated that all ALI cultures possessed a polarised epithelial barrier with permeability similar between transwell replicates (TEER >200 $\Omega \cdot \text{cm}^2$; SEM $\leq$ 25). Values obtained for MucilAir and SmallAir cultures were in line with values reported by the supplier (Epithelix Sarl) prior to shipping, validating the in-house TEER assay and indicating ALI culture integrity and successful maintenance upon receipt. Although TEER values were variable between donors, significant differences were observed between ALI types (Figure 33). Values calculated for commercially obtained ALI derived from bronchial epithelial cells (MucilAir) were slightly increased (~30% from a mean of 316 to 420 $\Omega \cdot \text{cm}^2$) compared with in-house derived ALI (B-Ali; P=0.0244). Further, CF MucilAir culture values were approximately 50% higher than normal MucilAir (P=0.0029). The most notable difference, however, was between MucilAir and SmallAir cultures derived from the same donors, with SmallAir cultures displaying an approximate two-fold (100%) increase in $\Omega \cdot \text{cm}^2$.

($P < 0.0001$). These differences indicate that the permeability of airway ALI cultures may be affected by donor and type of ALI culture, and perhaps most influenced by the airway epithelial cell location from which they are derived (e.g. bronchial versus bronchiolar).

Upon weekly inspection and washing, cilia beating and mucus production was confirmed in all airway ALI cultures (2.11.1). Unsurprisingly, mucus from CF MucilAir cultures represented a more cohesive layer across the apical epithelium that was difficult to reduce/remove with washing, compared with the 'mobile' mucus observed in normal MucilAir cultures. This cohesive mucus was not unique to cultures from CF donors, but was also produced by non-CF B-ALI cultures (donors 1 and 4). This phenotype was also independent of rate of mucus production with mucus from all B-ALI cultures apparent within 24hrs of removal, in contrast to MucilAir and SmallAir cultures only visible before or during wash procedure (Table 9).

The proportion of each of the major airway epithelial cell types in the ALI cultures was determined. Immunohistochemistry (IHC) of transwell cryosections (Section 2.11.7 & 2.7.2) was performed in parallel for markers specific for basal (Cytokeratin 5), ciliated (β -Tubulin), goblet (Mucin 5AC) and club (CC-10) cells (Figure 34). Basal and ciliated cells were abundant in all airway ALI cultures. The frequency of goblet and club cells varied; no goblet cells were detected in donor 5 MucilAir or donor 6 SmallAir cultures despite the presence of mucus (Table 9) and no club cells were found in B-ALI cultures from donor 2. All cultures comprised a pseudostratified layer of airway epithelium, although the B-ALI cultures were noticeably thicker than MucilAir and SmallAir cultures, due to multiple layers of basal cells as determined by IHC.

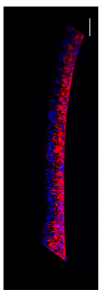
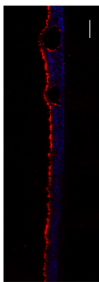
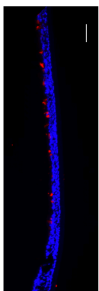
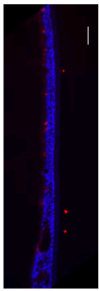
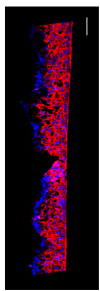
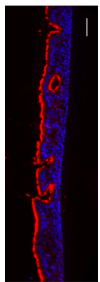
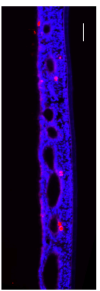
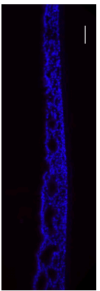
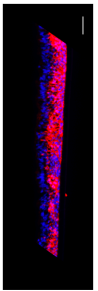
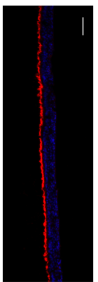
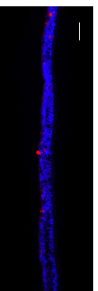
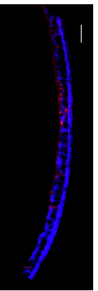
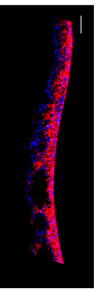
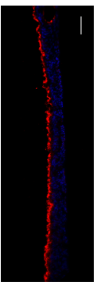
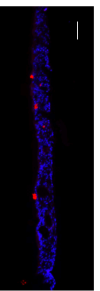
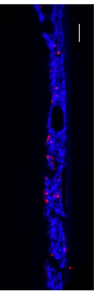
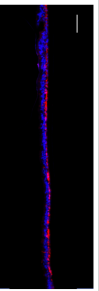
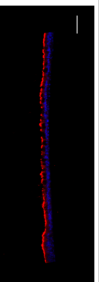
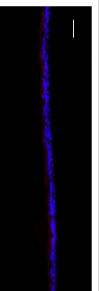
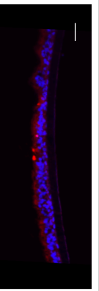
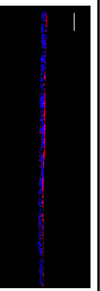
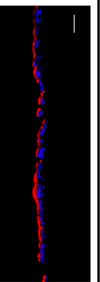
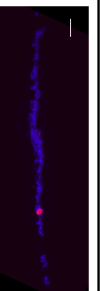
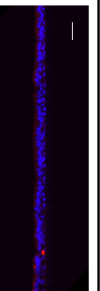
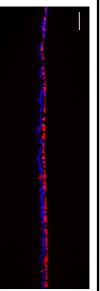
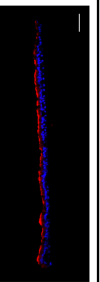
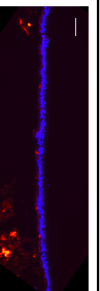
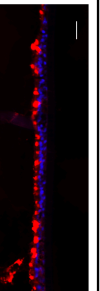
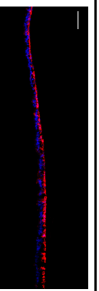
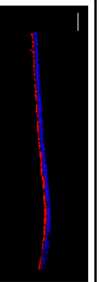
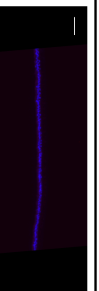
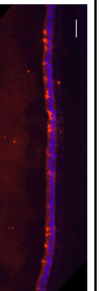
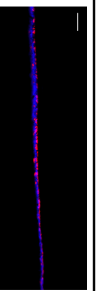
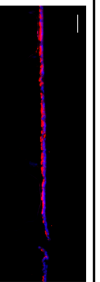
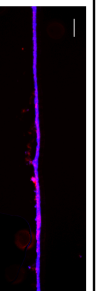
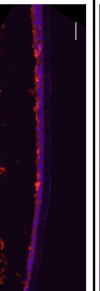
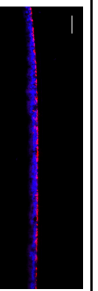
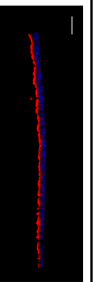
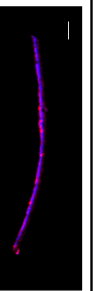
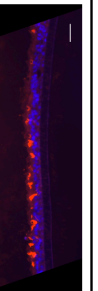
Donor	Type	Basal cells (Cytokeratin 5)	Ciliated cells (β -Tubulin)	Goblet cells (Mucin 5AC)	Club cells (CC10)
1	B-ALI	 Yes	 Yes	 Yes	 Yes
2	B-ALI	 Yes	 Yes	 Yes	 ND
3	B-ALI	 Yes	 Yes	 Yes	 Yes
4	B-ALI	 Yes	 Yes	 Yes	 Yes
5	Muci/Air	 Yes	 Yes	 ND	 Yes
6	SmallAir	 Yes	 Yes	 Yes	 Yes
	Muci/Air	 Yes	 Yes	 Yes	 Yes
	SmallAir	 Yes	 Yes	 ND	 Yes
7 (CF)	Muci/Air	 Yes	 Yes	 Yes	 Yes
8 (CF)	Muci/Air	 Yes	 Yes	 Yes	 Yes

Figure 34- Airway epithelial cell types in human ALI culture models derived from different donors. Immunohistochemistry staining of ALI culture transwell cryosections with cell type-specific antibodies for basal (Cytokeratin 5), ciliated (β -Tubulin), goblet (Mucin 5AC) and club (CC10) cells, shown in red. Nuclei counter stained blue (DAPI); scale bars represents 100 μ M. ND: not detected. Representative images shown from four cryosections per two transwells stained per donor and cell type (n=8).

5.2.2. Use of ALI cultures to model transduction of human airway cells

To investigate transduction of the different airway ALI culture types, rSIV pseudotyped with F/HN expressing EGFP, a vector previously shown to transduce bronchial-derived ALI cultures (Griesenbach et al., 2012), was delivered as a positive control.

5.2.2.1. Detecting transduction using LV expressing EGFP

B-ALI cultures from donor 2 were transduced with a range of TU doses of rSIV.F/HN.hCEF.EGFP (positive control) as well as rHIV.F/HN.CMV.EGFP, to assess the impact of alternative LV platform (SIV versus HIV), or transgene promoter (CMV versus hCEF), on transduction. Native EGFP fluorescence was visualised in culture and observed to gradually increase, until day 14, when all cultures were imaged (2.11.5, Figure 35) and processed for cryosectioning.

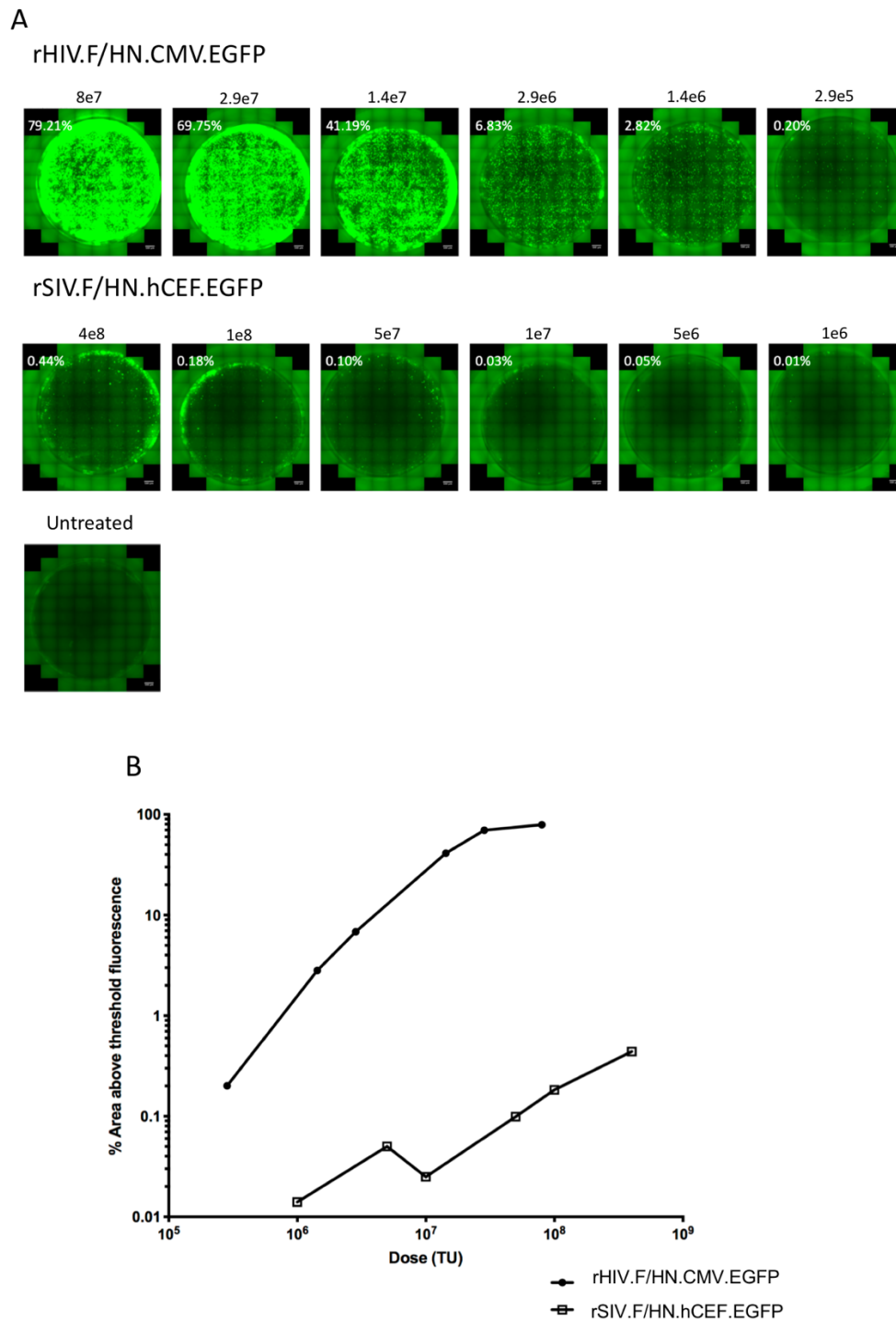


Figure 35- Dose related increase in EGFP fluorescence in B-ALI cultures following transduction with F/HN pseudotyped LV. Native EGFP detection from B-ALI cultures (donor 2) at 14 days following transduction with a range of TU of rHIV.F/HN.CMV.EGFP (vGM182 Wave 96), or rSIV.F/HN.hCEF.EGFP (vGM107 Wave 121), or left untreated. (A) Cultures were imaged to capture fluorescence from entire transwell (scale bars represents 500 μ M) and (B) EGFP fluorescence levels quantified by % area above threshold fluorescence (determined using untreated ALI culture as described Section 2.11.6). Representative image of untreated ALI culture used to set threshold is also shown.

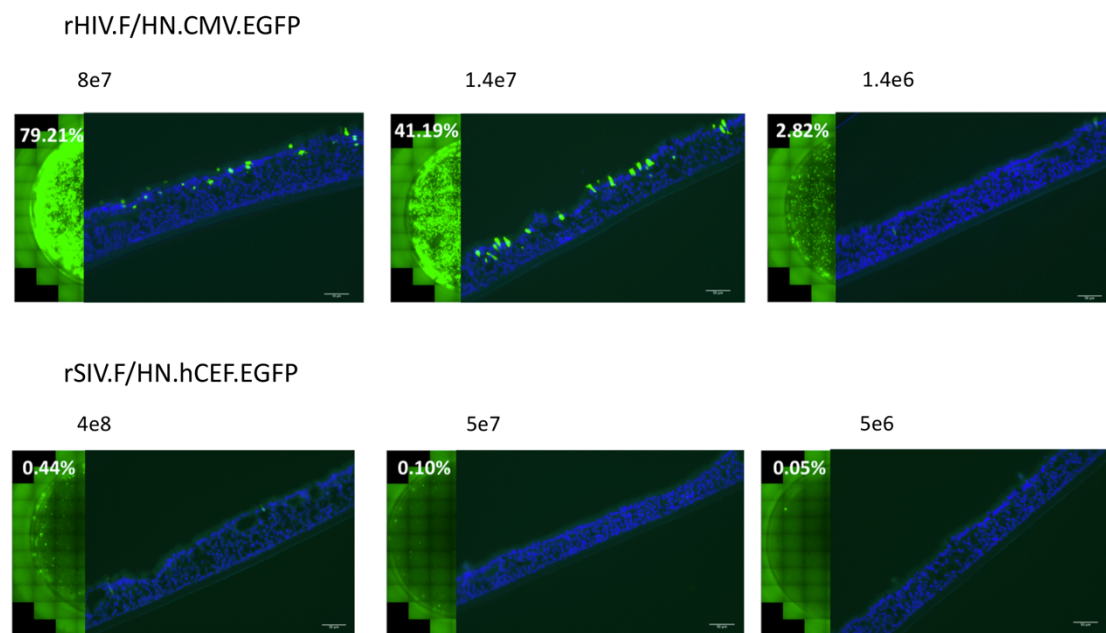


Figure 36- Dose related increase in native EGFP-expressing cells detected in cryosections of B-ALI cultures following transduction with F/HN pseudotyped LV. Representative images of transwell cryosections generated from B-ALI culture (donor 2) 14 days following transduction with rHIV.F/HN.CMV.EGFP (vGM182 Wave 96) or rSIV.F/HN.hCEF.EGFP (vGM107 Wave 121) and imaged to detect native eGFP (green); nuclei counter stained blue (DAPI); scale bars represents 50 μ m. Native EGFP fluorescence detected in-culture from the corresponding transwell cryosection is also indicated.

A dose related increase in EGFP fluorescence was observed for both vectors, when quantified as described (2.11.6). Interestingly, fluorescence levels were much greater in cultures treated with rHIV.F/HN.CMV compared with the rSIV.F/HN.hCEF positive control (Figure 35 A; day 14), with the latter achieving a maximum percentage area above threshold fluorescence of <0.5 %, while delivery of 5-fold less rHIV.F/HN.CMV achieved 79% (Figure 35B). Many EGFP-expressing cells were identified in transwells treated with 1.4e7TU of rHIV.F/HN.CMV, consistent with the high levels of fluorescence quantified, but very few EGFP-expressing cells were identified in any ALI culture cryosection treated with rSIV.F/HN.hCEF (Figure 36). Together, these data suggest that low levels of native EGFP detected from B-ALI cultures treated with

rSIV.F/HN.hCEF are likely a result of lower transduction efficiency (fewer cells) rather than the different transgene promoters used (hCEF and CMV for SIV and HIV vectors, respectively).

To assess native EGFP expression over time, B-ALI cultures treated with 2.9×10^7 TU rHIV.F/HN.CMV.EGFP or 1×10^8 TU rSIV.F/HN.hCEF.EGFP were maintained in culture, rather than processed for cryosections. At 10 and 20 weeks after treatment, the transwells were imaged under the same settings as those used at 2 weeks (day 14) and EGFP expression quantified as before.

High levels of EGFP fluorescence from the rHIV.F/HN.CMV treated culture were observed at week 10 and 20 (Figure 37), although fluorescence was somewhat reduced compared with 2 weeks (Figure 35), falling from 69.75% to 45.01% by week 10 and to 39.17% by week 20. Nevertheless, EGFP expression over a 20-week period suggests transgene expression from transduced human airway cells can persist for extended periods, in agreement with published reports of stable (3 months) expression observed in human ALI cultures transduced with rSIV.F/HN.CMV (Griesenbach et al., 2012). Any modest reduction in expression could be due to turnover of transduced cells. Similar kinetics were observed in ALI cultures treated with rSIV.F/HN.hCEF.EGFP (Figure 37), suggesting this observation was independent of the CMV or hCEF transgene promoter.

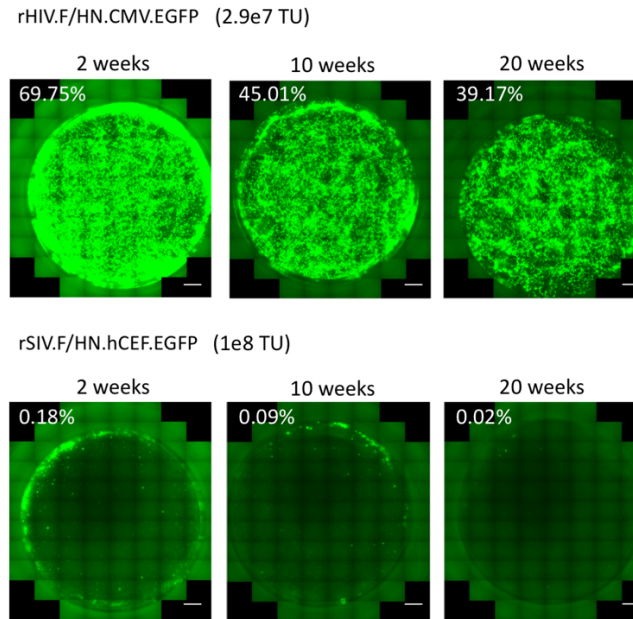


Figure 37- Native EGFP fluorescence from B-ALI cultures at 2, 10 and 20 weeks after transduction with F/HN pseudotyped LV. Native EGFP fluorescence from the same B-ALI cultures (donor 2) following transduction with rHIV.F/HN.CMV.EGFP (vGM182 Wave 96) or rSIV.F/HN.hCEF.EGFP (vGM107 Wave 121) was imaged at 2, 10, or 20 weeks (under the same settings). The percentage area above threshold fluorescence calculated at each timepoint is indicated. Scale bars represent 500µM.

5.2.2.2. Impact of donor and type of airway ALI culture on transduction efficiency

Since efficient transduction of donor 2 B-ALI cultures was observed with rHIV.F/HN.CMV, this vector configuration was selected to investigate the impact of alternative donors and ALI culture type on transduction efficiency by F/HN, with equivalent TU of VSVg pseudotyped LV as a negative control to confirm polarised receptor expression and integrity of the cell layer.

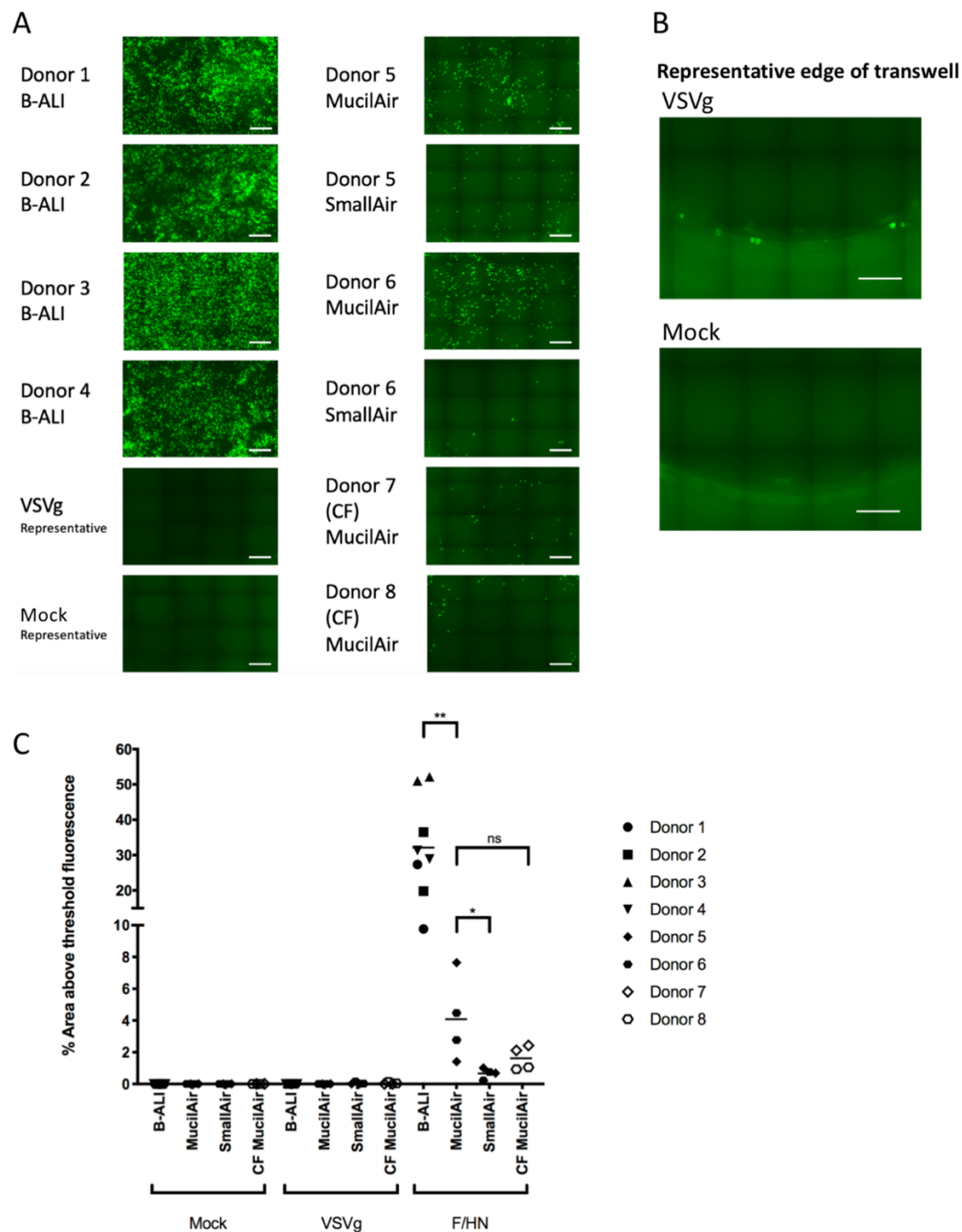


Figure 38- The impact of donor and airway ALI culture type on transduction by rHIV.CMV pseudotyped with F/HN or VSVg. Native EGFP detection in airway ALI cultures 14 days after treatment with 7.5×10^7 TU of rHIV.F/HN.CMV.EGFP (vGM182 Wave 96), or rHIV.VSVg.CMV.EGFP (DJ001; negative control) or buffer only (Mock). (A) Representative stitched centre-of-transwell images are shown (n=2 biological replicates per donor and culture type; B-Ali culture n=4 donors; MucilAir, SmallAir, and CF MucilAir n=2 donors); scale bar represents 500m. (B) Representative images of native EGFP expression observed at edges of rHIV.VSVg.CMV.EGFP treated cultures (excluded in quantification analysis; Section 2.11.6) compared with no EGFP expression in mock-treated transwells; scale bar represents 500μM. (C) Quantification of EGFP

detected (percentage area above threshold fluorescence) in ALI cultures transduced, or mock-treated (buffer only). Each symbol represents a biological replicate; each shape represents a single donor; the bar represents the mean. **, $P=0.004$ (rHIV.F/HN treated B-ALI vs MucilAir), *, $P=0.0286$ (MucilAir vs SmallAir), ns: not significant ($P=0.1143$; MucilAir vs CF MucilAir) as determined using Mann-Whitney U test.

Following rHIV.F/HN delivery, EGFP was detected in all airway ALI culture types from all donors (Figure 38). In contrast, no EGFP fluorescence could be quantified in airway ALI cultures treated with equivalent TU of rHIV.VSVg except for low levels at the transwell edges (Figure 38 B), suggesting overall ALI culture integrity (Jonsdottir et al., 2019). Although the percentage area of EGFP fluorescence varied between donors (median of $n=2$ replicates ranging between 19 and 52% area of EGFP quantified from rHIV.F/HN treated B-ALI cultures), there was no significant difference detected ($p=0.1619$ Kruskal-Wallis; B-ALI donors 1-4; $n=2$). There was approximately 2-fold less EGFP fluorescence from CF compared with non-CF MucilAir cultures, although not statistically significant ($P=0.1143$ Mann-Whitney U test; Figure 38). Significant differences in EGFP fluorescence were detected, however, between ALI culture types. A mean of only 4% area EGFP was detected in MucilAir in contrast to the 32 % in B-ALI cultures, despite the derivation of both culture types from bronchial cells ($P=0.004$). In SmallAir cultures derived from bronchiolar cells of donors 5 and 6, <1% area EGFP fluorescence was quantified, 5-fold lower than MucilAir cultures ($P=0.0286$). These results suggest that differences between the types of ALI generated, rather than the source of airway cells, can significantly affect transduction via F/HN, possibly indicating differences in the availability of receptors.

5.2.2.3. Impact of LV platform and transgene promoter on native EGFP detection

The low levels of EGFP fluorescence from rSIV.F/HN.hCEF delivered to bronchial B-ALI cultures (donor 2, Figure 35) was unexpected given the published reports of transducing (also bronchial)

MucilAir cultures with this vector (Alton et al., 2017; Griesenbach et al., 2012). This could be due to factors such as the specific ALI culture type or donor, and so these were investigated, along with the potential impact of the transgene promoter (CMV or hCEF) and LV platform (rHIV or rSIV). Bronchial-derived ALI cultures were treated with 7.5×10^7 TU of rHIV.F/HN.CMV or rSIV.F/HN.CMV to assess the impact of LV platform, and B-ALI cultures were treated with equivalent TU of rSIV.F/HN.hCEF as well as the maximum feasible dose of 1.2×10^7 TU rHIV.F/HN.hCEF to assess the impact of the hCEF transgene promoter. Imaging in culture revealed clear donor-independent differences in both the abundance and intensity of EGFP fluorescent cells between the different F/HN pseudotyped LV configurations across all bronchial ALI cultures assessed (Figure 39). The number of EGFP-expressing cells detected was patently greater with both rHIV configurations compared with the analogous rSIV configurations, despite treatment with an equivalent or greater TU dose (Figure 39 A). In addition, for both rHIV and rSIV, the CMV promoter resulted in a much greater intensity of native EGFP fluorescence than the hCEF promoter (Figure 39 A). This impact of using either an alternative promoter or LV platform was further quantified by percentage area (2.11.6), and a significant difference detected between all LV pairs for comparison using Mann-Whitney U test (Figure 39 B). As well as emphasising the significance of LV platform and transgene promoter on native EGFP detection, this indicates the quantification method by percentage area (2.11.6) able to measure differences in native EGFP expression, by both amount and intensity. These results suggest that although hCEF appears to drive lower levels of EGFP expression in these cultures, rHIV results in a greater transduction efficiency versus equivalent TU of rSIV. This result is in contrast to the efficient detection of rSIV.F/HN.hCEF transduction across all models investigated so far in Chapters 3 and 4 (Figure 13 & Figure 30).

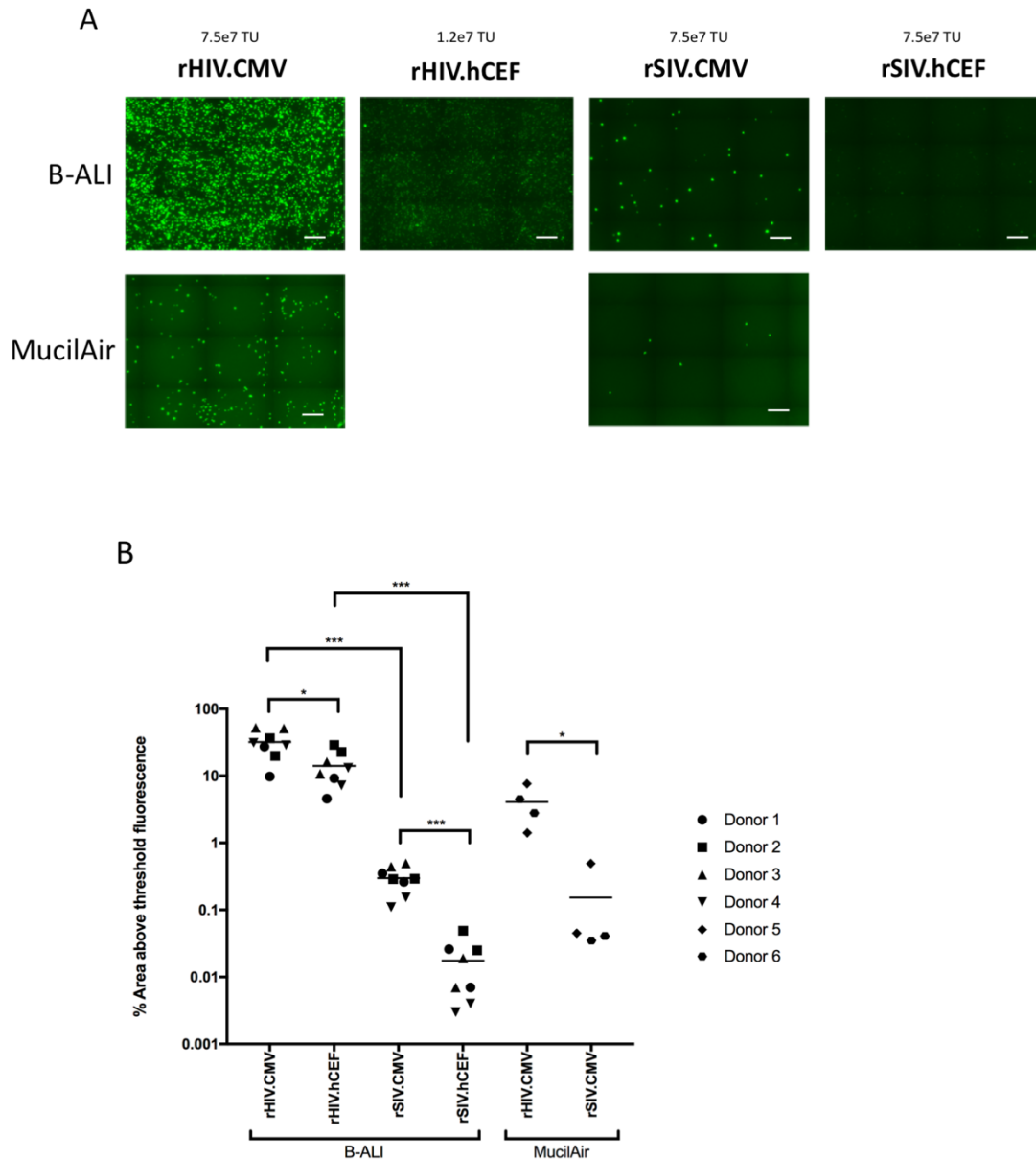


Figure 39- Impact of transgene promoter and vector platform on EGFP detection in bronchial ALI cultures. Native EGFP detection of B-ALI or MucilAir cultures at 14 days following treatment with F/HN pseudotyped LV expressing EGFP. Cultures treated with either 7.5e7 TU (rHIV.F/HN.CMV.EGFP (vGM182 Wave 96), rSIV.F/HN.CMV.EGFP (B-ALI: vGM010 Wave 111), or rSIV.F/HN.hCEF.EGFP (vGM107 Wave 121)) or 1.2e7 TU (rHIV.F/HN.hCEF.EGFP (vGM193 Wave 113)). (A) Representative stitched centre-of-transwell images (B-ALI donor 3, MucilAir donor 6) are shown; n=2 biological replicates per donor and type (B-ALI n=4 donors; MucilAir n=2 donors); scale bar represents 500µM. (B) Quantification of EGFP detected. Each symbol represents a biological replicate; each shape represents a single donor; the bar represents the mean. B-ALI: *, P=0.0148 (rHIV.CMV vs rHIV.hCEF). ***, P=0.0002 (rSIV.CMV vs rSIV.hCEF, P=rHIV.CMV vs rSIV.CMV, P= rHIV.hCEF vs rSIV.hCEF). MucilAir: *, P=0.0286 (rHIV.CMV vs rSIV.CMV). Significance determined using Mann-Whitney U test.

5.2.2.4. Identification of transduced airway cell types

To identify transduced cell types, cryosections were prepared from B-ALI cultures (all donors) transduced with 7.5e7 TU of rHIV.F/HN.CMV.EGFP and stained using IHC for airway cell-type specific markers as before (Section 5.2.1, Figure 34). Colocalization of antibody staining and native EGFP signal was determined for all four cell types, with a minimum of 100 EGFP-expressing cells identified per cell type for each donor (Figure 40 A); it should be noted that a limitation of this approach is that it does not normalise for the variation in abundance of cell types studied. Colocalization with β -tubulin was most frequently observed (22-26% of EGFP-positive cells), suggesting that of the four cell types investigated, ciliated cells were the most frequently transduced (Figure 40 B). EGFP colocalization with Cytokeratin 5 (basal cell marker) was slightly less frequent in all four donors (14-18%). Colocalization of EGFP with Mucin 5AC stained goblet cells was least frequent across donors (8-11%), though it should be noted that goblet cells were relatively infrequent in the B-ALI cultures used, while basal and ciliated cells were abundant (Figure 34). Club cells, present in B-ALI culture donors 1, 3 and 4 only, were also transduced (Figure 40). Although colocalization with EGFP in club cells was variable between donors (8-20%), the frequency was similar to goblet cells, which were similarly sparse in occurrence in the B-ALI culture (Figure 34). This suggests efficient targeting of all four cell types by the F/HN pseudotype despite their different frequency and distribution across the cultures. It should be noted that the percentages of native EGFP and cell type marker staining colocalization amounted at maximum to ~76% (donor 3), this could be due to a combination of factors such as insufficient IHC sensitivity to identify all cells expressing the markers, transduction of other cell types for which staining was not included, and insufficient optical resolution (widefield microscopy was utilised in these studies).

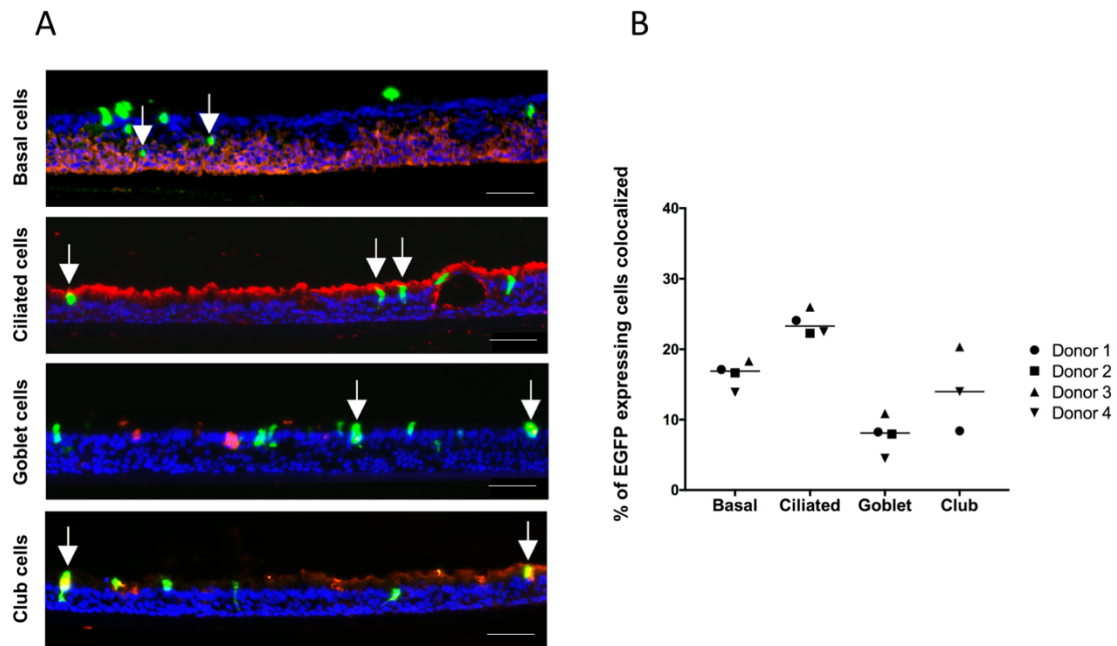


Figure 40- Detection of airway cell transduction by rHIV.F/HN in B-ALI cultures. Cryosections generated from B-ALI cultures transduced with 7.5×10^7 TU rHIV.F/HN.CMV.EGFP (vGM182 Wave 96) were stained using IHC for basal (Cytokeratin 5), ciliated (β -Tubulin), goblet (Mucin 5AC) and club cells (CC10), and colocalization with native EGFP determined. (A) Examples of colocalization (white arrows) in B-ALI from donor 1. Nuclei are stained blue (DAPI); scale bar represents $50 \mu\text{M}$. (B) Quantification of EGFP-expressing cells that colocalized with cell type immunostaining was calculated from a minimum of 100 EGFP-positive cells per B-ALI culture donor ($n=3-4$) for each cell type; no club cells were identified in donor 2, the bar represents the median.

5.2.3. Use of ALI cultures to compare transduction by alternative pseudotypes

Having established that the different human airway ALI culture types could be used to model LV transduction, they were subsequently used to compare the efficiency and airway cell type tropism for the alternative LV pseudotypes. Both B-ALI and SmallAir cultures were selected for these studies as they demonstrated the greatest diversity in TEER, cell type and rHIV.F/HN transduction efficiency (Figures 33-34 & 38) with potential to reflect properties of proximal and distal airway epithelium respectively.

5.2.3.1. Comparing transduction efficiency using airway ALI

B-ALI and SmallAir cultures were treated with equivalent TU doses of rHIV expressing EGFP. Due to limited availability, ShanghaiN1 pseudotyped LV was not included. In both ALI culture types, the F/HN pseudotype resulted in the greatest levels of native EGFP fluorescence (Figure 41), with between 300- and 75-fold greater percent fluorescence achieved in B-ALI cultures, compared with Rostock ($p<0.0001$) and ShanghaiN9 ($p=0.0333$) pseudotyped equivalents.

In B-ALI cultures, the Rostock pseudotype did not result in visible or quantifiable EGFP fluorescence, except for a single transwell. For the ShanghaiN9 pseudotype EGFP fluorescence was detected in all donors treated, although at a very low level ($<0.5\%$, Figure 41 B). This is in contrast, however, with the many EGFP-expressing cells present in B-ALI cultures treated with rHIV.F/HN.hCEF.EGFP (Figure 41 A). This indicates that when exposed to equivalent TU doses of each pseudotyped LV, the F/HN pseudotype is significantly more efficient at transducing the B-ALI cultures. Although EGFP expression was lower than previous treatment of B-ALI with this vector, this was anticipated with a 10-fold lower TU dose used (median of 3.8% versus 11.9%; Figure 39 B).

In SmallAir cultures, low levels of EGFP were anticipated from rHIV.F/HN, since SmallAir cultures were the least efficiently transduced by F/HN (Figure 38). Despite this inefficiency, F/HN was the only pseudotype to result in readily quantified eGFP expression (0.1-0.2%). Overall, F/HN was the most efficient pseudotype for transduction of the human ALI culture types evaluated.

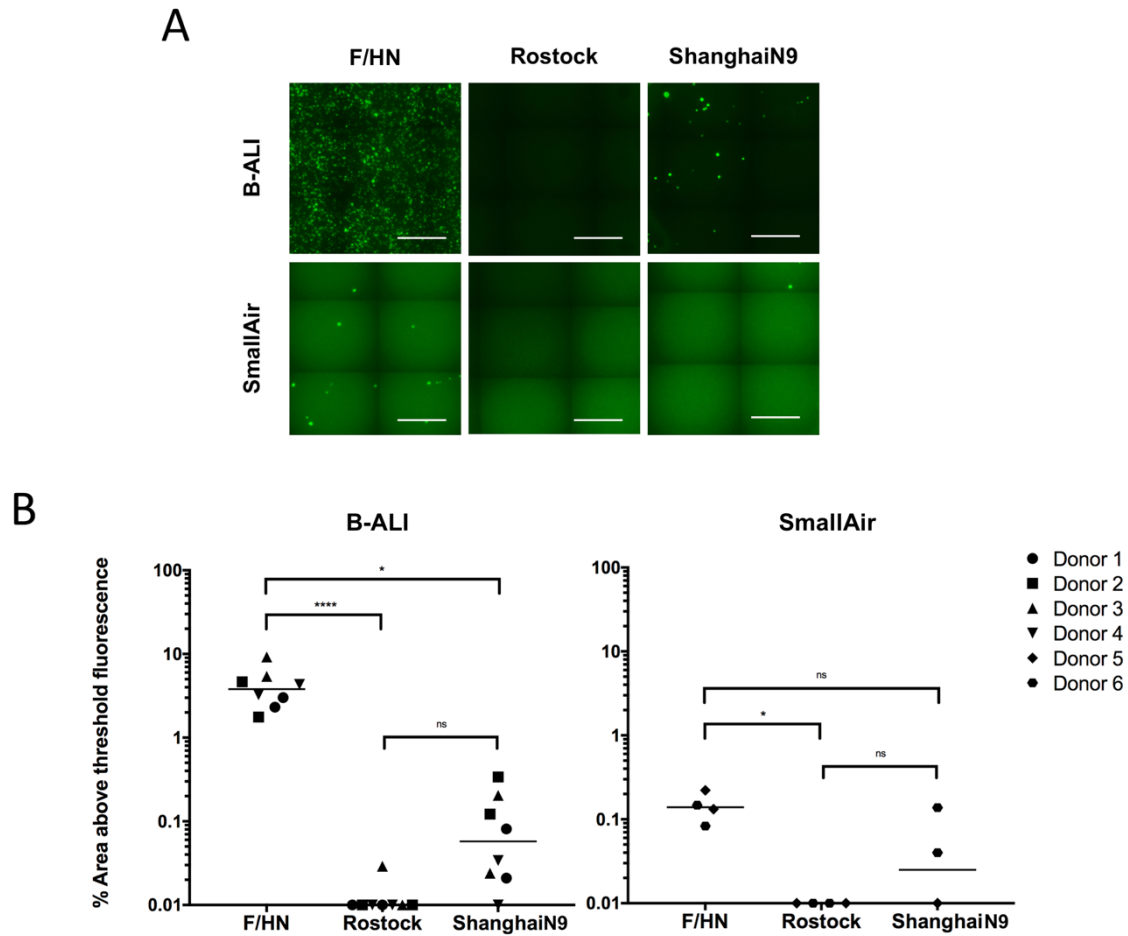


Figure 41- Transduction by alternatively pseudotyped rHIV expressing EGFP in B-Ali and SmallAir cultures. Equivalent TU doses of rHIV.F/HN, rHIV.Rostock or rHIV.Shanghai expressing EGFP were delivered apically to B-Ali (7.5e6 TU per transwell) and SmallAir (4.7e6 TU per transwell) cultures and examined 14 days later. (A) Detection of native EGFP in representative, stitched centre-of-transwell images. Scale bar represents 500 μ M. (B) EGFP quantified from LV transduced cultures of (Left) B-Ali (****, $p < 0.0001$; rHIV.F/HN versus rHIV.Rostock; and *, $P = 0.0333$; rHIV.F/HN versus rHIV.Shanghai), or (Right) SmallAir (*, $p = 0.0245$; rHIV.F/HN versus rHIV.Rostock). Each symbol represents a biological replicate; each shape represents a single donor; bar represents the median. Significance determined using Kruskal–Wallis followed by Dunn’s Multiple Comparison test; ns, not significant. B-Ali cultures treated with rHIV expressing EGFP under hCEF promoter (vGM193, vGM194 and vGM192). SmallAir cultures treated with rHIV expressing EGFP under CMV promoter (vGM182, vGM222, vGM220).

5.2.3.2. Comparing airway cell type transduction

To determine whether low efficiency transduction with Rostock and ShanghaiN9 pseudotypes was due to specific cell type-targeting, B-ALI cultures were transduced with the maximum feasible TU dose of rHIV.CMV.EGFP to increase detection of EGFP-positive cells, for colocalization in cryosections. Due to limited availability of concentrated vector, and untreated B-ALI cultures, evaluation of rHIV.Rostock (6e6 TU) and rHIV.ShanghaiN9 (5.7e5 TU) was only possible in a single donor (B-ALI donor 2; n=2 transwell replicates). Treatment resulted in 2.3% and 9.3% area of transwell EGFP fluorescence for Rostock and ShanghaiN9 respectively (Figure 42 A), greater than that achieved with rHIV.hCEF vectors (Figure 41 B), maximising the potential detection of EGFP expressing cells in subsequent cryosections for IHC colocalization. Although ALI cultures from this donor were lacking club cells (Figure 34), all three remaining cell types observed in this donor were transduced by both rHIV.Rostock and rHIV.ShanghaiN9 (confirmed by native EGFP colocalization – data not shown).

Quantifying EGFP colocalization with basal, ciliated and goblet cell markers was challenging with so few EGFP-positive cells per cryosection compared with F/HN (Figure 40). Despite imaging 10 cryosections (5 per transwell) per cell type for each pseudotype, insufficient EGFP-positive cells per cell type (<100) were available to assess cell type targeting for Rostock. Quantification was feasible for ShanghaiN9 pseudotype, however, showing the greatest colocalization with ciliated cells (40%) and fewest with goblet cells (14%) (Figure 42 B), a similar cell-type transduction profile to F/HN. The colocalization for all three cell types was higher than that recorded for F/HN (Figure 40); for example, nearly 2-fold more EGFP-positive cells were ciliated cells from ShanghaiN9 treated transwells. This could indicate a greater transduction efficiency of these cell types, however, with only one B-ALI donor investigated and limited detection of EGFP-expressing cells, this can only be speculated. Despite the limits in quantification, both Rostock and ShanghaiN9 pseudotypes were capable of transducing all three

cell types suggesting that the low transduction efficiency compared with F/HN is unlikely to be due to restricted cell type-targeting.

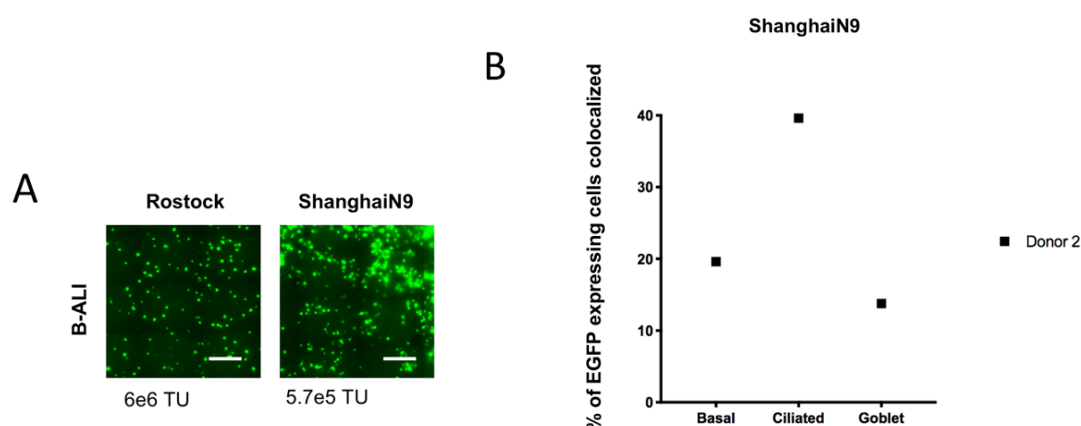


Figure 42- Cell types transduced in B-ALI cultures with maximum TU dose of rHIV.Rostock or rHIV.ShanghaiN9. (A) Detection of native EGFP in representative (n=2), stitched centre-of-transwell images of B-ALI 14 days following transduction with rHIV.CMV.EGFP pseudotyped with Rostock (6e6 TU vGM222, OBM368, 2.0-2.3% EGFP quantified above threshold) or ShanghaiN9 (5.7e5 TU vGM220, OBM370, 9.2-9.3% EGFP quantified above threshold). Scale bar represents 500µM. (B) Cell type targeting quantification in transwells treated with ShanghaiN9. Colocalization of EGFP and cell-specific markers was calculated from a minimum count of 100 EGFP-positive cells per cell type. No Club cells were identified in donor 2. Insufficient EGFP-positive cells counted from transwells treated with rHIV.Rostock to quantify cell type targeting (<100 EGFP- positive cells per cell type).

5.2.3.3. Transduction of Surfactant ALI culture model of parenchymal epithelium

The surfactant-ALI (SALI) cultures, modelling human lung parenchyma (Section 2.11.2 & 5.1.3), were also treated with an equivalent TU dose of rHIV.CMV.EGFP for each pseudotype. Imaging in culture at day 4 revealed efficient transduction by the F/HN pseudotype (~40%), compared with very low levels by either of the influenza-based pseudotypes, Rostock and ShanghaiN9, achieving ~1-2% and ~0.2% respectively (Figure 43).

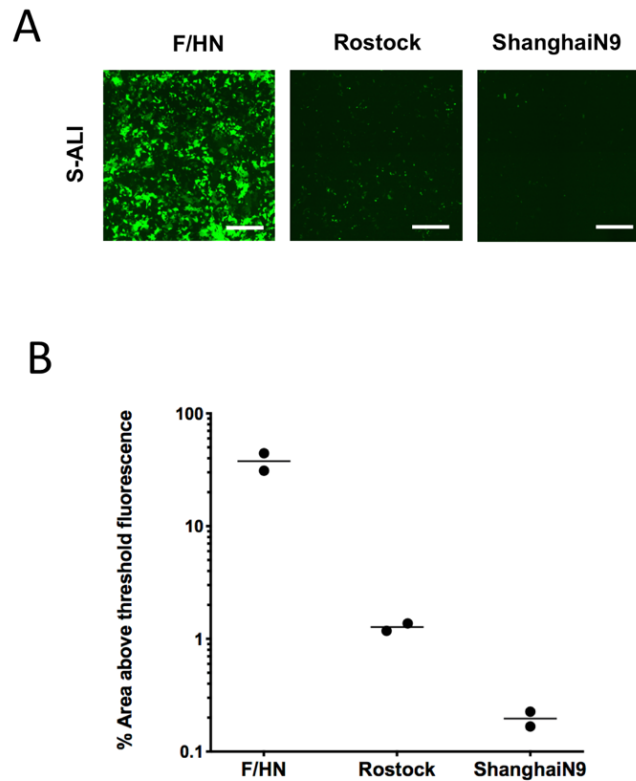


Figure 43- Transduction of SALI cultures with alternatively pseudotyped rHIV expressing EGFP. Equivalent TU doses of rHIV.CMV.EGFP pseudotyped with F/HN (vGM182), Rostock (vGM222) or ShanghaiN9 (vGM220) were delivered apically to surfactant ALI (SALI cultures (4.7e6 TU per transwell, n=2) and examined 4 days later. (A) Detection of native EGFP in representative, stitched centre-of-transwell images. Scale bar represents 500 μ M. (B) EGFP quantified by % area above threshold, each symbol represents a biological replicate; bar represents the median.

This indicates that the F/HN pseudotype is most efficient in transduction of this parenchymal SALI culture model, similar to its performance in airway ALI cultures (Figure 41). In contrast to the relative pseudotype efficiency in transducing airway ALI cultures, however, the Rostock pseudotype performed better than the ShanghaiN9 pseudotype in this model. This reversal in efficiency could indicate an alternative receptor by these pseudotypes in these models and requires further investigation to understand the importance and relevance to predicting transduction in human lung.

5.3. Discussion

5.3.1. Modelling the airway epithelium using different types of ALI cultures derived from different donors

Human ALI cultures are widely-used to model the human airway epithelium, but a diverse range of protocols and human cell sources have been used. In addition, ALI cultures are time-consuming to generate and expensive to maintain, such that different ALI culture types are rarely well-characterised or compared. Studies in this chapter characterised three ALI culture types and investigated their use in modelling LV transduction of the human lung. Characterisation of ALI cultures obtained from Epithelix Sarl, or generated in-house (B-ALI), indicated that all airway ALI cultures modelled a polarised, electrically resistant epithelium with a mucociliary phenotype, containing the major airway cell types. However, there were significant differences between ALI culture types and donors that were not expected.

As anticipated, a more cohesive mucus phenotype was observed in CF donor MucilAir versus normal MucilAir, with transduction efficiency by F/HN tending to be approximately 2-fold lower, although this failed to reach statistical significance (5.2.2.2, Figure 38). With transduction efficiency of healthy donor B-ALI cultures appearing unaffected by cohesive mucus production (Table 9, donor 1 or 4 versus 2 or 3; no significant difference characterised between donors; 5.2.2.2), by extension, it is likely that the cohesive CF mucus phenotype did not significantly impact the use or availability of receptors by rHIV.F/HN. In the context of a pseudotyped vector based on Sendai virus, minimal impact of mucus could be anticipated, with only a two-fold reduction characterised using sheep trachea for gene transfer by Sendai virus vector (Yonemitsu et al., 2000). However, reports of a general decrease in sialylation in the CF airway (Rhim, Stoykova, Glick, & Scanlin, 2001) and studies specifically suggesting that lower levels of α 2,3 sialylation is found on the membrane of epithelial cells expressing the common p.508del CFTR mutation (Dosanjh & Muchmore, 2009), could explain the reduced efficiency of F/HN transduction observed.

In contrast to the anticipated differences in mucus phenotype between ALI derived from CF versus healthy donors, an unexpected difference in club cell abundance was detected between donors, with no clear relationship observed between ALI type or cell origin. Despite SmallAir cultures reported to model the relatively high abundance of club cells observed in the small airways of the human lung (Huang et al., 2017), no difference in club cell abundance between MucilAir and SmallAir cultures from the same donors was observed (Figure 34). In contrast, many more club cells were observed in both MucilAir and SmallAir cultures from donor 6 than donor 5. This indication that modelling of club cells in ALI cultures is dependent on donor suggests the potential requirement to investigate multiple donors in order to ensure modelling of epithelium comprising of such cell types.

Although differences between bronchiolar (SmallAir) and bronchial-derived (MucilAir and B-ALI) cultures were expected, as they were derived from more distal and more proximal airway epithelial cells respectively, significant differences were also detected between MucilAir and B-ALI cultures; despite both types derivation from normal bronchial epithelia. The B-ALI cultures displayed significantly lower TEER (Figure 33), noticeably more basal cells (Figure 34) and were transduced by rHIV.F/HN with much greater efficiency than either MucilAir or SmallAir cultures (Figure 38). The B-ALI cultures also generally comprised fewer club cells than MucilAir or SmallAir cultures (with the exception of B-ALI donor 5), and produced more visible mucus (Figure 34).

Lower TEER values detected in B-ALI cultures correlate with the greater mucus production by this culture type. This relationship could be explained by mucus producing goblet cell abundance in these cultures, with the lack of tight junctions associated with these cells speculated to impact TEER (Pearce et al., 2018; Srinivasan et al., 2015). Additionally, the elevated TEER in SmallAir cultures could be explained by a reduced frequency of goblet cells expected in these cultures, reported versus MucilAir (Huang et al., 2017). In contrast to these observations, staining of cultures did not reveal any clear relationship to TEER or mucus production with a

generally similar abundance of goblet cells observed across all ALI cultures. This might indicate that the IHC against Mucin 5AC to indicate goblet cell abundance lacked sufficient sensitivity to detect all expressing cells, or alternatively the cells were not distributed across the cultured surface such that the 8 cryosections assessed per ALI culture did not capture a representative abundance.

Such staining insensitivity is supported by the lack of goblet cells detected in SmallAir cultures from donor 6 (Figure 34), despite mucus production being readily detected (Table 9). The large difference in efficiency of transduction by rHIV.F/HN between ALI culture types was unexpected, indicating a significant difference between the cultures. It is important to understand this difference in order to predict how transduction of such cultures may model *in vivo* transduction. The observation that ALI cultures with a lower TEER had a greater transduction efficiency with F/HN vectors (Figures 33 & 38) could imply that F/HN transduction was enhanced by 'leaky' tight-junction permeability; this seems unlikely, however, as ALI cultures with lower TEER were largely resistant to VSVg vector transduction – a vector type that has been consistently shown to require tight-junction permeability to access basolateral receptors (Cmielewski et al., 2010; L G Johnson, Olsen, Naldini, & Boucher, 2000; Kremer et al., 2007).

Alternatively, the difference in transduction efficiency between ALI culture types could reflect differential expression (or access to) receptors. With some evidence of alternative receptor subtypes expressed between airway cell types reported in the literature (5.1.2), a cell type transduction preference by rHIV.F/HN could have been anticipated given the $\alpha 2,3$ preference of Sendai virus. However, although many more basal cells were detected in B-ALI culture, there was no clear difference in airway cell types accessible at the apical surface detected between the ALI culture types (Figure 34), and additionally, no strong preference for transduction of a specific cell type by F/HN observed (Figure 40). Although EGFP colocalized most frequently with β -tubulin, suggesting ciliated cells could be most efficiently transduced by

F/HN (~24%), EGFP colocalization with goblet and club cell markers was ~ 10% despite the low frequency of these cell types compared with the apparently more abundant ciliated and basal cells. This could suggest a greater relative efficiency for targeting goblet and club cell types than ciliated and basal cells. To resolve this however, cell type numbers would need quantifying - requiring confocal microscopy of all cryosections to deduce accurately. Although there was no strong cell type preference detected in B-ALI cultures this could be unique to this culture type if, for instance, the receptors used by F/HN are expressed on all cell types in B-ALI cultures, but differentially expressed in the cell types present in MucilAir and SmallAir. Such a difference between these cultures could explain the different efficiencies, or alternatively, expression of receptors could be generally lower, independent of cell types. An unexpected difference in relative expression of receptors between ALI culture types, not attributed to the epithelial cell source/location (given this difference between B-ALI and MucilAir cultures both derived from bronchial HBEC,) may explain the contrasting cell-type specific receptor expression reported in the literature (5.1.2), despite similarity of cultures investigated (derivation from tracheal/tracheobronchial cells).

Characterisation of these airway ALI, although revealed a potential importance to derive cultures from multiple donors in order to capture the diversity of cell types represented in airway epithelia, highlighted significant differences between the types of cultures, even between those derived from an equivalent bronchial (versus bronchiolar) cell source. Understanding how these cultures differ (e.g. receptor availability), how this results in significant differences in transduction efficiency, and how these differences may best represent human airway epithelium, will be important in predicting the potential of these pseudotype potential in humans. Differences between B-ALI and MucilAir observed here in particular suggest an impact of protocols to generate and maintain Epithelix Sarl versus B-ALI cultures (following Stemcell PneumaCult ALI protocol). However, given the ability of all cultures to model a polarised epithelium of major human airway cell types, investigation of different ALI cultures

may represent subtle differences between human epithelia, both anatomically and between individuals.

5.3.2. Impact of LV platform and transgene promoter on transduction

Fewer EGFP-expressing cells were observed and quantified in all ALI cultures treated with equivalent or similar doses of rSIV.F/HN compared with rHIV.F/HN (Figure 39). In contrast, although EGFP-positive cell numbers appeared equivalent between cultures transduced independent of the CMV or hCEF promoter (Figure 39A), a greater percentage fluorescence above threshold was quantified from LV using the CMV promoter (Figure 39B), possibly due to a greater fluorescence intensity of these EGFP-expressing cells compared with LV using the hCEF (Figure 39A). This difference in fluorescence intensity of EGFP-expressing cells was also visible at 10 and 20 weeks (Figure 37) and, although different LV platforms and TU doses were used, indicates the difference in fluorescence intensity arising from these promoters is independent of the detection timepoint. It is possible that transcription factors required to facilitate expression of the EF1 α promoter (as found in the hCEF transcriptional element (Hyde et al., 2008)) are limited in the cells of transduced B-ALI cultures, or that inhibitory/silencing factors are expressed. Interestingly, in the murine lung *in vivo*, the CMV promoter is inferior to the EF1 α promoter in terms of transgene persistence (Gill et al., 2001) and is generally more vulnerable to silencing (Brooks et al., 2004; Qin et al., 2010; Teschendorf, Warrington, Siemann, & Muzyczka, 2002).

Despite the greater percentage area fluorescence above threshold quantified with the CMV promoter (Figure 39 B), a significant difference of at least ~100-fold was detected between rSIV.F/HN and rHIV.F.HN, regardless of promoter used (Figure 39 B). This underlines the magnitude of impact on transduction efficiency by the LV platform, with the rSIV transduction being much less efficient, and possibly highlighting a restriction of the rSIV platform to transduce the human ALI cultures. This result is in contrast to the detection of rSIV.F/HN.hCEF.EGFP

transduction in the parenchyma of human PCLS (Table 8, Figure 30 B), yet no EGFP detected following rHIV.F/HN.CMV.EGFP treatment. This can be explained however by the lower rHIV TU treated (5.45×10^7 versus 3×10^8 TU of rSIV). Interestingly, only one mouse treated with rHIV.F/HN.CMV achieved quantifiable detection of EGFP (7.98×10^7 TU) versus the reliable detection in mouse airway following rSIV.F/HN.hCEF delivery at similar TU (Figure 13 & Figure 25 ; 1×10^8 TU). Together, these results suggest a species-specific restriction of the rSIV platform in *human* airway cells, and this deserves further investigation. A restriction specific to rSIV fusion, capsid, enzymes, RNA/cDNA sequences involved in facilitating uncoating, reverse transcription, integration or escaping innate immune response, could be hypothesised. Although rSIV transduction of human lung cells was observed using PCLS (Table 8), the efficiency may not be optimal, and these cells could express lower levels of restriction factors affecting rSIV.

5.3.3. Use of airway and parenchymal ALI cultures to compare transduction by pseudotyped LV

The greater efficiency of F/HN pseudotyped rHIV in transducing both B-ALI and SmallAir cultures as well as SALI cultures, when treated with equivalent TU, suggests that in all ALI models investigated, the F/HN receptors were of greater availability than those used by Rostock or ShanghaiN9 pseudotypes. Additionally, there was no evidence that this difference in transduction was the result of cell type-specific targeting by the influenza-based pseudotypes, in contrast to the relatively indiscriminate transduction shown by F/HN. Although only confirmed in one B-ALI donor (Figure 42), both the Rostock and ShanghaiN9 pseudotypes were able to target all three cell types present in those ALI cultures. That said, some indication that ShanghaiN9 may target ciliated cells with greater efficiency than F/HN (comparing Figure 40 and Figure 42 B), could correlate with the expected greater affinity for $\alpha 2,6$ sialylated receptors characterised for this HA - reported to be enriched on human ciliated cells (in human trachea) (Baum & Paulson, 1990). However, with characterisation limited to a single B-ALI donor, and the

receptor expression on such cells in dispute as discussed (5.1.2), such tropism can only be speculated and the limited detection of transduction of any cells, including ciliated cells, indicates that specificity or preference for this cell type is not the limit to efficiency.

The similar relative transduction efficiency between the three pseudotypes in B-Ali and SmallAir cultures, suggests that the receptors are similar in proportion between these culture types, even if possibly varying in abundance. In contrast, the relative efficiency of Rostock and ShanghaiN9 in SALI cultures was reversed, suggesting that SALI cultures may contain different receptors than airway ALI cultures, leading to different relative transduction efficiencies between Rostock and ShanghaiN9 pseudotypes. Although it is possible that transduction by the influenza-based pseudotypes is limited in these cultures compared with F/HN, independently of cellular receptors, the reversed efficiency of ShanghaiN9 and Rostock suggests that the transduction by these pseudotypes is dependent on specific differences in ALI culture type. Importantly, use of the same vector batch and TU dose to transduce both the SmallAir and SALI cultures rules out any confounding effect of inconsistencies between vector preparations.

5.3.4. Conclusion

All human airway ALI cultures demonstrated many characteristics to support modelling of human airway epithelium. The F/HN pseudotype showed greater efficiency in transducing all pulmonary ALI cultures investigated than the Rostock and ShanghaiN9 pseudotypes, suggesting that the receptor used by F/HN is common across these ALI cultures and is of greater availability, or used with greater efficiency. This was also observed in SmallAir cultures despite an overall reduction in transduction efficiency with all pseudotypes. Although ShanghaiN9 was more efficient than Rostock at transducing airway ALI cultures, Rostock LV appeared to transduce SALI cultures (modelling parenchymal epithelium) with greater efficiency; this could imply differences in the receptors expressed (and used) between these cultures. Understanding the potential of each of these culture types to model receptors expressed on human airway

epithelium is paramount to understanding the utility of these cultures in predicting transduction of human lung cells. Additionally, understanding differences in receptor use for simple binding as opposed to mediating cell transduction, could help elucidate whether Rostock and ShanghaiN9 transduction inefficiency is a result of receptor interaction or another quality of the LV particles. These results additionally illustrate the importance of selecting an appropriate LV platform to facilitate evaluation of different pseudotypes for transduction. A restriction of rSIV transduction, as well as a limit in transgene expression using hCEF versus CMV, is indicated for *human* airway cells here.

6. Chapter 6- Investigating the use of sialylated receptors for lentiviral vector transduction

6.1. Introduction

In previous Chapters, efficient LV transduction mediated by the F/HN pseudotype was observed in multiple models suggesting that this pseudotype can either use a cellular receptor in common between these models or, alternatively, that F/HN is able to use a variety of receptors to mediate efficient transduction. Similarly, the relative inefficiency of transduction via Rostock and ShanghaiN9 pseudotypes in human ALI cultures, could be due to less availability of their preferred receptors. The use of lectins was investigated to further explore this issue.

6.1.1. Lectins to characterise receptor distribution between models

The term lectin is used to describe proteins that bind to glycans (carbohydrates such as simple sugars and oligosaccharides) with high specificity. Ubiquitously found across animals, plants, bacteria and viruses, glycans play diverse roles, from acting as simple viral receptors (such as the hemagglutinin pseudotypes used here), to more complex activities including cell adhesion, signalling and immune defence against pathogenic microorganisms (Sharon & Lis, 2004). Lectins derived from plants are a rich and inexpensive source of reagents that have been thoroughly characterised for oligosaccharide specificity, and are widely adopted as tools in glycobiology to characterise carbohydrate moieties (Cummings, Darvill, Etzler, & Hahn, 2017). Although antibodies against oligosaccharides have been generated, and can be of greater affinity, their wide availability has established plant lectins as the gold standard tool for glycohistochemistry.

The lectins commonly used to detect and differentiate sialylated glycans are derived from the plants *Sambucus nigra* (α 2,6 sialylation specific) and *Maackia amurensis* (α 2,3 sialylation specific). *Sambucus nigra* agglutinin (abbreviated to SNA) is a lectin that binds any residues with an α 2,6 sialylated terminal galactose moiety. Two lectins isolated from seeds of *Maackia amurensis*, however, are of greater specificity, differentiating beyond the terminal α 2,3

sialylation of galactose (see figure 9). Although specificity is now well characterised for each lectin (Figure 9) (Geisler & Jarvis, 2011), historically there has been substantial confusion, with single lectin isolates from *Maackia amurensis* used mistakenly to define the presence of (all) α 2,3 sialylated residues. Specific binding properties of the lectin isolates were first identified via hemagglutination of red blood cells (RBC), with the more potent isolate termed *Maackia amurensis* hemagglutinin (MAH or MAII) (Kawaguchi & Osawa, 1976), presumably due to the abundance of α 2,3 sialylated ganglio-series (often O-linked) glycans available on the RBC for which this isolate is specific. The other lectin isolate (with 86% amino acid sequence similarity) is referred to as MAM, MAL or MAI (Kawaguchi & Osawa, 1976) and is specific for α 2,3 sialylated LacNAc residues (neolacto-series, often N-linked). In characterising α 2,3 sialylated receptor distribution some studies use an undefined mixture of lectin isolates, whereas others often use only MAII, which is problematic given the potential abundance of α 2,3 sialylated LacNAc residues that remain undetected without use of MAI. The neglect in use of MAI could stem from some observed weak binding to non-sialylated targets (Geisler & Jarvis, 2011). Even when a combination of lectins is used to characterise sialylated receptor distribution, the protocol differences in tissue fixing, staining, antigen retrieval, detection and sectioning can result in flawed, or at best conflicting, conclusions (Geisler & Jarvis, 2011; Nicholls et al., 2007). One clear lesson learned from such studies is that glycohistochemistry should be performed in parallel, with common reagents, to allow for a true comparison of receptor distribution between tissues or models.

6.1.2. Hemagglutination to assay binding of virus targeting of sialic acid

First performed by Hirst in 1941 (Hirst, 1941), the agglutination of red blood cells (hemagglutination) is a well-established assay for quantification of influenza haemagglutinin or other lectin-containing preparations. The basis of the hemagglutination assay is the interaction of hemagglutinin on the viral surface with sialylated glycans on the surface of RBC in solution

resulting in a cross-linked lattice formation holding the RBC in suspension. With insufficient binding, however, the RBC settle out of suspension forming a (visible red) pellet. As this assay is dependent solely on the binding properties of a preparation, it may be used to characterise the LV pseudotypes binding without the additional and possibly confounding requirement for cell transduction. In addition, the assay could assess the potential for free glycans to block or compete with sialylated receptors on RBC for binding by haemagglutinin, offering the ability to compare relative binding affinity between glycans for each of the LV pseudotypes investigated in previous chapters.

6.1.3. Free glycans as cellular receptor analogues to investigate binding by viral glycoproteins

Hemagglutinin binding of glycans, either soluble or adhered to solid phase, is often used to study receptor interaction and binding affinity. Unconjugated or 'free' glycans (such as $\alpha 2,3$ or $\alpha 2,6$ sialylated LacNAc) are widely adopted as receptor analogues in crystallography to characterise receptor interactions (Yang, Carney, Donis, & Stevens, 2012). Although the absence of conjugation, or solid phase adherence, makes it difficult to control the orientation of glycan binding, free glycans are readily obtained and can be used to investigate receptor binding in RBC assays (described (6.1.2)). Sialylated ganglio-series glycans ($\alpha 2,3$ recognised by MAII) are readily procured from rich sources of ganglioside glycolipids such as brain tissue (e.g. ganglion cells (E. Klenk, 1942)). Procurement of sialylated LacNAc glycans (such as from neolacto- or lacto- series glycolipids) is more challenging however since expression of the enzymes required for synthesis are limited to specific cell types (Chou & Jungalwala, 1996). As these LacNAc glycans, recognised by the MAI and SNA lectins upon sialylation, are most abundant in the human airways however (1.5.3), investigating LV pseudotype binding to these receptor sequences is of most interest. Figure 44 shows the panel of free glycans obtained to investigate receptor binding.

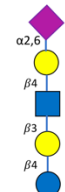
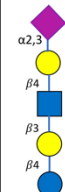
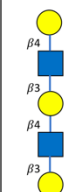
Glycan	6'-Sialyl-LacNAc (6'-Sialyl-N-acetylglucosamine)	LSTc α 2,6 sialylated di-LacNAc (Sialylacto-N-tetraose c)	3'-Sialyl-LacNAc (3'-Sialyl-N-acetylglucosamine)	LSTd α 2,3 sialylated di-LacNAc (Sialylacto-N-tetraose d)	Galacto-N-neopentaose (asialo)	LSTa α 2,3 sialylated lacto-N-biose (Sialylacto-N-tetraose a)	GM3 (3'-Sialyllactose)	GM1a	GD1a	Monosaccharide/ Disaccharide units:
Saccharide sequence										 Sialic acid (Neu5Ac)  Galactose  N-acetylgalactosamine  Glucose  N-acetylglucosamine  N-acetylglucosamine (LacNAc)
Associated glycosphingolipid class	Neolacto	Neolacto	Neolacto	Neolacto	Neolacto	Lacto	Ganglio	Ganglio	Ganglio	
Lectin Binding	SNA	SNA	MAI	MAI	-	-	-	-	MAII	

Figure 44- Panel of free glycans used to investigate receptor binding. The names and structure of free glycans used to investigate receptor binding are shown, including oligosaccharides that are commonly found on glycosylated proteins (N- or O- linked) and lipids such as in Figure 6. The saccharide sequence, associated glycosphingolipid class (Lacto-, neolacto-, or ganglio- series) and lectin used to recognise the glycan (SNA, MAI or MAII) are shown. Where no lectin is indicated, binding by one of these lectins is not anticipated (Geisler & Jarvis, 2011). α 2,6 and α 2,3 sialylated LacNAc (6'-Sialyl and 3'-Sialyl-LacNAc) trisaccharides represent the sequences recognised by SNA and MAI respectively (Figure 9). With an additional lactose disaccharide, LSTc and LSTd were obtained to investigate the impact of LacNAc glycan length on binding, LSTd modelling the glycosylation of Sialyl- 3-paragloboside (Figure 6). LSTa (β 1,3 glycosidic linkage between galactose and N- acetylglucosamine, rather than β 1,4 -neolacto) was also acquired to investigate the specificity for neolacto versus lacto sequences. GM3 was obtained to investigate impact of non-acetylated glucose and glycans representing the glycosylation in ganglio-series glycolipids GM1a and GD1a (Figure 6) were also procured. Galacto-N- neopentaose, served as a negative control, without sialylation.

6.1.4. Investigating use of cellular receptors *in vitro*

In addition to assessing receptor binding to specific glycans as described above, the use of receptors by Sendai and influenza viruses to mediate cell infection has also been investigated, with the opportunity to assess *functional* receptor use rather than simply receptor binding. To do this, however, the cells must be carefully selected, since cells (eg RBC) from different species can express alternative sialylated receptors that lead to different hemagglutinating titres (Ito et al., 1997). For example, a lack of agglutination by hPIV1 and hPIV3 was observed in *equine* RBC

due to the cells lacking glycolipids of branched sialylated LacNAc, whereas expression in human, cow and guinea pig RBC facilitated hemagglutination (T. Suzuki et al., 2001). The cells commonly used to propagate and investigate influenza virus infectivity also demonstrate differential cellular receptor expression. For instance, MDCK cells are regularly used to propagate human influenza virus to allow retention of HA with high affinity for 2,6 sialylated moieties, in contrast to propagation in chicken embryo chorioallantoic membrane leading to HA with affinity for 2,3 sialylation (Gambaryan, Robertson, & Matrosovich, 1999). This is a result of both α 2,3 and α 2,6 sialylated receptor expression in MDCK cells. Use of MDCK cells could facilitate investigation into the use of cellular receptors by pseudotyped rHIV whereas MDBK cells, originally used in studies characterising Sendai virus receptor use (1.5.1.1), cannot be used due to rHIV restriction (P. L. Sinn et al., 2012). In particular, MDBK cells were treated with sialidase to cleave all sialic acid from the surface of the cells (generating asialo cells), and subsequently treated with specific sialyltransferase enzymes to restore sialylation specific for α 2,3 or α 2,6 linkage (M. A.K. Markwell & Paulson, 1980). Only restoration of α 2,3 sialylation restored infection by Sendai virus suggesting at least preferential use of α 2,3 sialylated receptors. Although this suggests Sendai virus dependency on α 2,3 sialylation in MDBK cells, Sendai virus binding of α 2,6 sialylated glycolipids has also been detected (T. Suzuki et al., 2001) and could suggest the observed dependency in MDBK cells is specific to that cells line, or merely reflect insufficient restoration of α 2,6 sialylation to detect any restored infection.

Additionally, an MDCK cell line stably transfected with the human 2,6-sialtransferase SIAT1 gene (MDCK-Siat1) has been developed to facilitate study of human influenza neuraminidase inhibitors, and shown to express two-fold more α 2,6 and two-fold less α 2,3 residues than parent MDCK cells (M. Matrosovich, Matrosovich, Carr, Roberts, & Klenk, 2003). This was determined using the lectins MAI and SNA, and so change in sialylation of abundant α 2,3 sialylated LacNAc (bound by MAI) implied. Rich in both α 2,3 and α 2,6 sialylated LacNAc, similar to MDBK cells (Ogawa & Galili, 2006), and becoming recognised in human lung (1.5.3), these

MDCK cell lines could therefore be appropriate to investigate use of these receptors by lung-targeting LV.

6.1.5. Sialidase and Sialyltransferase enzymes

Studies described above often utilise sialidase enzymes to cleave sialic acid from the surface of the cells and sialyltransferase enzymes to restore sialylation specific for $\alpha 2,3$ or $\alpha 2,6$ linkages. Sialidase enzymes, such as those added to cells exogenously to generate asialo cells prior to re-sialylation, have broad specificity. Most commonly used are sialidase isolated from *Arthrobacter ureafaciens* (known as sialidase A) (Jia et al., 2020; Walther et al., 2013) or *Vibrio cholerae* (Bell et al., 2011; M. A.K. Markwell & Paulson, 1980) which cleave sialic acid irrespective of the glycosidic linkage. Alternatively, sialidase from *Streptococcus pneumoniae* (sialidase S, also known as Nan B) is widely used to selectively cleave $\alpha 2,3$ sialylated residues (Chokhawala, Yu, & Chen, 2007). Although sialidase S possesses some activity to cleave alternative ($\alpha 2,6/\alpha 2,8$) subtypes of sialylation, it has a much greater affinity (5-10 fold) for cleaving $\alpha 2,3$ residues (G. Xu et al., 2008). Sialidase S is thus widely used to categorise the identity of glycan sialylation (Jia et al., 2020). Exogenous sialidase has also been useful to investigate receptor use *in vivo* (Bell et al., 2011), where airway transduction by recombinant AAV9 was significantly enhanced after intranasal delivery of sialidase enzyme to mice, 1 hour prior to intranasal dosing of vector. This study was able to confirm the hypothesis that rAAV9 utilises non-sialylated galactose terminating residues, which are exposed following sialidase delivery (Bell et al., 2011).

Sialyltransferases, which are used to restore sialylation, have a more complex range of specificity. The β -galactoside $\alpha 2,3$ sialyltransferase is specific to sialylation of galactose bound to N-acetylgalactosamine ($\beta 1,3$; e.g. terminal sialylation of GD1a in Figure 44) (Rearick, Sadler, Paulson, & Hill, 1979). The $\alpha 2,3$ -sialyltransferase from *Pasteurella multocida* has a broader substrate specificity including LacNAc (Yu et al., 2005), and also some $\alpha 2,6$ -sialyltransferase activity that is much less efficient and requires an acidic pH. In MDBK cells, the β -galactoside

α 2,6 sialyltransferase purified from bovine colostrum (Paulson, Beranek, & Hill, 1911) was used and was unable to restore Sendai virus infectivity (M. A.K. Markwell & Paulson, 1980). There is speculation, however, that α 2,6 sialyltransferase enzymes from eukaryotic sources have a narrower substrate range compared with bacterial sources (Kang et al., 2015). The α 2,6-sialyltransferase from *Photobacterium damsela* is one such bacterial sialyltransferase with flexible substrate specificity (Yu et al., 2006).

6.1.6. Aims

To understand the potential of pseudotype receptor use to explain transduction patterns observed across multiple models, the receptor availability and distribution between models using lectin staining, as well as the use of specific or subtypes of receptor by each pseudotype, was investigated. Further, characterising the relationship to receptors available in human lung, the aim was to resolve the potential of each pseudotype for transduction in human lung/airway.

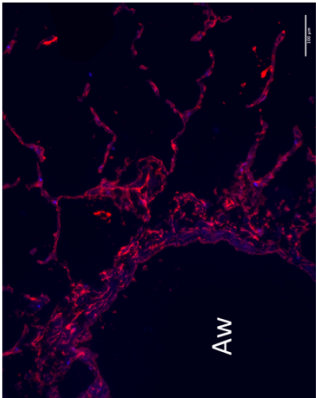
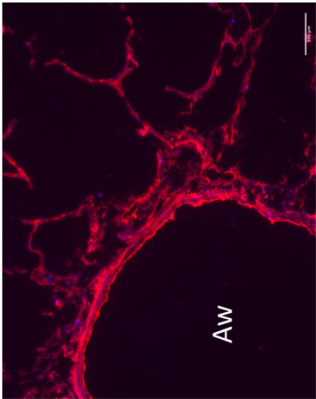
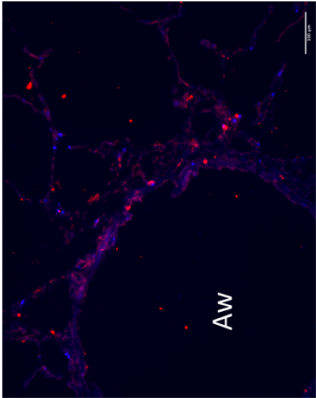
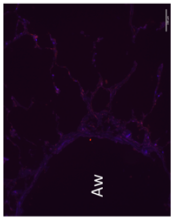
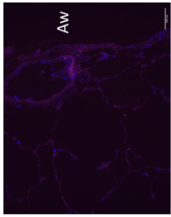
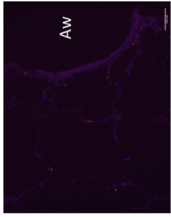
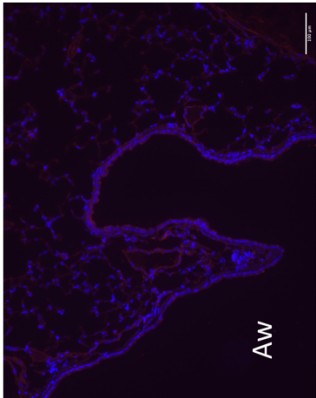
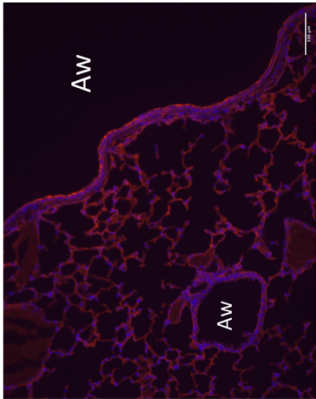
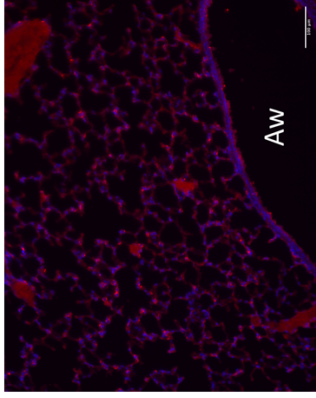
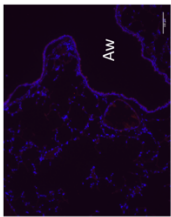
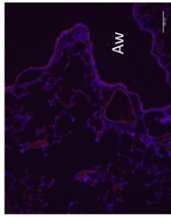
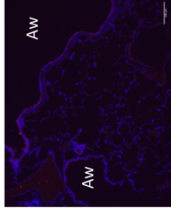
6.2. Results

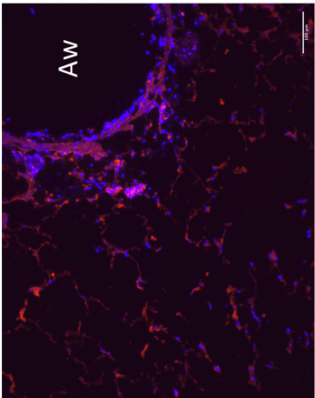
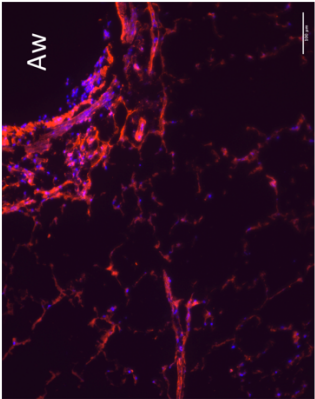
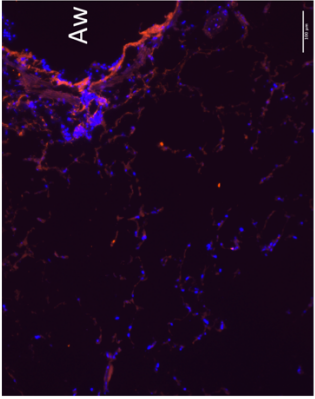
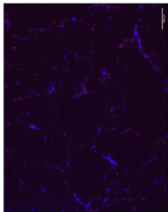
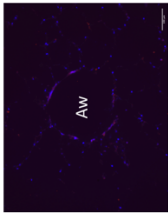
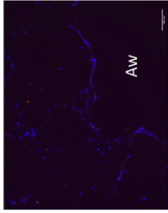
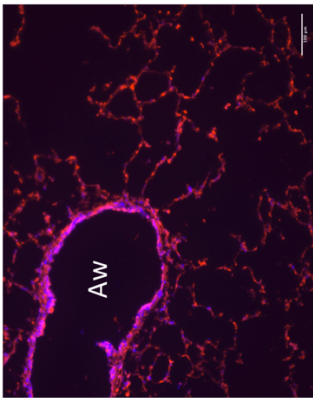
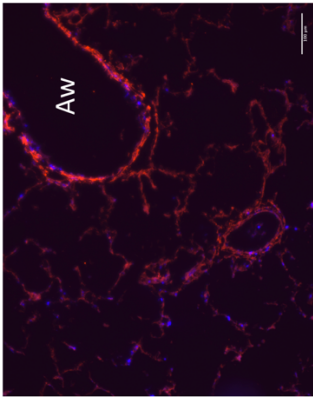
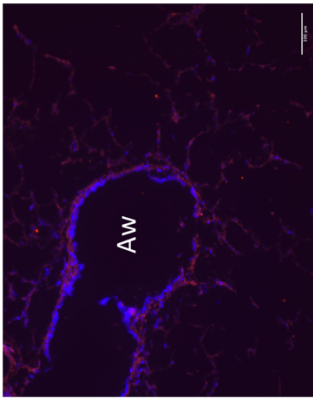
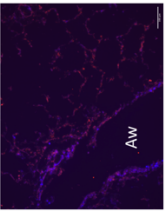
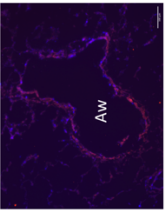
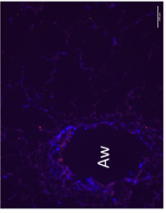
6.2.1. Characterisation of sialylated receptor distribution in lung tissue from different species

The PCLS generated as described in 2.10.1 from several species were used for lectin staining in parallel. Cryosections (7 μ m) from untreated (and uncultured) PCLS were prepared (2.10.5) from mouse, ferret, pig and macaque tissue. Additionally, consecutive (7 μ m) cryosections were generated from PCLS of whole human lung following *ex vivo* lung perfusion (EVLP) (kind gift from Dr Gerry McLachlan, Roslin Institute, Edinburgh, UK), with the expectation that these sections would capture human airways, in contrast to PCLS from patient lobectomies (2.10.1), where airways were essentially absent. Consecutive cryosections containing visible airways were used wherever possible; as expected, airways captured in human, pig, macaque and ferret sections

were all small/bronchiolar in nature due to lung structure and feasibility of sectioning, whereas larger (bronchial) airways were more often captured in mouse lung cryosections.

To control for non-specific lectin binding, sections from each species were pre-treated with sialidase A (6.1.5) to remove all sialylated residues prior to performing antigen retrieval (2.7.1) and lectin staining (2.7.3) on all sections in parallel. Individual cryosections from the same PCLS were treated with biotinylated SNA specific for α 2,6 sialylation, as well as biotinylated lectins MAI and MAII (6.1.3) specific for α 2,3 sialylated receptors. A streptavidin-conjugated secondary Alexa Fluor was used for detection, with equivalent sensitivity under the same imaging settings (Figure 45). Although MAI lectin was previously shown to bind weakly to some non-sialylated residues (Geisler & Jarvis, 2011), here, detection was minimal in all control PCLS sections pre-treated with sialidase A, indicating negligible staining of non-sialylated residues, and in agreement with more recent reports of MAI use (Jia et al., 2020).

PCLS	SNA		MAI		MAII	
Human (EVLP AFFA448)	No treatment					
	Sialidase A treated control sections					
Mouse (BALB/c)	No treatment					
	Sialidase A treated control sections					

PCLS		SNA	MAI	MAII
Ferret	No treatment			
	Sialidase A treated control sections			
Pig	No treatment			
	Sialidase A treated control sections			

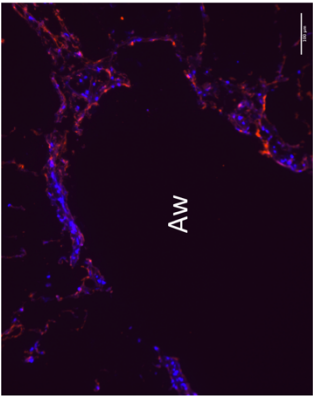
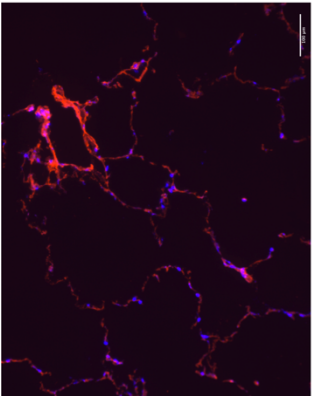
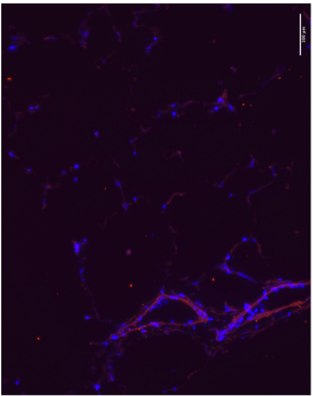
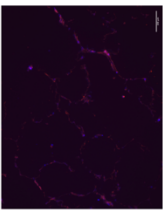
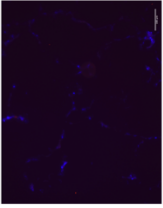
PCLS	SNA	MAI	MAII
	No treatment		
Rhesus Macaque (Macaca mulatta)			
	Sialidase A treated control sections		

Figure 45- Receptor distribution in lung tissue from different species. Cryosections (7µM) from PCLS (with and without sialidase A pre-treatment) were stained in parallel using biotinylated plant lectins (SNA, MAI and MAII) as detailed (2.7.3) and imaged using the EVOS Auto 2 microscope (same exposure and gain settings). Representative images were selected to include the same airways (Aw) where possible. Nuclei are counterstained blue (DAPI), scale bar represents 100µM.

6.2.1.1. Characterisation of sialylated receptor distribution in human lung tissue

In human sections (Figure 45), lectin staining was positive with both MAI and SNA, but poor with MAII (MAI>SNA>MAII) indicating a receptor distribution similar to published reports (Jia et al., 2020; Nicholls et al., 2007). The intense MAI staining of the airway and parenchyma observed was also consistent with published studies using this isoform on human tissue (Nicholls et al., 2007), and with abundant α 2,3 sialylated LacNAc glycans isolated from human lungs (Jia et al., 2020; Walther et al., 2013). Although less intense than MAI, SNA staining (Figure 45) was present in both airway and parenchyma, consistent with extensive literature describing α 2,6 sialylated receptor distributed throughout human respiratory tract. Low level SNA staining in the parenchyma, though in agreement with weak detection by some (Nicholls et al., 2007) may represent enhanced detection versus others that report absence (Ibricevic et al., 2006). As expected, the minimal MAII staining observed reinforces the importance of using both MAI and MAII lectins to assess α 2,3 receptor (subtype) distribution. The absence of MAII staining in the airways (Figure 45) is consistent with both airway staining (Nicholls et al., 2007) and the absence of detection of glycans terminating with sialylated galactose bound to N-acetylgalactosamine (the ganglio-series) in human lung (Jia et al., 2020; Walther et al., 2013). The very low level of MAII staining in the parenchyma (Figure 45) is also consistent with reported MAII staining in alveolar epithelia (Nicholls et al., 2007). Altogether these results in human lung sections were similar to published lectin staining and consistent with reported receptor distribution.

6.2.1.2. Characterisation of sialylated receptor distribution in murine lung tissue

Overall, the lectin staining of murine sections (Figure 45) was positive for both MAI and MAII, observed in both airway and parenchyma, indicating a broad distribution of α 2,3 receptors. The relative intensity and ubiquitous distribution of MAI lectin staining in the airway and parenchyma was similar to human PCLS, suggesting α 2,3 sialylated LacNAc residues in common between human and mouse lung. Furthermore, staining was poor with SNA (MAI>MAII>SNA), (Figure 45) consistent with the reported lack of α 2,6 sialylated glycans in mouse lung (Ibricevic et al., 2006). There were, however, qualitative differences with MAII staining of murine parenchyma localised to cuboidal, potentially ATII, cells (Figure 45). In the literature, one study reported only diffuse MAII staining of mouse parenchyma (Ning et al., 2009), but another showed colocalization of MAII with pro-surfactant protein C (an ATII cell marker) in the mouse lung (Ibricevic et al., 2006).

6.2.1.3. Characterisation of sialylated receptor distribution in ferret lung tissue

Ferret lung stained positively with all three lectins (Figure 45), although the quality of the cryosection morphology rendered the staining unclear in the apical airway. Despite this, relative staining intensity across the airway and parenchyma was similar to human (MAI>SNA>MAII). Also similar to human tissue, only low levels of parenchymal staining were observed with MAII, although, some MAII staining of the ferret airway was detected (Figure 45). Overall, the positive MAI and SNA lectin staining in ferret sections supports the use of ferret airway to model human airway with expression of α 2,3 and α 2,6 sialylated LacNAc, and potentially also indicates availability of α 2,3 sialylated ganglio-series receptors (MAII) on ferret airway. This staining,

however, is in contrast to a study that failed to detect α 2,3 sialylated receptors in the proximal airways and parenchyma (Q. Xu et al., 2010).

6.2.1.4. Characterisation of sialylated receptor distribution in pig and macaque lung tissue

Of all the species investigated here, the greatest intensity of SNA staining (α 2,6 sialylated receptors) was observed in pig lung (airways and parenchyma) (Figure 45), and together with the strong MAI staining of pig airway and parenchyma (SNA=MAI>MAII), indicates a receptor distribution generally similar to human tissue (MAI>SNA>MAII). Also similar to human PCLS, MAII staining in pig was restricted to the parenchyma with no staining observed in the apical airway epithelia (Figure 45). This lack of MAII lectin staining is surprising given the distal nature of airways captured in the pig PCLS and staining patterns previously reported in porcine bronchiolar airways (Nelli et al., 2010). Overall, however, the results indicate that the pig lung (and those airways captured) are highly representative of the distribution in human lung and airways (Figure 45).

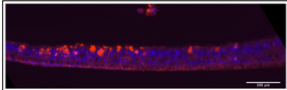
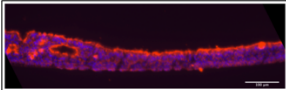
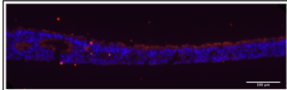
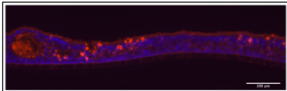
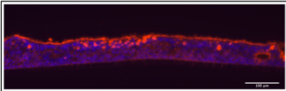
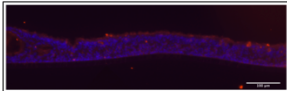
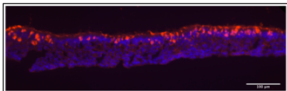
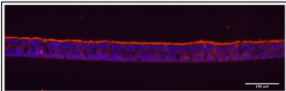
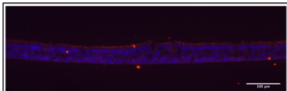
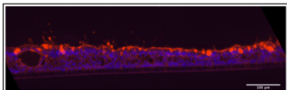
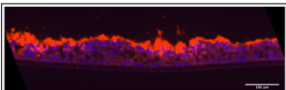
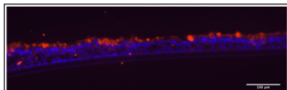
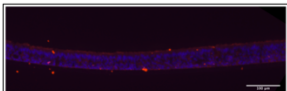
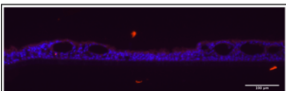
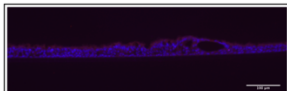
Cryosections from rhesus macaque lungs were challenging to capture and interpret, and tended to show poor morphology. Airways were only identified in cryosections with SNA staining, which was very weak in the airway and minimal in parenchyma. Some positive staining with MAI was observed in the parenchyma and in structures that could represent airway, but minimal MAII staining was detected (MAI>SNA>MAII) (Figure 45). The lack of α 2,6 receptors detected using SNA could suggest these airways in rhesus macaque are most similar to chimpanzee bronchioles, similarly lacking in SNA staining (N. M. Varki et al., 2011) in contrast to African green monkey (Matsuoka et al., 2014).

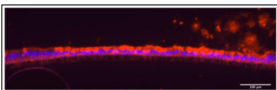
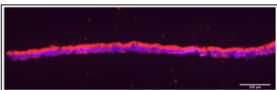
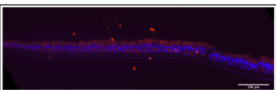
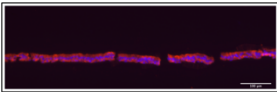
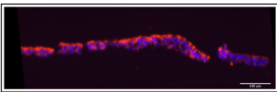
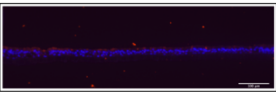
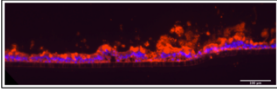
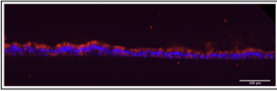
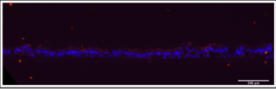
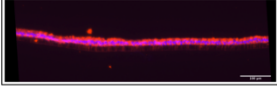
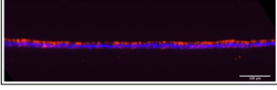
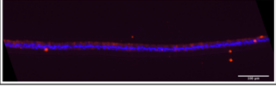
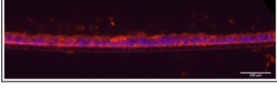
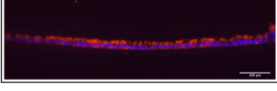
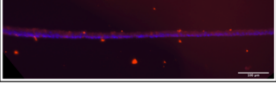
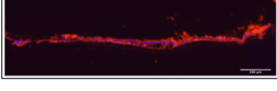
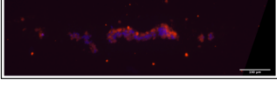
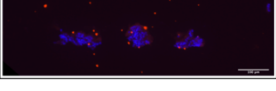
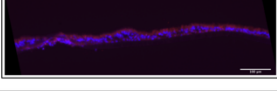
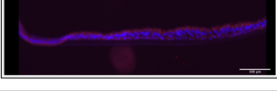
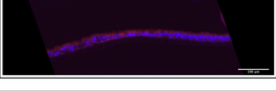
Overall, well-controlled and parallel lectin staining of lung tissue from different species (Figure 45) confirmed previously reported distribution of receptor subtypes. The observed differences between species emphasises the importance of selecting an appropriate model in

which to investigate transduction. These results promote use of pig lung tissue to model the receptor distribution and abundance in human lung, whilst highlighting the stark contrast in receptor distribution modelled using mouse.

6.2.2. Characterisation of sialylated receptor distribution in ALI models

Cryosections from ALI cultures were stained using lectins (also using antigen retrieval (2.7.1)), with sections pre-treated with sialidase A serving as negative controls. Figure 46 shows positive staining of all airway ALI cultures with SNA and MAI, although the intensity varied between donors and culture types. Very little MAII staining was observed, except for B-ALI donor 4. Overall, this indicates that the sialylated receptor subtypes in the human ALI cultures are similar to those in the human (proximal and distal) airway and, importantly, also reflect the scarcity of (MAII-stained) α 2,3 sialylated ganglio-series receptors. This is of course in contrast to observations in the murine airway.

A	In house ALI HBEC donor	SNA	MAI	MAII
	Donor 1			
	Donor 2			
	Donor 3			
	Donor 4			
	Donor 1 (Sialidase A treated control sections)			

B	Epithelix donor	Epithelix ALI type	SNA	MAI	MAII
	5	MucilAir™			
		SmallAir™			
	6	MucilAir™			
		SmallAir™			
	7 (CF)	MucilAir™			
	8 (CF)	MucilAir™			
	5	MucilAir™ (Sialidase A treated control sections)			

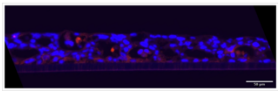
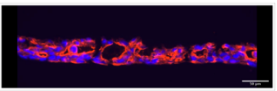
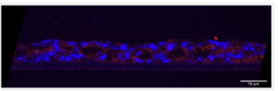
C	In house S-ALI cultures	SNA	MAI	MAII
	H441			

Figure 46- Receptor distribution across ALI models. Cryosections (7µM) from B-ALI (A), Epithelix MucilAir and SmallAir (B), and SALI (C) cultures were stained (with and without sialidase A pre-treatment) in parallel using biotinylated plant lectins (SNA, MAI and MAI) as detailed (2.7.3) and imaged using the EVOS Auto 2 microscope (same exposure and gain settings). Representative images shown from a minimum of 2 transwells stained per donor and lectin. Nuclei are counterstained blue (DAPI), scale bar represents 100µM.

Results in Chapter 5 suggested that transduction profiles did not necessarily relate to cell type distribution (5.2.2.4 & 5.3.1), despite (disputed) cell-type specific receptor distribution patterns characterised as discussed (5.1.2). In B-ALI cultures from donor 1 and 3 there did appear to be a clear qualitative difference in staining pattern between MAI and SNA; a continuous apical pattern of MAI staining indicative of ciliated cell expression versus more punctate staining with SNA (Figure 46). This could support studies suggesting α 2,3 (MAI) is specific to ciliated cells and α 2,6 (SNA) to non-ciliated cells (Mikhail N. Matrosovich et al., 2004). However, also observed in B-ALI was punctate staining of donor 2 and 4 with MAI and some continuous staining across donor 4 with SNA, opposing cell-type specificity in B-ALI cultures (Figure 46). This variability could suggest an ALI donor specific impact on the cell-type specificity of receptor subtype presentation and explain the inconsistency of cell-type specific expression reports.

The relative intensity of lectin staining in B-ALI cultures (Figure 46) appears similar to the airway of human PCLS (MAI>SNA>MAI) (Figure 45), which could indicate that B-ALI cultures are the most appropriate for modelling receptor distribution in human airway. However, the punctate SNA staining in B-ALI cultures was dissimilar to the linear pattern observed on the apical airway epithelium using human PCLS (Figure 45). A greater intensity of, and possibly more apical, SNA staining was observed in Epithelix ALI cultures as well as reduced and less continuous MAI staining (with the exception of MucilAir donor 5). This could indicate a reduced availability of α 2,3 sialylated LacNAc (MAI stained) receptors available for transduction in the Epithelix ALI culture (versus greater availability of α 2,6), and could help explain the reduced rHIV.F/HN

transduction efficiency observed in Epithelix cultures compared with B-ALI cultures (Figure 38). A greater abundance of SNA staining in Epithelix cultures could additionally support (alongside the qualitative staining of B-ALI donor 1 and 3) the enrichment of α 2,6 in non-ciliated cells described by Mikhail N. Matrosovich et al. (2004), considering the overall greater abundance of non-ciliated club cells detected in Epithelix ALI (Figure 34). Further, the continuous MAI staining of Epithelix cultures from MucilAir donor 5 could correlate with the exceptional infrequency of club cells (more similar in frequency to B-ALI), by reduced interruption of α 2,3 sialylated ciliated cell staining.

Two pairs of MucilAir and SmallAir cultures from the same donor were obtained commercially to assess the possible impact of the epithelial cells being derived from either bronchial or bronchiolar epithelium, respectively. Only minimal differences in lectin staining intensity were observed between MucilAir and SmallAir cultures, compared with the differences between these cultures and B-ALI cultures. Also, no clear difference in lectin staining was observed between healthy and CF MucilAir cultures (SNA>MAI>MAII). Although cryosection morphology was not maintained for CF MucilAir donor 8, staining intensity did not suggest a different receptor expression profile in either culture to normal MucilAir.

Interestingly, the SALI cultures, presumed to model the distal lung epithelia, stained strongly with MAI similar to airway ALI cultures processed in parallel (though staining appeared less apically localised) (Figure 46 C). However, almost no staining using SNA or MAII lectins was detected (MAI>SNA>=MAII). The strong MAI staining in SALI cultures is consistent with intense MAI staining of human parenchyma in PCLS (Figure 45) and the greater proportion of (MAI-stained) α 2,3 sialylated LacNAc (N-Glycan) receptors observed in human alveoli using chromatography and mass spectrophotometry (Sriwilaijaroen et al., 2018). Although SNA staining in SALI cultures (Figure 46 C) appeared less intense than in human parenchyma (Figure 45), SNA staining was absent from human parenchyma in some studies (Ibricevic et al., 2006). The difference in SNA staining patterns observed between the SALI cultures (little/no staining)

and B-ALI cultures (abundant staining), is consistent with a hypothesis that the ShanghaiN9 pseudotype more efficiently utilises SNA labelled α 2,6 receptors than the Rostock pseudotype (6.3.1), explaining the different relative transduction efficiency (5.3.3).

6.2.3. Use of hemagglutination to assay free glycan binding by pseudotyped

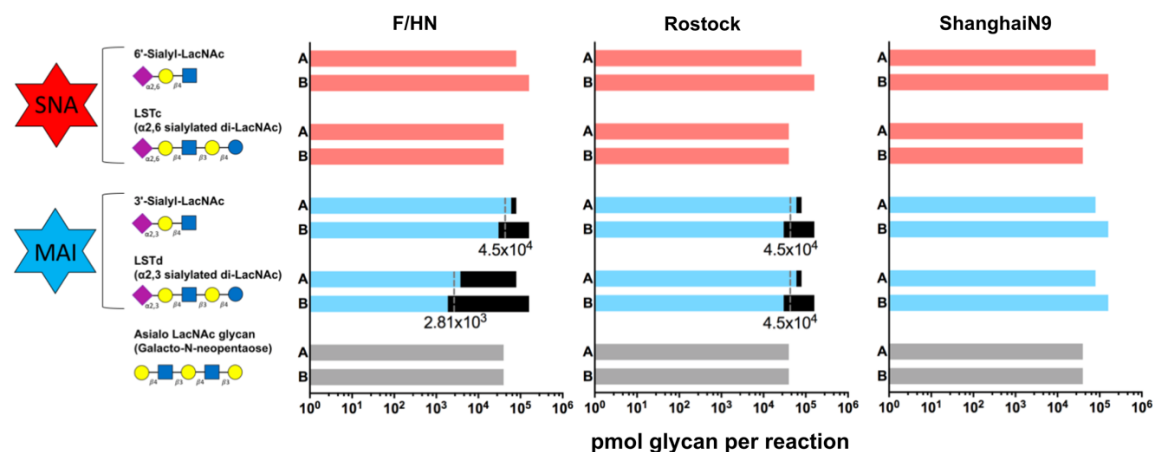
LV

Hemagglutination assays are a well-established tool to quantify virus expressing hemagglutinin glycoproteins. Here, rather than directly quantifying virus, the assay was used to determine the 'minimum volume' of LV preparation required to hemagglutinate (chicken) RBC, allowing an assessment of how hemagglutination could be inhibited by different glycans. This 'minimum volume', known as the HA unit, was determined as described (2.12.3) for rHIV preparations with F/HN and influenza-based pseudotypes prior to assaying for glycan inhibition. The HA unit was determined in two independent experiments using different batches of RBC (A and B). The results showed a consistent two-fold greater volume of ShanghaiN9 pseudotyped rHIV (vGM220 Wave 191) was required than the F/HN (vGM182 Wave 96) and Rostock (vGM222 Wave 193) pseudotypes for both RBC batches A and B. This observation is consistent with twice the concentration of sialic acid binding activity being available per unit volume of the F/HN and Rostock LV preparations than the ShanghaiN9 preparation. It should be noted however, that the HA unit determined for each LV preparation differed by two-fold between each RBC batch (Figure 47), possibly due to different levels of cellular receptors between the different batches of RBC.

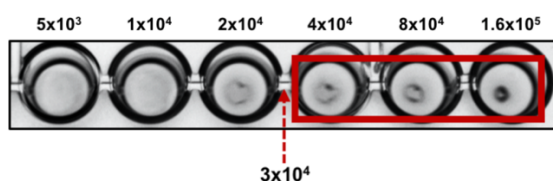
Serial dilutions of each of the glycans under investigation (Figure 44) were first incubated with the RBC to confirm that they would not directly hemagglutinate the RBC, independent of vector. Agglutination was not detected at any of the concentrations investigated (up to 4×10^4 and 1.6×10^5 pmol/reaction) except for glycan GD1a, which hemagglutinated RBC at 1.25×10^4 pmol/reaction or above. GD1a is a glycan with two sialic acid residues per molecule, each

of which might bind lectins on the erythrocyte surface to maintain the RBC in suspension. Unfortunately, this limited the investigation of the receptor preference for the α 2,3 sialylated ganglio-series (MAII) receptors, and the opportunity to relate LV binding to that characterised for Sendai virus and FPV/Rostock H7N1 using GD1a in solid phase (1.5.2.1).

To assay inhibition of hemagglutination by the remaining glycans, serial dilutions of each were prepared in dPBS and incubated (37°C, 1hr) to allow binding with one HA unit of LV (as determined immediately prior for each RBC batch). Following RBC addition, hemagglutination was again used to determine (2.12.3) interaction between the pseudotyped LV and the free glycans, competing with sialylated receptors on RBC to inhibit hemagglutination.



Example Inhibition:



HA unit (dilution factor):

	F/HN vGM182 Wave 96	Rostock vGM222 Wave 193	ShanghaiN9 vGM220 Wave 191
A:	1280	1280	640
B:	2560	2560	1280

Figure 47- Use of sialylated glycans to inhibit hemagglutination by pseudotyped LV. Hemagglutination of red blood cells (RBC) by a single HA unit of pseudotyped rHIV, prepared in the presence of one of a panel of glycans (Figure 44) at a range of concentrations shown. For each glycan investigated, the corresponding lectin for detection is also indicated (left). Each colored bar represents the extent of hemagglutination of RBC in a single experiment (n=2; using separate RBC batches; A and B) performed in parallel across all pseudotypes. The length of each bar represents the maximum pmol/reaction investigated for each glycan. Where inhibition of hemagglutination occurred, the pmol/reaction observed to inhibit is indicated by the black portion of the bar, starting at the calculated lowest pmol required (mean value of highest pmol/reaction hemagglutinated and lowest pmol inhibited). The mean pmol required to inhibit hemagglutination across both RBC batches is indicated by annotated grey dashed lines. Inhibition was determined by observation of RBC pellets matching control reactions in the absence of vector. An example of hemagglutination inhibition (red box) and determined lowest pmol glycan required to hemagglutinate (RBC batch B, 3'-Sialyl- LacNAc, rHIV.F/HN) is shown below, along with the HA units (dilution factor in 50μL TSSM) determined and used for each pseudotyped rHIV.CMV.EGFP preparation and RBC batch. Not shown: LSTa, GM3 and GM1a glycans were investigated up to a maximum of 2.5e4 pmol/reaction but no inhibition of hemagglutination was detected.

As expected, there was no inhibition of hemagglutination detected by the control asialo LacNAc glycan in either experiment across all concentrations (up to 4e4pmol/reaction, Figure 47). In addition, no inhibition was detected using LSTa, GM3 or GM1a glycans, investigated to a maximum of 2.5e4pmol/reaction. In contrast, hemagglutination by both rHIV.F/HN and rHIV.Rostock was inhibited by each of the α 2,3 sialylated LacNAc glycans (LSTd and 3'-Sialyl-LacNAc), but not by the equivalent α 2,6 sialylated glycans (LSTc and 6'-Sialyl-LacNAc). Importantly, no inhibition of rHIV.ShanghaiN9 was detected using any of the glycans investigated. This suggests that both the F/HN and Rostock pseudotypes bind α 2,3 sialylated LacNAc receptors, whereas the ShanghaiN9 pseudotype either does not bind, or has a weaker affinity for this receptor subtype. The concentration of LSTd required to inhibit rHIV.F/HN hemagglutination was 16-fold less than that required to inhibit rHIV.Rostock in both experiments (RBC batches A and B) with a mean of 2.81e3 and 4.5e4 pmol/reaction respectively (Figure 47), suggesting that F/HN binds with greater affinity to α 2,3 sialylated LacNAc receptors with preceding lactose or LacNAc disaccharide unit repeats than either Rostock or ShanghaiN9. The higher concentration of 3'-Sialyl-LacNAc required to inhibit F/HN hemagglutination, compared with LSTd, suggests HN binding may be enhanced via interaction with the additional disaccharide (lactose) unit, whereas the Rostock pseudotype interaction appears independent of the LacNAc receptor length, with the same concentration required (Figure 47). The two independent experiments utilised different RBC batches to control for possible batch-dependant effects on the sensitivity of the assay to detect hemagglutination inhibition by each glycan. Indeed, the pmol/reaction of glycans LSTd and 3'-Sialyl-LacNAc determined to inhibit hemagglutination (both F/HN and Rostock) differed two-fold between experiments with RBC batch A versus B, possibly suggesting a difference in cellular receptors on the RBC batches and so different concentrations of free glycans were required to compete (Figure 47). Overall these results indicate that the F/HN pseudotype has a higher affinity for α 2,3 sialylated LacNAc

receptors of at least one disaccharide unit (LacNAc or lactose) repeat, compared with Rostock and ShanghaiN9 pseudotypes.

6.2.4. Detection of glycan binding by inhibition of LV transduction

Hemagglutination by the LV pseudotypes was only inhibited using relatively high glycan concentrations, therefore, glycan inhibition of LV transduction was also investigated, in case it was a more sensitive assay of glycan binding. Adherent HEK293T cells were transduced with rHIV.F/HN expressing EGFP (vGM182, 6.62e5 TU/ well of approximately 20,000 cells) in the presence of a range of glycan concentrations. Preliminary experiments were performed to select the TU dose of LV that would achieve approximately 50% (EC50) maximum transduction under these conditions (15% EGFP-expressing cells compared with ~30% using maximum feasible TU). This TU dose was prepared in a dilution of each glycan as indicated (Figure 48) and incubated at 37°C for 1hr to allow binding, prior to exposure of cells (50µL of LV & glycan added to 50µL HEK293T cell media (2.2.1)). After 1hr the cells were washed with dPBS before fresh media was replaced, two days following transduction, cells were analysed by FACS (minimum of 10,000 cells counted per condition; Section 2.6.2) to determine the percentage of EGFP-expressing cells.

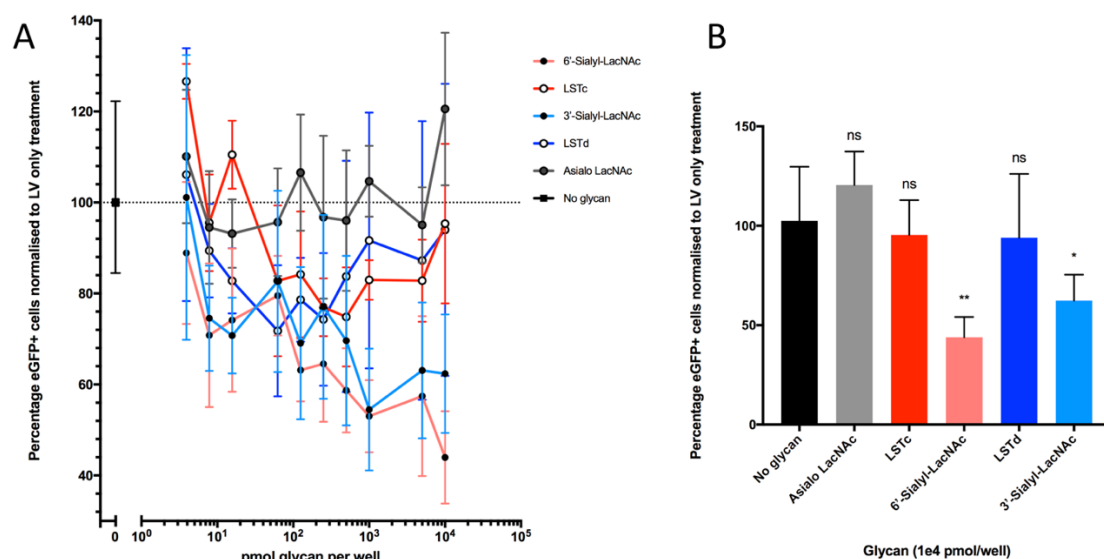


Figure 48- Inhibition of rHIV.F/HN transduction of HEK293T cells with sialylated glycans. (A & B) Adherent HEK293T cells were transduced with rHIV.F/HN.CMV.EGFP (vGM182, Wave 96, 6.62e5 TU/ well of ~20,000 cells, 1hr exposure) in the presence of each glycan at different concentrations. LV was pre-incubated with glycan for 1hr prior to transduction. The percentage of EGFP-expressing cells was determined by FACS 2 days later (n=4). Cells treated with LV (1hr) in the absence of glycan (No glycan; n=39) were used to normalise the percentage EGFP-positive cells determined in the presence of each glycan (15.86% = 100%, dashed line). All error bars indicate standard deviation. (B) Values determined using 1e4 pmol glycan/well were tested for significance using Kruskal–Wallis followed by Dunn’s Multiple Comparison test versus No glycan control. **, p=0.003; 6’-Sialyl- LacNAc and *, p=0.0284; 3’-Sialyl-LacNAc. ns: not significant.

Some variability was observed in the percentage EGFP-expressing cells between replicates (standard deviation shown; n=4, Figure 48), but in contrast to the asialo LacNAc negative control glycan, equivalent concentrations (1e4 pmol/well) of both 3’- and 6’-Sialyl-LacNAc glycans resulted in a statistically significant reduction in EGFP-expressing cells compared with no glycan addition (Figure 48), and a trend for inhibition was also observed over the other concentrations evaluated. Interestingly, both glycans showed a similar potency of inhibition, achieving a maximum inhibition of approximately 50%, although this was unexpected, as hemagglutination inhibition by this LV was only detected using 3’-Sialyl-LacNAc (Figure 47). Furthermore, no trend was apparent in reduction of EGFP-expressing cells with increasing glycan

concentration for LSTd (or LSTc), despite LSTd resulting in the most potent inhibition of F/HN hemagglutination; this is possibly a result of the high inter-replicate variability due to multiple washing/media replacement steps disturbing transduced cells. There may also be confounding effects of the glycans interacting directly with the cells, potentially facilitating rather than inhibiting (electrostatic) interactions between vector and cell (Sampaolesi, Nicotra, & Russo, 2019). The inhibition of transduction by 6'-Sialyl-LacNAc seen here, however, may simply be highlighting the limited sensitivity of the hemagglutination assay.

6.2.5. Detection of cellular receptor use by pseudotyped LV

Assessment of free glycan binding may reveal the specific saccharide sequences bound by the various pseudotypes, but use of the cellular receptors to mediate cell transduction may differ. In addition, the binding by pseudotypes required high glycan concentrations. Furthermore, the assays failed to fully resolve rHIV.F/HN use of α 2,6 sialylated receptors as binding of 6'-Sialyl-LacNAc was implicated only through inhibition of transduction and not hemagglutination. Following the observation that Sendai virus infection of MDBK cells was possible after re-sialylation with an α 2,3 and not α 2,6 sialyltransferase (M. A.K. Markwell & Paulson, 1980)(6.1.4 and 6.1.5), we used a similar approach to determine the use of cellular receptors by pseudotyped LV.

6.2.5.1. Characterisation of receptor expression between cell lines

To characterise receptor subtypes on MDBK cells (M. A.K. Markwell & Paulson, 1980) and the MDCK-based cell lines discussed above (6.1.4), the cells were fixed and stained using biotinylated lectins (SNA, MAI and MAII) and conjugated Alexa Fluor 594 and subsequently imaged in parallel. MDBK, MDCK and MDCK-Siat1 cells were cultured to between passage 8-13, seeded into 96-well plates and, when at least 50% confluent, fixed briefly and stained as described (2.7 and 2.7.3) without antigen retrieval in order to minimise cell detachment. Cells

stained with a variable intensity and pattern of distribution for each lectin, in a cell line-specific manner (Figure 49). As with staining of PCLS (Figure 45) and ALI cultures (Figure 46), pre-treatment of each cell line with sialidase A prior to lectin staining served as a negative control, showing minimal/background fluorescence only ((Figure 49); representative image), and confirming that observed staining was specific to sialylated residues.

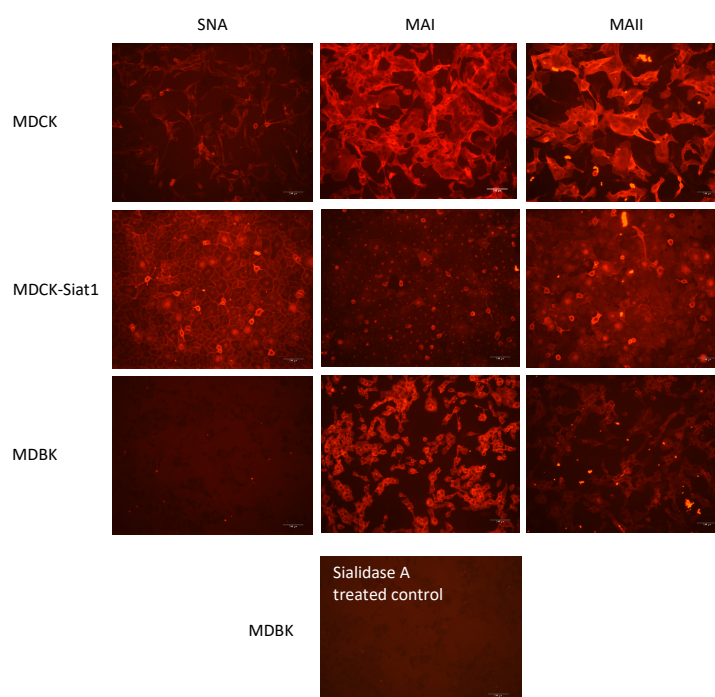


Figure 49- Receptor expression on MDCK, MDCK-Siat1 and MDBK cells. Cells were stained in parallel using biotinylated plant lectins (SNA, MAI and MAII) and imaged using the EVOS Auto 2 microscope (same exposure and gain settings) to determine relative levels of receptor subtypes between cell lines. Also shown, is a representative image of ‘background only’ fluorescence detected from cells pre-treated with sialidase A before staining (MDBK, MAI representative images). Scale bar represents 100μM.

As expected, the parental MDCK cell line stained strongly with both (MAI and MAII) lectins detecting α 2,3 sialylated residues, and lower levels of α 2,6 receptor expression (SNA), consistent with others using the same lectins (at the same concentration) (Q. Xu et al., 2010). Again as expected, the MDCK-Siat1 cell line stained with a similar intensity to MDCK cells using MAII, however an alternative intensity of SNA (greater) and MAI (lower) staining, indicative of

the alternative LacNAc substrate sialylation (greater α 2,6) alluded to by M. Matrosovich et al. (2003)(6.1.4). In contrast to both the MDCK based cell lines, MDBK cells did not stain with SNA, indicating a relative lack of α 2,6 sialylated receptors. MDBK cells stained with a similar intensity using MAI to MDCK cells, yet of the three cell lines investigated, stained least with MAII (in addition to SNA). A lack of α 2,6 sialylated receptor expression on these untreated MDBK cells could indicate this cell line is inappropriate to investigate the potential of α 2,6 sialylation to restore Sendai virus transduction of these cells, as carried out by M. A.K. Markwell & Paulson (1980), with no expression of α 2,6 sialylation evident in untreated cells. In order to investigate the potential use of α 2,3 or α 2,6 sialylated cellular receptors by the LV pseudotypes, the MDCK-Siat1 cell line was therefore selected based on expression of all receptor subtypes recognised by these lectins.

6.2.5.2. Use of sialidase enzymes A and S to investigate receptor dependence of pseudotyped LV transduction

MDCK-Siat1 cells (in 96-well plates) were incubated with either sialidase A (from *Arthrobacter ureafaciens*; 50mU/mL) or sialidase S (from *Streptococcus pneumoniae*; 200mU/mL) (6.1.5) for up to 60 minutes as described (2.12.2). Following treatment, the cells were then either fixed immediately and stained with lectins to confirm impact of sialidase treatment on receptor availability (Figure 50 A), or transduced with rHIV.F/HN.CMV.EGFP (3.8e5 TU/well of approximately 20,000 cells; Figure 50 B). Exposure of cells to LV was limited to 10 minutes in order to minimise potential for endogenous replenishment (re-sialylation) of receptors on the cells following removal of the sialidase. Cells were lectin stained without antigen retrieval (as 6.2.5.1), but the procedure was simplified by detection of SNA binding via conjugated FITC (2.7.3) and co-staining with biotinylated MAII to detect changes in receptor availability (α 2,6 and α 2,3 respectively). As expected, staining of MDCK-Siat1 cells (untreated) was in line with that determined using each lectin discretely (Figure 50 A versus Figure 49). Cells treated with

sialidase S generally stained with both lectins at a similar intensity to the untreated cells, despite an anticipated reduction in MAII staining via cleavage of $\alpha 2,3$ sialylated receptors. This could indicate either insensitivity of the method to detect reduced receptor availability, or a lack of ganglio-series $\alpha 2,3$ sialylated receptor cleavage by sialidase S. In contrast, sialidase A treated cells stained minimally with both lectins indicating receptor cleavage.

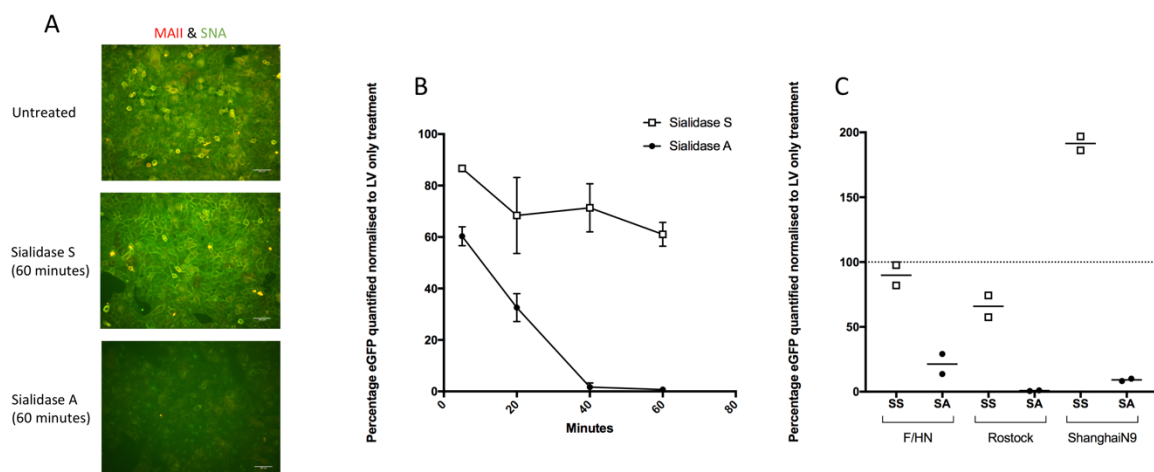


Figure 50- Effect of sialidase A and sialidase S on MDCK-Siat1 cell receptor availability and transduction by pseudotyped rHIV. MDCK-Siat1 cells were treated with Sialidase A (50mU/mL) or Sialidase S (200mU/mL) and either stained using lectins (A) or transduced with pseudotyped rHIV expressing EGFP, quantifying expression at 48hrs (B and C), n=2 replicates. (A) Staining of cells $\pm$ sialidase treatment for 60 minutes using biotinylated MAII (red) and FITC labelled SNA (green) to detect $\alpha 2,3$ and $\alpha 2,6$ sialylated receptors respectively. Cells were imaged immediately using the EVOS FL Auto 2 microscope. Scale bar represents 100 μ M. (B) Cells treated with sialidase A or S for up to 60 minutes prior to transduction with rHIV.F/HN.CMV.EGFP (3.8e5 TU/well vGM182 Wave 96, 10-minute exposure) were imaged to detect EGFP fluorescence at 48hrs and expression quantified by percentage area (2.6.3) normalised to cells transduced in the absence of sialidase treatment (5.2% area fluorescence above threshold= 100%). Mean value shown, error bars represent standard deviation. (C) Cells treated with sialidase A or S for 40 minutes prior to transduction with 5e5 TU/well of rHIV.CMV.EGFP pseudotyped with F/HN (vGM182 Wave 187), Rostock (vGM222 Wave 193) or ShanghaiN9 (vGM220 Wave191). Cells were imaged and EGFP quantified by percentage area (2.6.3) normalised to transduction in the absence of sialidase treatment (100%= 9.2% F/HN, 4.6% Rostock, 5.7% ShanghaiN9). Each symbol represents a single replicate, line represents median.

EGFP was quantified at 48 hours following transduction with rHIV.F/HN, using images captured from transduced wells and measuring % area above background fluorescence as described (2.6.3). Although this method cannot enumerate transduced cells, FACS facility closure due to COVID-19 restricted the methods available to quantify transduction in these studies. Cells transduced with rHIV.F/HN after treatment with sialidase A showed a reduction in EGFP quantified from as little as 5 minutes (reduced to 60% of EGFP quantified following transduction of cells without sialidase pre-treatment), with EGFP detection abolished from 40 minutes (Figure 50 B). In contrast, treatment with sialidase S achieved only partial reduction (by approximately 40%) in EGFP. This suggests that F/HN transduction of these cells is dependent on the sialylated receptors expressed on these cells (all cleaved by sialidase A), however transduction was only reduced following cleavage of receptors by sialidase S. With no evidence for complete cleavage of α 2,3 sialylated receptors by sialidase S, it is unclear whether the remaining transduction following sialidase S treatment is a result of F/HN use of α 2,6 sialylated receptors or use of α 2,3 sialylated receptors of lower abundance. Based on these results it was concluded that 40 minutes pre-treatment was sufficient to achieve the maximal receptor cleavage using either sialidase enzyme on these live cells.

In order to confirm this cellular receptor dependency for F/HN transduction, and compare with the influenza-based pseudotypes, the experiment was repeated using an equivalent TU dose (5×10^5 TU/well) of rHIV.CMV.EGFP for F/HN, Rostock and ShanghaiN9, and the effect of pre-treatment sialidase A or S (with a 40 minute incubation) on transduction efficiency in MDCK-Siat1 cells was investigated (Figure 50 C).

EGFP fluorescence was reduced following rHIV.F/HN transduction of sialidase A and S pre-treated cells, similar to the reduction achieved previously at this timepoint (Figure 50 B). However, EGFP detection was not abolished in sialidase A pre-treated cells as before, indicating some F/HN transduction (<30% versus no pre-treatment), which could suggest incomplete cleavage of sialic acid receptors at 40 minutes. Importantly, a large reduction in EGFP was

observed for both rHIV.Rostock and rHIV.ShanghaiN9, indicating dependency for both pseudotypes on sialic acid for transduction (EGFP levels were either abolished or reduced to ~10% with sialidase A). Sialidase S reduced EGFP fluorescence with F/HN (to 82-98%) and with Rostock pseudotypes (to ~65%) but, in contrast, sialidase S pre-treatment resulted in nearly two-fold increased EGFP fluorescence after transduction with rHIV.ShanghaiN9 (186-197%). Crucially, this suggested that the sialidase S cleavage of α 2,3 receptors on MDCK-Siat1 cells improved access to the receptors used by the ShanghaiN9 pseudotype to mediate transduction. A subtle increase in SNA staining observed could support greater α 2,6 receptor access for binding by lectins or hemagglutinin (Figure 50 A). Interestingly, mild sialidase treatments have previously been associated with enhanced binding by hemagglutinating viruses due to a potential 'receptor unmasking' effect (M. A.K. Markwell & Paulson, 1980; Wassilewa, 1977). Such an unmasking effect could explain the enhanced transduction by the ShanghaiN9 pseudotype observed here, particularly given this pseudotypes hypothesised use of α 2,6 sialylated receptors (6.2.2 & 6.3.1).

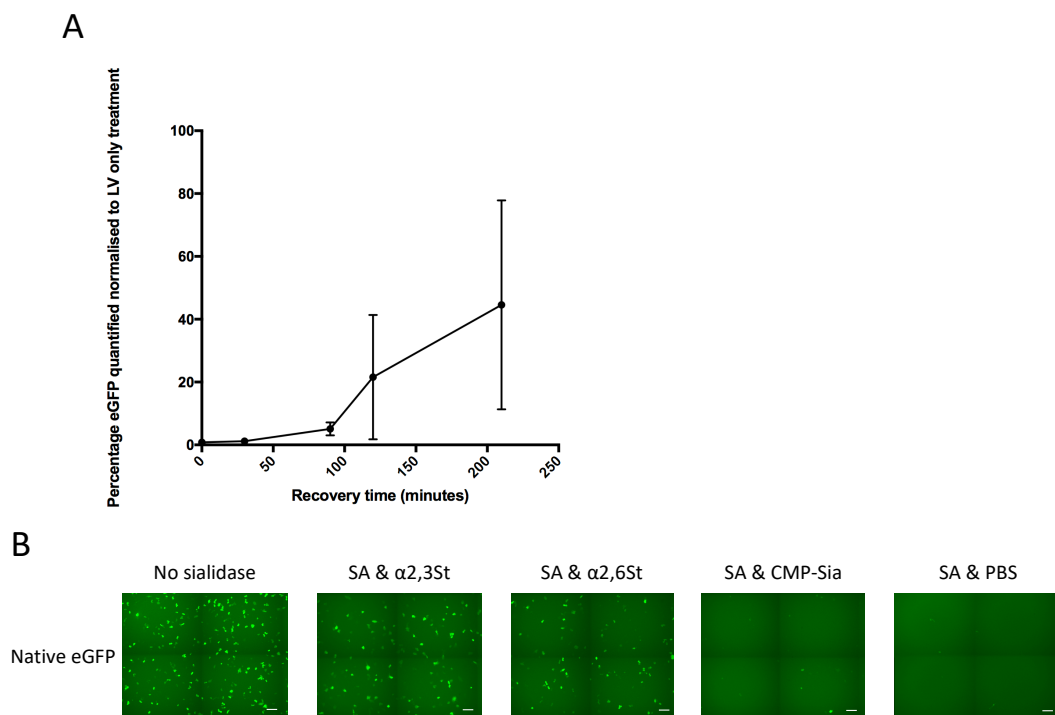
6.2.5.3. Restoration of F/HN transduction using α 2,3 and α 2,6 sialyltransferase in MDCK-Siat1 cells

Following treatment of MDCK-Siat1 cells with sialidase S, both MAb staining (α 2,3) as well as the expected SNA staining (α 2,6) were observed, thus the use of receptor subtypes by the F/HN pseudotype remained unclear. The potential for restoration of F/HN transduction by re-sialylation of receptor sub-types was therefore investigated, using sialyltransferases with specificity for different receptor sub-types (6.1.5 Section). Cells were first treated with Sialidase A (50mU/mL at 37°C, 40 minutes) to cleave all sialic acid residues to generate asialo MDCK-Siat1 cells, which were then allowed to recover (for between 30 and 210 minutes in dPBS) before being transduced with rHIV.F/HN expressing EGFP (Figure 51 A). No increase in EGFP fluorescence was detected between 0 and 30 minutes of recovery time suggesting that the

asialo nature of the cells was maintained, without naturally-occurring endogenous enzymes restoring sialylation during this time. Furthermore, very little recovery of F/HN transduction was observed at 90 minutes (<10% EGFP fluorescence without sialidase A) (Figure 51 A), although recovery became more apparent as incubation continued up to 210 minutes (Figure 51). Nevertheless, as there was negligible (endogenous) restoration of receptors used by rHIV.F/HN within 30 minutes of sialidase A treatment, the use of exogenous sialyltransferase enzymes to restore transduction within this time frame could be readily investigated.

Cells were therefore subjected to treatment with sialidase A as before and subsequently treated with either α 2,3-sialyltransferase from *Pasteurella multocida* or α 2,6-sialyltransferase from *Photobacterium damsela* (6.1.5) as described (2.12.2). As negative controls, cells were treated with PBS only, or with the activated sialic acid substrate (CMP-Sia:cytidine-5'-monophosphate-sialic acid), for 20 minutes before transduction (10 minutes LV exposure, Figure 51 B & C). In parallel, treated cells were lectin staining to determine receptor availability (Figure 51 D). As expected there was no evidence of EGFP-expression in asialo cells transduced following recovery in PBS (SA & PBS) and only very low levels of EGFP in asialo cells treated with CMP-Sia substrate before transduction (<2% compared with no sialidase). Endogenous sialyltransferase enzyme activity must therefore be minimal during this time period, suggesting sialyltransferase activity is rate-limiting step in restoration of receptors for F/HN transduction under the experimental conditions used. Lectin staining further confirmed the expected asialo status in cells at the point of transduction (PBS only control) with minimal MAII and SNA staining, in contrast to untreated cells (no sialidase, Figure 51 D). Importantly, asialo cells treated with α 2,3 or α 2,6 sialyltransferase enzymes prior to transduction, showed restoration of EGFP expression to a median of 42.3% and 29.6%, respectively. This partial restoration of transduction using either enzyme could suggest rHIV.F/HN transduction is mediated by either α 2,3 or α 2,6 sialylated receptors in these cells, although lectin staining in parallel did not indicate a clear increase in a specific receptor subtype. Both MAII and SNA staining was observed in cells treated

with $\alpha 2,3$ sialyltransferase, indicating the presence of both subtypes, possibly due to the low level $\alpha 2,6$ -sialyltransferase activity reported for this enzyme (Yu et al., 2005). In addition, cells treated with the $\alpha 2,6$ sialyltransferase did not stain with MAII (for $\alpha 2,3$) as expected, but showed only low levels of SNA lectin staining. However, these ambiguous staining patterns could simply reflect the relative insensitivity of the lectin staining protocol to detect the levels of receptor availability required for transduction. Despite the ambiguity in lectin staining, the increased EGFP detection in $\alpha 2,6$ sialyltransferase treated cells, indicates an increased availability of $\alpha 2,6$ sialylated receptors used by rHIV.F/HN to mediate transduction of MDCK-Siat1 cells without a dependency on $\alpha 2,3$ sialylated residues alone. However, the greater levels of EGFP expression restored using $\alpha 2,3$ -sialyltransferase (to ~40% of transduction without sialidase treatment) also supports the notion of preferential use of $\alpha 2,3$ sialylated residues by the F/HN pseudotype.



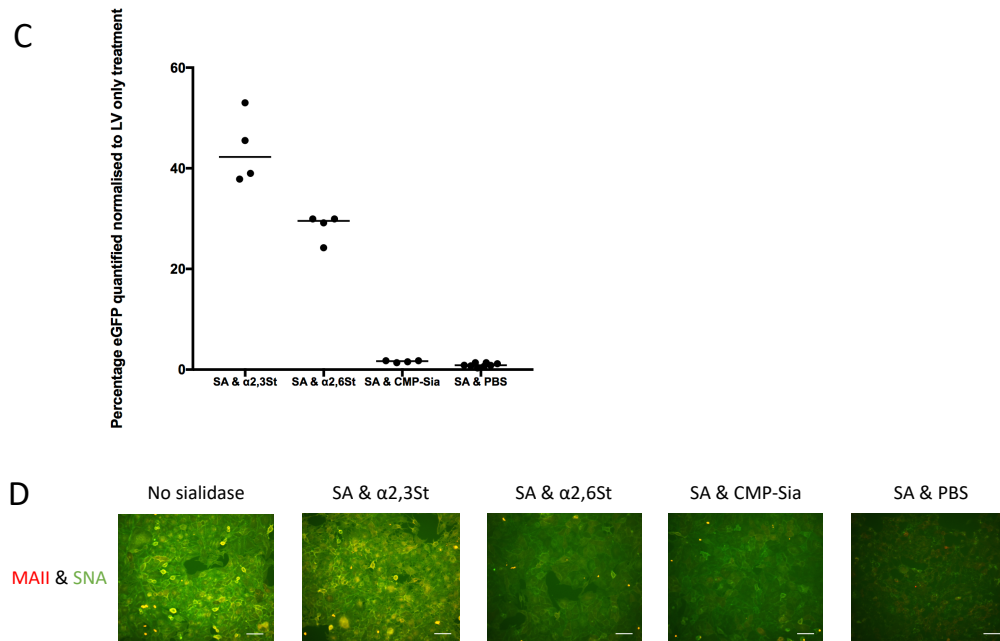


Figure 51- Restoration of rHIV.F/HN transduction of asialo MDCK-Siat1 cells after treatment with exogenous sialyltransferase. MDCK-Siat1 cells were pre-treated with sialidase A (50mU/mL for 40 minutes). Cells were then transduced with rHIV.F/HN.CMV.EGFP (3.8e5 TU/well vGM182 Wave 96, 10 minute exposure)(A, B & C), either after variable time points of cell recovery in dPBS (A), or following treatment with sialyltransferase enzymes (B & C). In parallel, cells treated with sialyltransferase enzymes were lectin stained to determine receptor availability at the point of transduction (D). (A) Cells treated with sialidase A were allowed to recover in dPBS for up to 210 minutes before transduction and were imaged to detect EGFP fluorescence at 48hrs. EGFP expression was quantified by percentage area (2.12.2) normalised to cells transduced without sialidase pre- treatment (5.2% area fluorescence above threshold= 100%, n=2 replicates). Mean value shown, error bars represent standard deviation. (B,C & D) Cells treated with sialidase A (SA) were subsequently treated for 20 minutes with either α2,3-sialyltransferase and CMP-Sia (SA & α2,3St), α2,6-sialyltransferase and CMP-Sia (SA & α2,6St), CMP-Sia only (SA & CMP-sia), or allowed to recover in dPBS (SA & PBS) before transduction (n=4 replicates, B & C), or fixation and lectin staining (n=2, D). EGFP expression was captured using the EVOS FL Auto 2 microscope at 48hrs following transduction (representative images shown- 'Native EGFP'- B) and quantified by percentage area (2.12.2) normalised to cells transduced without sialidase pre-treatment (C, each symbol represents a single replicate, line represents median, 4.4% area fluorescence above threshold quantified from 'no sialidase' pre-treatment cells = 100%). Alternatively, cells were fixed in parallel to transduction and receptor availability determined using biotinylated MAII (red) and FITC labelled SNA (green) to detect α2,3 and α2,6 sialylated receptor availability (D). Scale bars represent 100μM.

6.2.6. *In vivo* sialidase studies

The above experiments indicate that the F/HN pseudotype can use both $\alpha 2,3$ and $\alpha 2,6$ sialylated residues as receptors *in vitro*, but the receptors used *in vivo* are unknown. Adopting an approach similar to that performed by Bell et al. (2011), the impact of intranasal sialidase administration, 1 hour prior to intranasal delivery of rSIV.F/HN, was investigated for its effect on transduction *in vivo*.

6.2.6.1. Cleavage of sialylated receptors

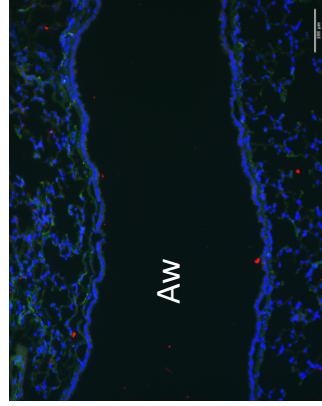
Mice were treated with sialidase A or S (200mU) or buffer only (2.9.1), and sacrificed 1 hour after delivery and the lungs immediately fixed and processed for cryosection staining using lectins.

In mice treated with buffer only, lectin staining was minimal for SNA but widespread with MAI and MAII, with greater intensity in apical airway epithelium and parenchyma, respectively (Figure 52). This distribution was consistent with that captured for the mouse airway and parenchyma using untreated mouse lung tissue previously (Figure 45), confirming receptor distribution is not altered by buffer administration alone. In contrast, mice treated with sialidase A intranasally were largely negative for SNA, MAII and MAI staining throughout the lung lobes, similar to background levels detected following *ex vivo* sialidase A treatment; and suggesting highly efficient cleavage of sialylated residues throughout the mouse lung and widespread distribution of the enzyme via intranasal delivery.

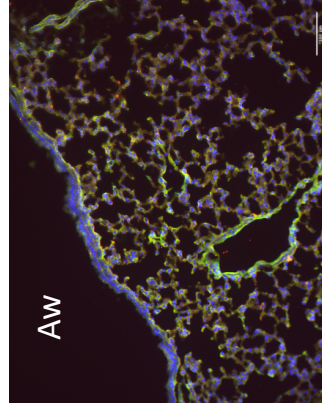
Following sialidase S delivery, the intense MAI staining of the apical airway seen in control (buffer only) mice was absent, suggesting cleavage of $\alpha 2,3$ residues in the airway, but in the parenchyma, MAI staining was of similar intensity to buffer only treated mice. In contrast, MAII staining was minimal throughout the lung suggesting efficient, widespread cleavage similar to that achieved with sialidase A, and indicating that sialidase S is more efficient at cleaving MAII ($\alpha 2,3$ sialylated ganglio-series) than MAI stained ($\alpha 2,3$ sialylated LacNAc) residues. SNA staining

was of unexpected intensity, greater than control (buffer only) mice, indicating increased availability of, or access to, α 2,6 sialylated residues after sialidase S. Increased SNA staining intensity was also observed *in vitro* after sialidase S treatment of MDCK-Siat1 cells (Figure 50 A). It was therefore concluded that intranasal sialidase treatment could be used *in vivo* to investigate receptor distribution and transduction by rSIV.F/HN.

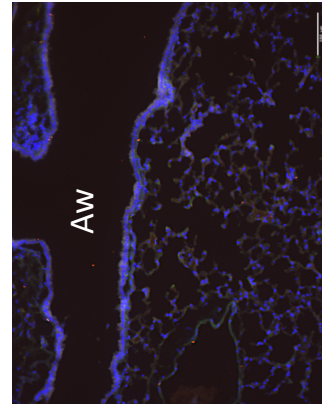
Ex vivo Sialidase A treated
cryosections (negative control)



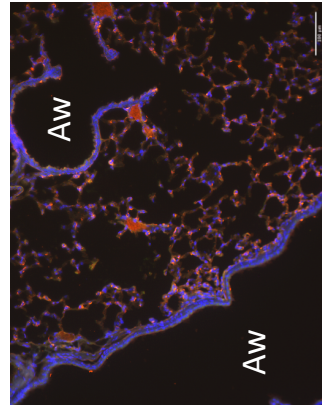
Sialidase S



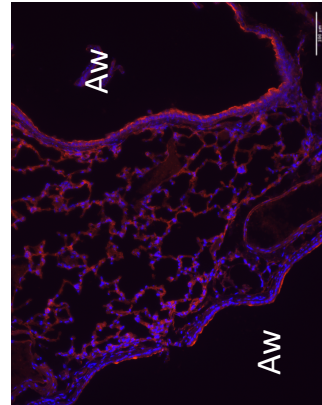
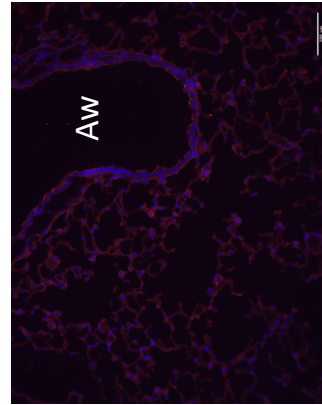
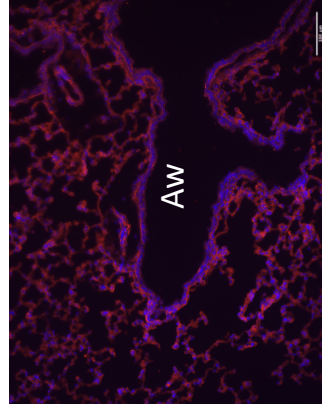
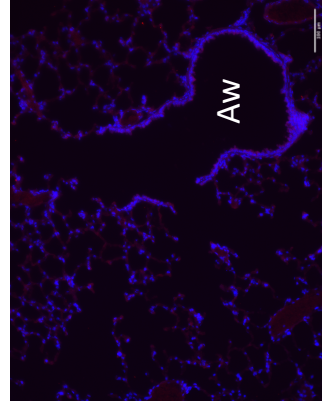
Sialidase A



Buffer only



MAII & SNA



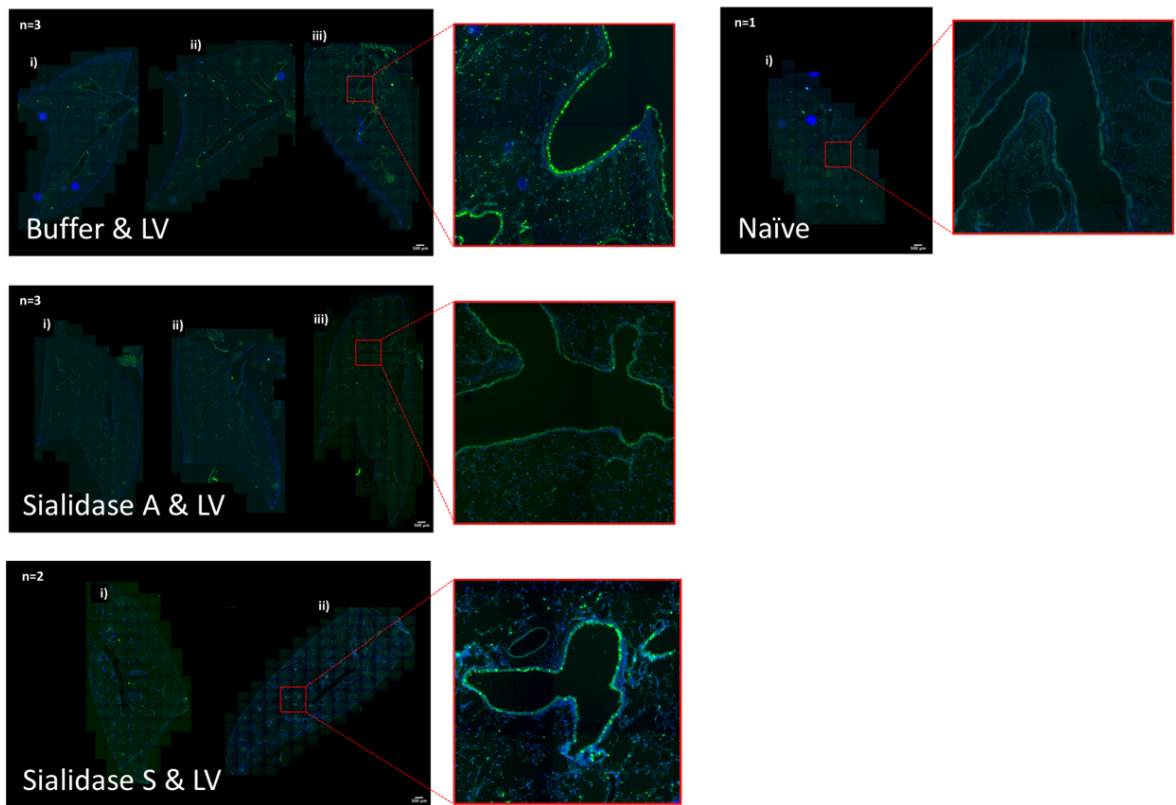
MAI

Figure 52- Effect of intranasal sialidase treatment on receptor availability in mouse lung at 1 hour. Mice were treated with 200mU of sialidase A, sialidase S or reaction buffer only by intranasal administration (100µL, n=2), and subsequently sacrificed at 1 hour to determine receptor availability. Cryosections generated from lung tissue, immediately fixed following culling, were stained using either biotinylated MAII (red) and FITC labelled SNA (green) or Biotinylated MAI (red). To control for non-specific lectin staining, cryosections of lung from untreated mice, but treated with sialidase A ex vivo as described (2.7.3) were additionally stained in parallel. Sections were imaged using the EVOS Auto 2 microscope (same exposure and gain settings). Representative images shown of stained cryosections (n=2 per mouse) and lectin staining. Nuclei are counterstained blue (DAPI), scale bar represents 100µM, Aw: Airway.

6.2.6.2. F/HN *in vivo* transduction is dependent on sialic acid

To investigate the impact on rSIV.F/HN transduction, mice were pre-treated intranasally with sialidase A, sialidase S or buffer only as before, and subsequently administered 1e8 TU rSIV.F/HN.hCEF 1 hour later, and expression of reporter transgene determined 7 days after dosing. Mice treated with LV expressing EGFP only were sacrificed at day 7 and lungs processed to detect native EGFP in cryosections. Alternatively, mice treated with LV expressing firefly luciferase (EGFPLux) in a multi-cistronic vector configuration were subjected to intranasal administration of D-Luciferin 7 days after LV dosing and bioluminescent imaging used to determine the luciferase transgene activity *in vivo* as described (2.9.3).

A



B



C

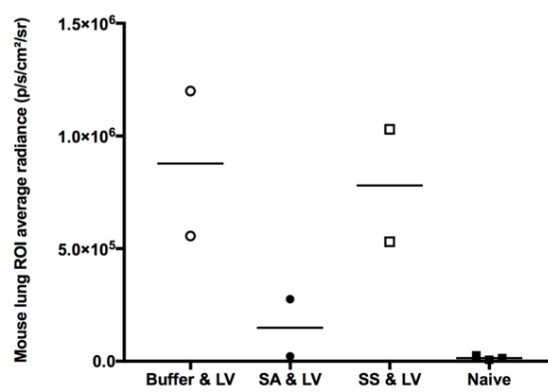


Figure 53- Effect of intranasal delivery of sialidase on receptor availability in mouse lung. Mice were treated with 200mU of sialidase A (SA), sialidase S (SS) or reaction buffer only by intranasal administration (100µL, n=2), and after 1 hour, treated with 1e8 TU rSIV.F/HN.hCEF (LV) with reporter expression determined 7 days later. (A) Mice transduced with rSIV.F/HN.hCEF.EGFP (vGM107 Wave 121, n=2- 3) were sacrificed at day 7 and lungs processed to determine EGFP expression and distribution in whole lung lobe in 7µM cryosections. Nuclei are counterstained blue (DAPI), scale bar represents 500µM. i), ii) and iii) correspond to sections from individual mice. (B & C) Mice transduced with rSIV.F/HN.hCEF.EGFPLux (vGM141 Wave 62, n=2) were administered D-Luciferin 7 days after vector dosing and imaged using an IVIS Lumina LT Imaging System to visualise the distribution of luciferase transgene activity (B) and quantify activity in the mouse lung (C, average radiance across lung ROI). Luciferase activity (radiance) was measured in photons/second/cm²/steradian.

High levels of EGFP expression were detected in both the airway and parenchyma of mice pre-treated with buffer only or sialidase S (Figure 53 A). In contrast, there was almost no EGFP expressing cells in cryosections from mice pre-treated with sialidase A. In agreement with EGFP transgene detection from these mice, luciferase activity in the lungs of mice that received the EGFPLux transgene was evident in the lungs and nose of mice in buffer only and sialidase S pre-treated groups, yet luciferase activity determined in mice pre-treated with sialidase A was greatly reduced, similar to naïve background detection levels in the lung, and absent in the nose (Figure 53 B & C). In conjunction with the sialylated receptors observed at this timepoint for transduction, absent/minimal transgene detection in mice pre-treated with sialidase A confirms that F/HN *in vivo* transduction of the murine lung is dependent on sialic acid, similar to *in vitro* findings.

High transgene expression levels in mice pre-treated with sialidase S appeared of similar abundance and distribution to mice pre-treated with buffer only (EGFP in airway and parenchyma and luciferase activity in nose and lung; Figure 53). This indicates that F/HN may be able to use α 2,6 sialylated receptors to transduce the mouse airway cells, although this cannot

be confirmed given the incomplete cleavage of α 2,3 receptors indicated by residual MAI and MAII staining.

6.2.7. Airway ALI sialidase studies

Manipulation of receptors for *in vitro* and *in vivo* transduction highlights the potential for the F/HN pseudotype to utilise both α 2,3 or α 2,6 sialylated receptors. However, use of the receptors expressed on human airway cells, lacking the MAII stained (Figure 46, α 2,3 sialylated gangliosides), as well as the 'non-human' sialic acid (N-glycolylneuraminic acid, 1.5) receptors, is unknown. With an abundance of α 2,6 sialylated receptors observed in B-ALI cultures, this model may be useful to investigate the impact of α 2,3 cleavage by sialidase S on F/HN transduction in human airway cells. Additionally, it may be possible to investigate the receptors used by Rostock and ShanghaiN9 pseudotypes to mediate transduction of B-ALI cultures (Figure 41); and to confirm the effect of sialidase S treatment to enhance transduction by ShanghaiN9, as observed in MDCK-Siat1 cells (Figure 50).

6.2.7.1. Cleavage of sialylated receptors on B-ALI cultures

To investigate the impact of receptor cleavage by sialidase A or S on LV transduction, B-ALI cultures were apically administered sialidase A or S for 1 hour as described (2.11.4), before fixation and cryosection staining with lectins (MAI and SNA).

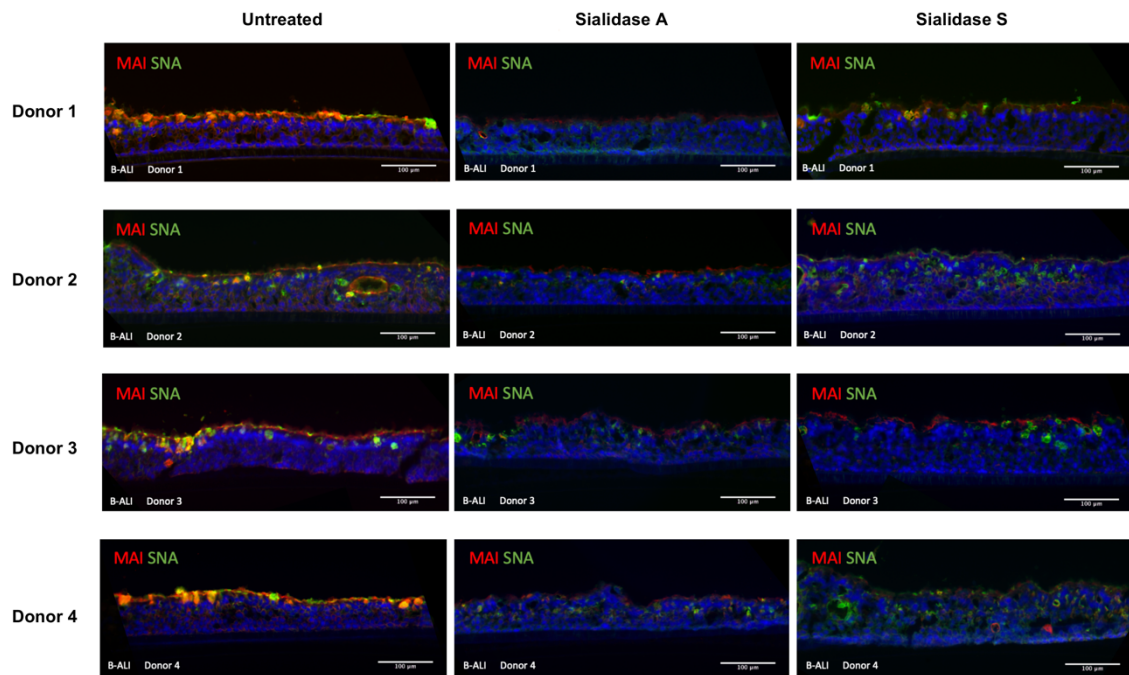


Figure 54- Effect of apical sialidase treatment on receptor subtypes in B-ALI cultures. B-ALI cultures, untreated, or treated apically with sialidase A or sialidase S for 1 hour, were fixed immediately and processed as cryosections to determine receptor availability by lectin staining. Cryosections were imaged using EVOS FL Auto 2 microscope (same exposure and gain settings) to detect biotinylated MAI (red) and FITC labelled SNA (green). Representative images shown from a minimum of two transwell cryosections stained per donor and lectin; nuclei are stained blue; scale bar represents 100µM.

Staining of untreated B-ALI cultures (Figure 54) from all four donors revealed a similar profile and intensity to staining with biotinylated MAI and SNA lectins (Figure 46). The difference in continuous/linear apical staining pattern observed with MAI contrasting with punctate/globular SNA staining in some donors (Figure 46, donors 1 and 3) was reproduced, and was particularly clear in donor 3 with minimal colocalization of the MAI and SNA staining. This adds support to the notion that these cultures may express the receptor subtypes in a cell type-specific manner similar to that observed by others (Mikhail N. Matrosovich et al., 2004). In contrast, cryosections from all transwells pre-treated with sialidase A stained only minimally with MAI or SNA compared to untreated, indicating cleavage of both $\alpha 2,3$ and $\alpha 2,6$ sialylated receptors as anticipated. Cryosections from sialidase S pre-treated cultures however all stained

with SNA (at similar intensity to untreated controls), with a reduced intensity of MAI staining, suggesting sialidase S cleavage of α 2,3 receptors only. Although MAI staining on sialidase S treated transwells from donor 3 was apparent, the intensity was lower compared with untreated. Detection of MAI staining may additionally be enhanced specifically in this donor, with a lack of SNA colocalization characterised. Overall, this staining supports the use of apical sialidase treatment of B-ALI cultures to investigate the impact of receptor availability on transduction by pseudotyped LV.

6.2.7.2. Pseudotype dependency on sialic acid to mediate transduction of B-ALI cultures

B-ALI cultures were treated with sialidase A or S, or left untreated, and then transduced with rHIV.CMV.EGFP pseudotyped with either F/HN, Rostock or ShanghaiN9. As expected, EGFP was detected 14 days after transduction in all cultures without sialidase pre-treatment (LV only, Figure 55 A).

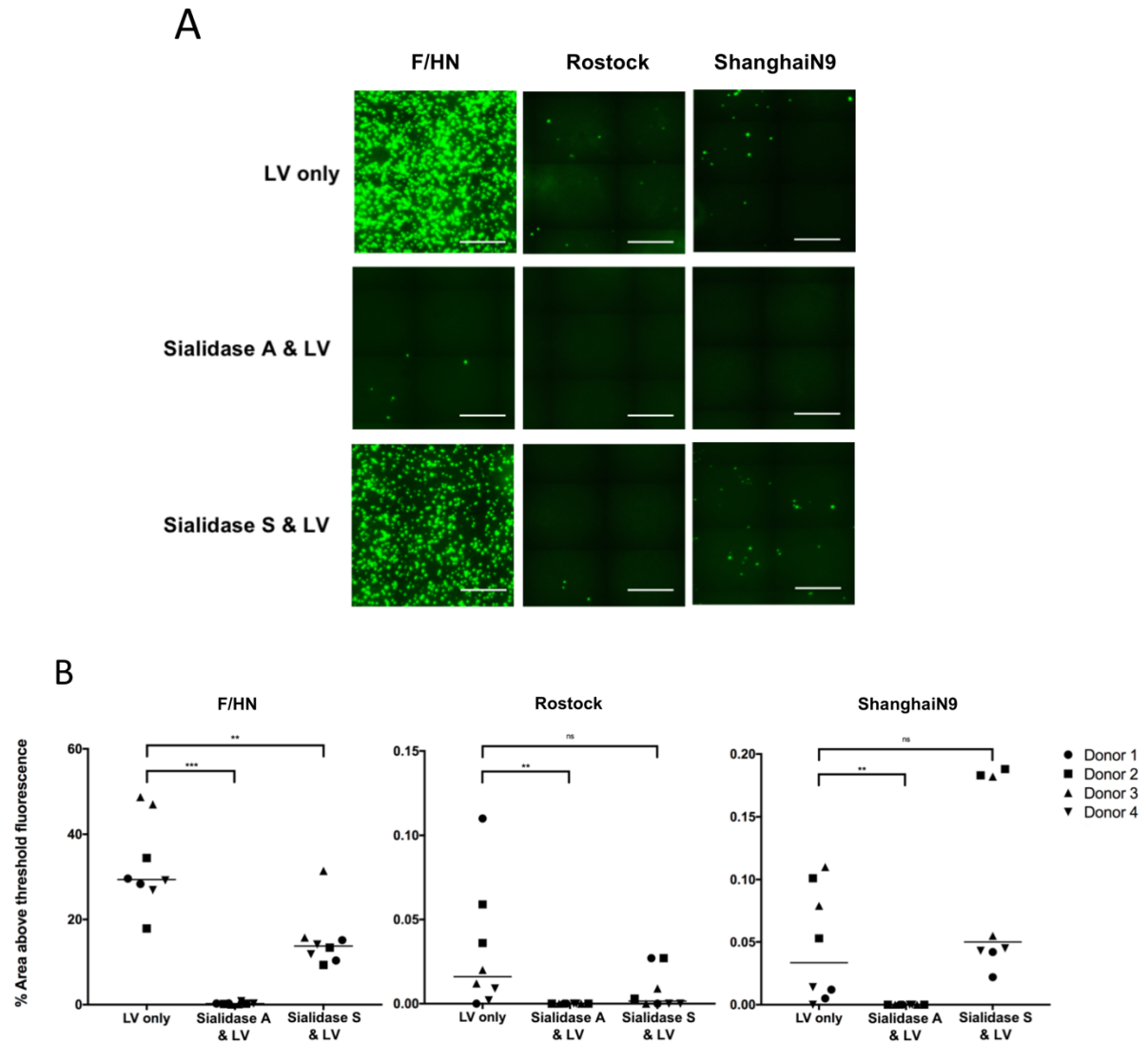


Figure 55- Effect of apical sialidase treatment on transduction of B-ALI cultures by rHIV pseudotypes. B-ALI cultures, untreated (LV only), or treated apically with sialidase A or sialidase S for 1 hour, were transduced with LV: either rHIV.F/HN (7.5e7 TU, vGM182 Wave 96), rHIV.Rostock (5.1e8 TU, vGM194 Wave 114) or rHIV.ShanghaiN9 (7.5e6 TU, vGM192 Wave) expressing EGFP. 14 days after transduction, cultures were imaged to detect EGFP fluorescence. (A) Representative stitched centre-of-transwell images (donor 3) shown; scale bar represents 500 μ M. (B) Quantified EGFP fluorescence determined from transwell images captured. Each symbol represents a biological replicate; each shape represents a single donor; bar represents the median. LV only versus sialidase A & LV; ***, $p=0.0002$ (F/HN), **, $p=0.0014$ (Rostock and Shanghai). LV only versus sialidase S & LV; **, $P=0.003$ (F/HN). Significance determined using Mann-Whitney U test; ns, not significant.

The transduction efficiency with Rostock and ShanghaiN9 pseudotypes was again much lower than for F/HN, in line with earlier experiments (Figure 41) indicating transduction efficiency in this model is not equal, even at equivalent TU. Importantly, B-ALI cultures pre-treated with sialidase A showed little to no evidence of transduction by any of the pseudotypes. Compared with untreated B-ALI cultures (LV only), a statistically significant (at least 100-fold) reduction in EGFP fluorescence was quantified from rHIV.F/HN ($p=0.0002$), and a complete absence of fluorescence from rHIV.Rostock and rHIV.ShanghaiN9 ($p=0.0014$) (Figure 55 B). This indicates that transduction by all pseudotypes is dependent on the sialylated receptors available. Sialidase S pre-treatment of B-ALI cultures prior to rHIV.F/HN transduction resulted in a significant, (approximately 2-fold) reduction in EGFP fluorescence compared with no pre-treatment (LV only), indicating use of $\alpha 2,3$ sialylated residues in B-ALI cultures by F/HN pseudotype. This is a similar (fold) reduction to that observed in MDCK-Siat1 cells investigating the impact of sialidase S treatment on F/HN mediated transduction (Figure 50 C).

Although reduced EGFP fluorescence was also observed for rHIV.Rostock with sialidase S pre-treatment, EGFP-positive cells were too few in number to draw firm conclusions or be statistically significant. Intriguingly, sialidase S pre-treatment did not change EGFP fluorescence resulting from rHIV.ShanghaiN9 transduction (Figure 55), indicating that the ShanghaiN9 pseudotype transduces B-ALI cultures independent of $\alpha 2,3$ sialylated (mostly MAI) receptors in this model. Alternatively, access to receptors used by rHIV.ShanghaiN9 (potentially $\alpha 2,6$ residues) is enhanced following sialidase S treatment, an observation that would support the increased transduction by this pseudotype observed in MDCK-Siat1 cells (Figure 50 C).

Overall, these results suggest that despite different transduction efficiencies observed in B-ALI cultures (Figure 41), all three pseudotypes depend on sialylated receptors to mediate transduction in this model. Additionally, although the lectin staining itself was somewhat variable between donors, the reduced transduction efficiency in sialidase S treated B-ALI

cultures supports the use of α 2,3 sialylated receptors (MAI stained; α 2,3 sialylated LacNAc) by the F/HN pseudotype.

6.2.8. Investigating use of lectins to block receptors mediating transduction of SALI cultures by F/HN pseudotype

Given the lectin staining/receptor profile characterised for SALI cultures, which revealed only expression of MAI stained receptors in abundance (Figure 46), it was thought that this model might be appropriate to confirm the use of α 2,3 sialylated LacNAc receptors to mediate F/HN transduction. Highly efficient transduction of this model (Figure 43) already supports F/HN use of this receptor, but to confirm this receptor is responsible for the observed transduction it was hypothesised that pre-treatment of SALI cultures with unconjugated MAI lectin could block F/HN interaction with this receptor subtype and reduce transduction. To investigate, SALI cultures, untreated or pre-treated (as described 2.11.4) with MAI lectin diluted to a range of concentrations were transduced with rHIV.F/HN.CMV.EGFP as before (5.2.3.3). MAII and SNA were also used to pre-treat SALI cultures in order to confirm that any potential blocking effect achieved by MAI was receptor-specific and that there were insufficient MAII and SNA receptors available in this model to facilitate transduction.

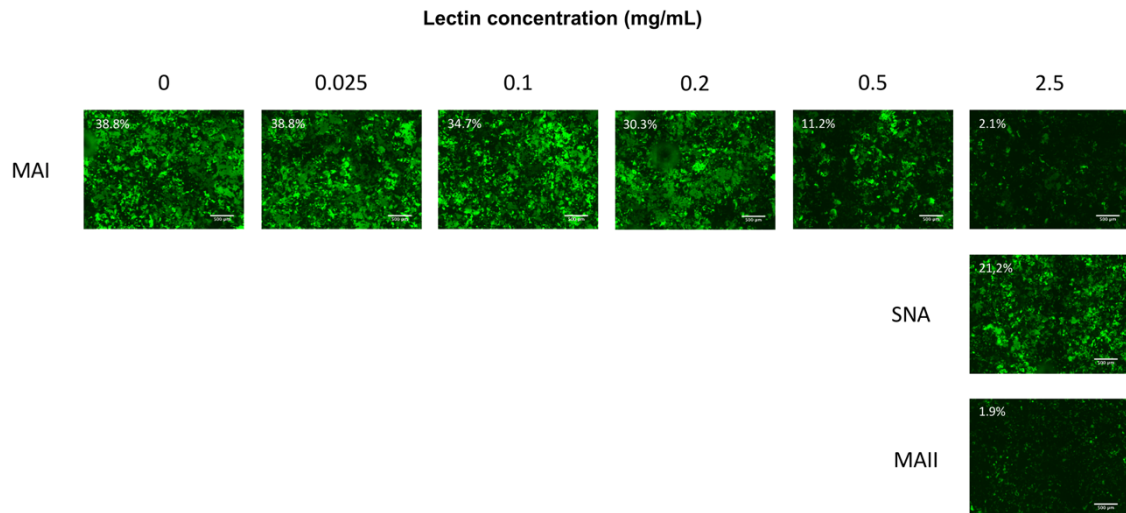


Figure 56- Concentration dependent lectin blocking of F/HN transduction in SALI cultures. SALI cultures, untreated, or pre-treated with unconjugated lectins (MAI, SNA or MAII) at various concentrations for 1 hour, were transduced apically with 7.5×10^7 TU of rHIV.F/HN.CMV.EGFP (vGM182 Wave 96) and cultures imaged to detect eGFP fluorescence 4 days later. Representative, stitched centre-of-transwell images are shown and EGFP quantified by % area above threshold indicated, scale bar represents $500 \mu\text{M}$.

EGFP expression captured and quantified by percentage area above fluorescence threshold (as described 2.11.5 and 2.11.6) 4 days following transduction revealed a concentration dependant block by MAI (Figure 56). In the absence of lectin 38.8% of the cells were transduced while 2.1% transduction was observed following MAI pre-treatment at the maximum concentration (2.5 mg/mL). SALI cultures pre-treated with an equivalent concentration of SNA resulted in much greater EGFP detection at 21.2%, in line with the very low levels of SNA staining in this model (Figure 46). However, this is a reduction compared with cultures transduced in the absence of lectin and could indicate either some non-specific lectin binding at this concentration, or the presence of $\alpha 2,6$ receptors on the SALI cultures mediating transduction by F/HN. In addition, there was an unexpected (equivalent) blocking achieved by MAII lectin, similar to MAI at this maximum concentration, even though there was a low intensity of MAII staining compared with MAI (Figure 46). This indicates either a non-specific

sialylated receptor blocking by lectins at this concentration, or the presence and use of the α 2,3 ganglio-series receptors by F/HN to transduce SALI cultures.

6.3. Discussion

6.3.1. Alternative receptor distributions modelled and their relationship to human lung

Lectin staining was used to compare sialylated receptor distribution in different airway cell models in order to understand receptor use by the different LV pseudotyped vectors. The literature describes many studies of lectin staining, however they often provide contradictory and incomplete datasets. It was decided, therefore, to improve the consistency of the multiple tissue processing steps and to perform lectin staining in parallel wherever possible, to maximise comparability between studies. This should give greater confidence in interpretation of the findings.

In human lung tissue, lectin staining showed a relative intensity of MAI>SNA>MAII (Figure 45) in line with published reports using these lectins (Jia et al., 2020; Nicholls et al., 2007). A key difference observed between the lectin staining of human lung and the commonly used mouse lung model was the intensity of MAII staining; the minimal MAII staining in human lung is in contrast to the abundant MAII staining in mouse lung (MAI>MAII>SNA) (Figure 45) and highlights a crucial concern in the use of murine airway to model human tissue. Importantly, however, the robust MAI staining of both human and murine lung indicates the presence of a common receptor (α 2,3 sialylated LacNAc) shared between these two species, a finding that has not previously been reported using the MAI lectin isolate alone (J. P. Guo et al., 2011; Ibricevic et al., 2006; Ning et al., 2009). The presence of both MAI and MAII stained α 2,3 receptors in the mouse model, obscures the identification of the specific α 2,3 subtype being used as a receptor. In particular, the lack of MAII stained (α 2,3 ganglio-series) receptors in human lung could explain the relatively inefficient transduction by rHIV.Rostock in human ALI models (Chapter 5), despite

published studies demonstrating efficient airway transduction by this pseudotype in mouse (McKay et al., 2006; Patel et al., 2013). Indeed, a preferential use of the MAII stained α 2,3 sialylated ganglio-series receptors, rather than (MAI stained) α 2,3 LacNAc (neolacto-series) receptors, is supported by binding studies with FPV/Rostock H7N1 (1.5.2.1), where there was a 7-fold greater binding of using GD1a versus Sialyl-3-paragloboside (see Figure 6)(M N Matrosovich et al., 1997).

The strong SNA staining observed in human airway and parenchyma (Figure 45) is consistent with extensive literature describing widespread distribution of α 2,6 sialylated receptors throughout human respiratory tract. For this reason, the ferret, similarly rich in α 2,6, is widely used to model viral infections of human lung. Staining of ferret airways captured within PCLS here however indicates expression of all three receptor subtypes including the α 2,3 ganglio-series receptors (MAII), not found in human airway. This suggests that the transduction of ferret PCLS airway by F/HN (Chapter 4) cannot confirm use of receptor subtypes available in human airway. In pig PCLS however, the greatest intensity of SNA staining for α 2,6 sialylated receptors was observed in the airway and parenchyma (SNA=MAI>MAII) (Figure 45). This, together with the lack of MAII staining, suggests that the pig lung PCLS may be the most relevant model for evaluating LV transduction of human airway. If correct, the efficient F/HN transduction of pig airway and parenchyma in PCLS (Figure 30), as well as *in vivo* (Gerry Maclachlan, personal communication), supports the potential of this pseudotype for efficient transduction of human airway and lung. Further supporting this notion, along with the identified transduction of human parenchymal tissue *ex vivo* (4.2.2), is the abundance of MAI staining throughout the airway and lung parenchyma in all models investigated and transduced by F/HN (Figure 45) in line with the binding of α 2,3 neolacto receptors by Sendai virus from which F/HN is derived (T. Suzuki et al., 2001).

Lectin staining of human airway ALI culture transwell cryosections showed a relative intensity similar to that observed in the human airway (MAI>SNA>MAII). There were some

differences between B-ALI cultures generated in-house and Epithelix ALI cultures obtained commercially however (Figure 46 A & B), in particular, the relative MAI and SNA staining intensity of B-ALI cultures was most similar to the human airway stained here. Following human lung N-glycan characterisation using mass spectrometry (Jia et al., 2020), it is speculated that the α 2,6 sialylation subtype is most abundant, however without isolation and full characterisation of glycans expressed on airway ALI cultures, it is not possible to confirm which of these culture types most accurately represents the receptors in human lung. Importantly however, a lack of MAI staining in all these ALI models (Figure 46) suggests they are helpful to investigate transduction in the absence of α 2,3 ganglio-series receptors, which are abundant in many other models/species (De Graaf & Fouchier, 2014; Ibricevic et al., 2006; Ning et al., 2009), and tends to obfuscate the use of α 2,3 receptor subtype.

The greater intensity of SNA staining of Epithelix cultures (Figure 46 B) indicates a greater proportion of α 2,6 sialylated LacNAc glycans, in contrast to the greater proportion of α 2,3 sialylated LacNAc (MAI) in B-ALI cultures (Figure 46 A). This could explain low transduction efficiency in the Epithelix ALI model by LV pseudotypes derived from viruses that preferentially target α 2,3 sialylated receptors (F/HN & Rostock). Further, differences in lectin staining between ALI cultures suggest variable cell-specific receptor subtypes found on ciliated versus non-ciliated cells, which could explain inconsistencies in published reports (Ibricevic et al., 2006; Mikhail N. Matrosovich et al., 2004). This is also in line with the observed transduction of all cell-types in the airway ALI cultures, by all three pseudotypes (Figure 40 & 42).

One of the most important disease targets in the human airway is Cystic Fibrosis and there has been some suggestion that sialylated glycan expression in the CF airway is reduced compared with healthy airways (Rhim et al., 2001). Interestingly, there was no observed differences in lectin staining of CF and healthy MucilAir cultures, and also no impact on F/HN transduction efficiency (Figure 38), possibly as a result.

The absence of SNA staining in SALI cultures (Figure 46 C) evidences a clear difference in receptor profile compared with the airway ALI cultures (Figure 46 A & B), which could explain the reversed transduction efficiency of Rostock and ShanghaiN9 pseudotypes between these models (Figure 41 versus Figure 43). The greater availability of α 2,6 sialylated receptors in airway ALI cultures (Figure 46 A & B), correlates with greater transduction efficiency of the ShanghaiN9 pseudotype (Figure 41), supporting a hypothesis that α 2,6 sialylated residues could act as receptors for this pseudotype given the WT virus HA binding characterised (3.1.1)(Yang et al., 2013). With detection of Sendai virus affinity for α 2,6 neolacto receptors also described by Suzuki et al. (2001)(1.5.1.1), the reduced fold difference in transduction efficiency between F/HN versus Rostock in SALI cultures compared to B-ALI (40-fold from 300-fold), could additionally support the potential use of α 2,6 sialylated receptors in B-ALI by the F/HN pseudotype, in contrast to Rostock.

6.3.2. Different affinities and receptor use between pseudotypes

The use of hemagglutination inhibition to measure binding of free glycans facilitates comparison of various pseudotypes, but a number of factors need to be considered before one can equate hemagglutination inhibition with receptor binding mediating transduction. First, although all pseudotypes investigated bind to sialylated receptors (and thus possess RBC hemagglutinating activity), they can also cleave sialic acid to release this binding (via neuraminidase activity). This means that interpretation of hemagglutination inhibition studies may be more complicated for LV pseudotypes due to the unknown ratios of neuraminidase and hemagglutinin activities in a given pseudotype, which in turn could be affected by the incorporation of the neuraminidase and hemagglutinin glycoproteins during LV production. Secondly, the inhibition of hemagglutinin binding may be influenced by specific cellular receptors on the RBC competing with the free glycans, such that the relative affinity for the glycans versus RBC receptors could impact the inhibition. In contrast to horse/cow erythrocytes however, the chicken RBC used

here have been shown to facilitate hemagglutination by a broad range of avian and human influenza viruses, as they harbour a mixture of both $\alpha 2,3$ and $\alpha 2,6$ sialylated receptors (Ito et al., 1997). The low variability in results observed between batches of RBC supports the notion that there were minimal differences in the proportions of receptors expressed.

Binding of free glycans corresponding to $\alpha 2,3$ sialylated LacNAc receptors was demonstrated for both F/HN and Rostock pseudotypes, and is in agreement with binding characterised for the parent virus from which each pseudotype is derived (using Sialyl-3-paragloboside in solid phase (M N Matrosovich et al., 1997; T. Suzuki et al., 2001). Although binding of each pseudotype could not be compared with GD1a to confirm affinity relative to ganglio-series receptors, significant binding detected for $\alpha 2,3$ versus none for $\alpha 2,6$ sialylated LacNAc equivalent glycans is in agreement with the approximate 10-fold lower affinity of Sendai for $\alpha 2,6$ sialylated LacNAc equivalent glycans (T. Suzuki et al., 2001), and the minimal 6'-Sialyl-LacNAc binding detected for WT FPV/Rostock (M N Matrosovich et al., 1997). Although the relative preference between ganglio-series and neolacto-series $\alpha 2,3$ sialylated receptors described for Sendai virus is the opposite of that for FPV/Rostock (1.5.2.1), the relative affinity for these $\alpha 2,3$ (or $\alpha 2,6$) sialylated receptors between the viruses is unknown.

Here, hemagglutination inhibition was achieved with 16-fold less LSTd glycan for F/HN than Rostock (and was not achieved for ShanghaiN9) at matching HA units (Figure 47), indicating a greater affinity of F/HN for $\alpha 2,3$ sialylated LacNAc receptors and could explain the greater relative efficiency of this pseudotype across all ALI models investigated, given the abundance of MAI staining (Figure 46). Although no difference between F/HN and Rostock affinity for 3'-Sialyl-LacNAc (also modelling $\alpha 2,3$ sialylated LacNAc receptors) was observed, glycans in the human lung tend to occur most frequently with repeated (poly) LacNAc units (Jia et al., 2020), indicating LSTd as most representative of the receptors 2,3 sialylated LacNAc receptors that would be available.

Additionally, the relative efficiency between all three pseudotypes transducing SALI cultures (staining with MAI only), correlates with this relative affinity for LSTd. The hypothesised greater relative affinity of the ShanghaiN9 pseudotype for α 2,6 sialylated residues to explain the contrasting greater efficiency of ShanghaiN9 versus Rostock in the BALI cultures compared with SALI, could not be confirmed however. This could indicate either a failure to model the α 2,6 receptor used by ShanghaiN9, or be explained by a broader range yet lower affinity for the specific receptors modelled here.

The inability to detect binding of any of the pseudotypes to α 2,6 sialylated glycans using hemagglutination inhibition, was contrasted with inhibition of F/HN transduction by 6'-Sialyl-LacNAc (Figure 48). This could reflect a lack of sensitivity in the hemagglutination inhibition assay, an expected reasonable limitation for any assay dependant on detecting a block of particle binding activity in order to detect candidate glycan binding. Despite this, the binding affinities characterised using hemagglutination inhibition (Figure 47) clearly support the preferential use of α 2,3 sialylated receptors for transduction by both F/HN and Rostock pseudotyped LV. Use for transduction was indicated for F/HN by restored transduction of MDCK-Siat1 cells using α 2,3-sialyltransferase (Figure 51 B & C), and indicated for both pseudotypes in MDCK-Siat1 cells and B-ALI cultures by reduced efficiency after sialidase S treatment to remove α 2,3 sialylated receptors (Figure 50 C & Figure 55). Additionally, the lack of 3'-Sialyl-LacNAc or LSTd binding observed with ShanghaiN9 (Figure 47), is in agreement with no observed reduction in transduction efficiency by this pseudotype following sialidase S treatment (Figure 50 C & Figure 55).

The impact of sialidase S treatment on the receptor availability across the cell/models treated as determined using lectins was unclear. Although sialidase S is widely used in literature as a diagnostic tool to reliably cleave α 2,3 sialylated receptors (6.1.5), the impact of this activity on the availability or access of alternative receptors on cells may be complex. An expected reduction in α 2,3 sialylated receptors, indicated by reduced MAI/MAII staining, was observed

following treatment in mouse and B-ALI culture (Figure 52 & 54). However, an unexpected increase in SNA staining was observed in mouse and MDCK-Siat1 cells (Figure 52 & 50). This implies an increase in availability of, or access to, α 2,6 sialylated receptors for binding by SNA. This could be explained either by re-sialylation of cleaved receptors by endogenous α 2,6-sialyltransferase activity in the cells or, alternatively, by cleavage of α 2,3 sialylated residues that enhance lectin binding/access to α 2,6 residues. This greater access for binding could additionally explain the potential increase in ShanghaiN9 transduction efficiency observed in MDCK-Siat1 cells and some of the sialidase S treated B-ALI culture transwells (Figure 50, Figure 55). A receptor 'unmasking' effect using mild broad-specificity sialidase treatments has also been speculated by others previously (6.2.5.2 (Wassilewa, 1977)).

In the mouse airway, *in vivo* sialidase S treatment was shown to result in cleavage of both α 2,3 subtypes by poor/absent subsequent MAI and MAII lectin staining (Figure 52). This cleavage however did not correlate with any clear reduction in efficiency of transduction by rSIV.F/HN (Figure 53). This could indicate that minimal receptor numbers are required to achieve transduction or support the notion that F/HN is able to also use α 2,6 sialylated receptors to mediate transduction in the mouse airway. Even though SNA staining of the untreated mouse lung indicates absence of α 2,6 (Figure 45 & Figure 52- buffer only), it could be possible that α 2,6 residues are unmasked following sialidase S treatment, facilitating both the maintained F/HN transduction efficiency and SNA staining observed. This observation, together with the restored transduction of asialo MDCK-Siat1 cells following α 2,6-sialyltransferase treatment, supports the potential for F/HN to use α 2,6 sialylated receptors, in addition to α 2,3.

Furthermore, although the reduced efficiency of F/HN and Rostock in MDCK-Siat1 cells and B-ALI cultures after sialidase S treatment indicates both pseudotypes use α 2,3 sialylated receptors on these cultures, the residual transduction efficiency relative to cultures transduced without sialidase S pre-treatment, was greater for F/HN. This could reflect a contrasting

potential for F/HN to use of α 2,6 sialylated receptors, versus an inability by Rostock, in line with lack of FPV/Rostock binding to 6'-Sialyl-LacNAc (M N Matrosovich et al., 1997).

6.3.3. Conclusion

These data highlight the importance of selecting an appropriate model in which to assess pseudotypes that utilise sialylated receptors to mediate gene transfer. It was shown here that receptor subtypes are distributed differently depending on the species and model. The F/HN pseudotype binding and use of α 2,3 sialylated LacNAc receptors could explain its efficient transduction across all models investigated and indicates that this pseudotype has potential for efficient transduction of human lung. Although Influenza-based pseudotypes may offer alternative receptor targeting, the affinity determined using equivalent hemagglutinating units suggests no advantage for these pseudotypes over the F/HN in affinity for the sialylated LacNAc residues that are the most abundant in the human lung.

7. Chapter 7- Discussion

Since the birth of gene therapy as a concept to treat genetic diseases nearly 50 years ago (Friedmann & Roblin, 1972), the clinical development of such therapies have been focused on the many challenges faced in ensuring safety alongside efficacy (Dunbar et al., 2018).

Recombinant LV, have emerged as a promising vector delivery system (after extensive modifications to mitigate toxicity risks), whilst maintaining a number of key advantages over alternative viral vectors being developed. In particular, the lack of pre-existing immune response and the ability to introduce large transgenes permanently into the genome of a cell, independent of whether it is dividing or non-dividing, offers the potential to facilitate expression of transgene for the lifetime of the cells, without loss upon mitosis. Although LV's are becoming readily used *ex vivo* in the clinic (1.3), these vectors are yet to be fully exploited for *in vivo* therapies. Importantly, appropriate pseudotyping of LV to achieve efficient delivery to disease-relevant cell types, upon either systemic or localised delivery *in vivo*, will be crucial in achieving efficacy and providing additional safety.

The lung, as a target organ, offers non-invasive and localised access for *in vivo* delivery by LV. Utilising appropriate pseudotypes to mediate efficient and/or specific transduction of pulmonary cells would pave the way for LV to treat many diseases of the lung. Representing a leading cause of death in the UK, lung conditions are under the spotlight since the SARS-CoV-2 virus (COVID-19) pandemic due to the development of more severe disease in these patients (Office for National Statistics, 2021). Although many target lung conditions are not necessarily hereditary, the prevalence of chronic lung diseases (cancer, pulmonary fibrosis, emphysema, COPD, etc) inspires great interest in the potential for long-term expression of a therapeutic protein from cells transduced using LV.

Faced with the many barriers of the respiratory epithelium (1.4.1), LV pseudotyping for efficient targeting of lung cells has been a well-recognised endeavour. Of the LV pseudotypes

developed over the last 20 years, only those pseudotypes able to actively target the carpet of glycosylation (the glycocalyx) that defends the lung, and to exploit it as a receptor for cell entry, have demonstrated transduction efficiency in the absence of adjuvant *in vivo* (1.4.2). Otherwise, this highly charged glycan coating is inhibitory to achieving efficient gene delivery using viral vectors, such as adenovirus (Stonebraker et al., 2004). The F/HN and Rostock LV pseudotypes are based on the hemagglutinating (sialic acid-binding) Sendai virus and influenza virus, respectively; both preferentially binding α 2,3 sialic acid subtypes associated with restricted human transmission (1.5.1.1 & 1.5.2.1). Importantly, no pseudotype has been investigated to date that is based on a hemagglutinating virus with a recognised human pathogenicity, or a specific affinity for the α 2,6 subtype of sialic acid residue prevalent in human lung. Here, for the first time, the F/HN and Rostock pseudotypes have been compared in multiple human lung models, alongside a new pseudotype based on a human transmissible and pathogenic influenza virus strain (Shanghai).

7.1. Modelling LV transduction of the human lung

7.1.1. LV configuration is important for detecting transduction mediated by pseudotypes

In order to compare pseudotypes, LVs were generated based on recombinant HIV-1 (3.2.2), the key being the use of a common LV platform and transgene promoter to obtain meaningful data on pseudotype performance. To detect transduction, it is important to use an efficient LV and promoter for the target tissue, in this case lung cells. Therefore, rSIV and rHIV-1 based LV were compared with both hCEF and CMV promoters. It was found that although rSIV vectors could be used to reliably evaluate transduction in the mouse lung, both *in vivo* and *ex vivo* (Figure 13, Figure 28), rSIV transduction of human airway cells in B-AI cultures was significantly restricted, at least 100-fold lower than similar rHIV vectors at equivalent TU doses (Figure 39). This is the

first time that the transduction efficiency of these two LV platforms has been directly compared. Additionally, although robust detection of EGFP transgene in the mouse lung was mediated by the hCEF promoter (3.2.1.1 & 4.2.1.1), this promoter resulted in lower levels of native EGFP fluorescence compared with CMV in human airway cells (5.2.2.3). This was unexpected, given published reports of greater expression from hCEF than with CMV (and EF1 α) in ALI cultures transduced with rSIV.F/HN (Alton et al., 2017); although an unspecified dose was delivered to the ALI cultures in those studies (between 6e7 and 3e8 TU per culture) that could have confounded the comparison of transgene promoters.

The selection of an appropriate promoter to facilitate transgene expression is crucial but, more importantly, the implications of rSIV restriction in human airway cells could be significant for the use of rSIV-based vectors for gene delivery to the human lung. Investigation into the mechanism by which such restriction could occur will be fundamental to understanding how this might impact future clinical translation of this gene delivery platform. Experiments in MucilAir cultures confirmed a restriction (~20-fold) of rSIV transduction in airway ALI cultures (Figure 39 B); this is in line with a relatively poor rSIV.F/HN transduction of MucilAir cultures observed by others; large doses/high multiplicity of infection were required to detect transduction, even when using very sensitive (luciferase) assays of transgene activity (Alton et al., 2017; Griesenbach et al., 2012; Mitomo et al., 2010). The apparent rSIV restriction identified here (Figure 39 B) manifested as a reduced abundance of transduced (EGFP-expressing) cells across differentiated airway ALI cultures, however, the degree of restriction could also be related to airway cell type. For example, the restriction in the MucilAir cultures was of lower magnitude (Figure 39) and could imply the cells transduced in these cultures include proportionately fewer cells that are restrictive for rSIV transduction, warranting further investigation. Importantly, these types of restriction mechanisms might be overcome by saturating amounts of LV administered *in vivo* (Hatzioannou, Cowan, Goff, Bieniasz, & Towers, 2003).

7.1.1.1. rSIV is restricted in human airway cell cultures

Many known restriction factors, such as those discussed in Chapter 1 (1.2.3.1), could be responsible for a difference in performance of the rHIV and rSIV-based platforms in human airway cells. Factors such as APOBEC3 and BST-2, which are involved in LV production, are less likely to be involved in a species-specific restriction of rSIV-mediated gene delivery. However, restriction factors such as NONO or TRIM5 α that recognise lentivirus capsid, could trigger an rSIV-specific immune response, or prevent uncoating in these human airway cells, whereas the HIV capsid may escape recognition. It is known, for example, that human TRIM5 α is only weakly restrictive of HIV-1 (Stremlau et al., 2004), yet is restrictive against capsid from alternative species such as EIAV (F. Zhang et al., 2006). Although Zhang *et al* present evidence that human TRIM5 α does not itself restrict SIVagm (at uncoating), other TRIM proteins could be relevant, such as TRIMCyp, shown to potentially inhibit an SIVagm (Tantalus strain). TRIM34 has also recently been shown to restrict SIVagm (and SIVsmm) in human monocyte-like cells (Ohainle et al., 2020). As discussed previously (1.2.3.2), NONO affinity to interact with HIV-1 capsid is lower than HIV-2 which is poorly pathogenic (Lahaye et al., 2018), and therefore could similarly play a role in restricting host range. SIVagm, as a closer relative of HIV-2, might perhaps be similarly recognised by NONO with a greater affinity than HIV.

The lentiviral accessory proteins/genes removed from rSIV and rHIV gene delivery vectors could also be of different importance in overcoming host restriction in these cells. Although some of the activities related to Vif (suppressing APOBEC3G) and Vpu (promoting virion release in HIV) seem unlikely to impact the relative transduction/integration observed here, Vpr and Nef are involved in combatting many different antiviral processes. Vpr, for instance, plays an important role in facilitating integration in non-dividing cells (Heinzinger et al., 1994) and Nef enhances infectivity by downregulation of numerous inflammatory pathways (Wonderlich, Leonard, & Collins, 2011). Overall, there are several candidate human restriction factors, but their potential to restrict SIV and rSIV is not well characterised; these results

highlight the importance of this gap in our knowledge, with significant implications for adopting LVs based on non-human viruses. Evaluating rSIV vectors using various accessory genes to overcome/understand the restriction observed, could be valuable to inform the translational potential and optimal design of these LVs.

7.1.1.2. The EGFP transgene can be used to inform qualitative and quantitative transduction

Expression of EGFP transgene was detected using native fluorescence from transduced cells in all pulmonary models investigated, where the LV platform and promoter allowed. In the mouse lung following *in vivo* delivery, detection of native EGFP fluorescence was exploited, in combination with fluorescence microscopy image stitching, to assess the distribution of transduced cells throughout the lung. This method (3.2.1.2) mitigates notoriously unreliable IHC protocols to detect transduced cells, and also facilitated a systematic, high-throughput quantification using 'area of fluorescence' to conservatively estimate the percentage of cells transduced (Figure 14). In addition, this method allows for both qualitative and quantitative assessment of transduction using a single transgene, whereas traditional quantitative methods (often using substrate-dependent reporter transgenes) fail to inform on the distribution of, or specific cell types driving, expression. Further, using the percentage area method, quantification of parenchymal cell transduction could be achieved, whereas previously no estimates were available. Quantification by counting individual cells in airways has been used by others to estimate that approximately 15% of airway cells were transduced following an 8×10^8 TU dose of rSIV.F/HN (Alton et al., 2017). Here, using the same LV, although approximately 40% EGFP area was quantified from the airways of mice treated with $1-3 \times 10^8$ TU (Figure 13); using an alternative LV production batch, only ~7.5% EGFP area was quantified from a 1×10^8 TU dose; which was determined to be equivalent to ~13.6% of airway cells (Figure 14). This variability in airway

transduction could result from limited TU assay precision, but may also reflect the impact of different methods of LV production and titration, suggesting that comparison of LV transduction efficiency can only be realised using LV produced and titrated using similar protocols. Such variance is further implied by the lack of EGFP detected following delivery of rHIV.CMV and rHIV.hCEF vectors (Figures 16 & 18), despite the efficient transduction of mouse airway using a lower TU dose of rHIV.Rostock (Patel et al., 2013) as discussed (3.3.2). Following rSIV.F/HN delivery, the reduced EGFP percentage area quantified from parenchymal tissue versus airway (Figure 13 B) indicates stronger tropism for airway cells, not identified previously for the F/HN pseudotype.

7.1.2. Different models predict alternative aspects of human lung transduction

7.1.2.1. The mouse model can be used to investigate LV transduction and long-term expression *in vivo*

To predict transduction of human lung, the use of an *in vivo* model is widely considered the most useful for evaluating gene delivery and expression, comprising a rich diversity of target cell types. In addition, clusters of EGFP-positive cells identified in the airways of mice at 1-year following transduction (indicative of transduced cell proliferation; Figure 23), were also observed following polidocanol-induced regeneration of transduced airway epithelium (Figure 25 C & F). This supports the use of the *in vivo* mouse model in conjunction with polidocanol to investigate progenitor cell transduction and long-term expression.

In the context of modelling human infection by hemagglutinating viruses such as influenza, the mouse model is recognised as flawed, however, being rich in α 2,3 sialylated residues, yet lacking in the α 2,6 residues present throughout the human respiratory tract (Ibricevic et al., 2006). For this reason, use of the mouse model alone to evaluate transduction

by hemagglutinating pseudotypes was avoided. Although an abundance of a subtype of $\alpha 2,3$ sialylated receptors was recognised more recently in human lung (MAI-stained neolacto series), the potential overlap in the availability of these receptors between mouse and human required further characterisation in the mouse lung. Studies presented in Chapter 6 used MAI lectin to determine the presence of this subtype, indicate an abundance throughout the murine airway and parenchyma (Figure 45), in common with human. However, staining with MAII also showed a similarly widespread distribution of the alternative $\alpha 2,3$ (ganglio-series) subtype in mouse airway and parenchyma (Figure 45), which is almost absent in the human airways and only detected diffusely in the parenchyma. Thus, it is difficult to determine whether any hemagglutinating LV successful in transducing the murine airway, is using either MAI or MAII stained $\alpha 2,3$ receptors.

7.1.2.2. PCLS can model *in vivo* transduction of alternative species

The use of the PCLS *ex vivo* model was chosen to facilitate study of LV transduction of lung tissue from different species (including human) that would otherwise not be feasible to investigate *in vivo*: however, the ability of *ex vivo* transduction of PCLS to model the *in vivo* setting was not known at the outset of these studies. Importantly, a similar transduction efficiency and distribution was apparent for both rSIV and rHIV vectors in the mouse lung PCLS (Figure 28 & Table 8) compared to results obtained *in vivo* (Figures 11 & 16), indicating that transduction of PCLS can model *in vivo* transduction (4.2.1.1). Furthermore, the use of PCLS from different species revealed alternative patterns of sialylated receptor distribution; for example, identifying the availability of $\alpha 2,6$ sialylated receptors to model human lung, not present in mouse. It was shown that the tissue captured in pig PCLS best represented the availability of receptors in human lung (Figure 45 & 6.2.1.4). Conversely, the airways captured in ferret PCLS were less

representative of human airways than anticipated, with MAII staining detected (Figure 45). Though this MAII staining indicates a confounding presence of the $\alpha 2,3$ (ganglio-series) subtype of receptor in these ferret airways, as also seen in the mouse but not human (7.1.2.1), this could reflect the distal nature of airways captured within ferret PCLS. In contrast, no staining with MAII (or MAI) was detected in proximal ferret airway epithelia (bronchial or tracheal) by Q. Xu et al. (2010). This could indicate a distinction in the receptors available between proximal and distal airway epithelia, or, alternatively, the observations could simply reflect the challenging nature of receptor detection using lectin binding.

7.1.2.3. ALI cultures can model human airway epithelium sialylated receptor availability

Cultures of human airway cells grown at an air-liquid interface are widely used as a model of the human airway (5.1.1). These were used here specifically because PCLS generated from human lobectomy tissue did not capture discernible airway structures (4.2.2). Subtle variations between ALI culture types were anticipated due to small differences in ALI culture protocols. However, despite the minimal differences identified between the commercially obtained (Epithelix) MucilAir and SmallAir cultures (for example, the TEER; Figure 33 and the rHIV.F/HN transduction efficiency; Figure 38 only), numerous unexpected differences were observed compared with in-house generated B-ALI cultures. B-ALI cultures generally displayed a reduced club cell frequency, reduced abundance of $\alpha 2,6$ receptors (SNA stain), increased abundance of neolacto $\alpha 2,3$ receptors (MAI stain) and facilitated a much greater efficiency of transduction by rHIV.F/HN. Although a relative lack of club cells suggests B-ALI cultures may be more representative of proximal airway epithelia in humans, the receptor staining was most similar to that of the small airway epithelium captured in gifted PCLS generated from human EVLP tissue, and stained in parallel (Figure 45). The relatively poor F/HN transduction efficiency in

Epithelix MucilAir and SmallAir cultures could be due to the reduced availability of (MAI) neolacto α 2,3 receptors (compared with B-ALI cultures); therefore transduction of Epithelix cultures with any hemagglutinating LV particle utilising this receptor subtype may be limited. Further, differences in lectin staining (both quantitative and qualitative) could reflect differences in receptor expression due to a specific culture type or donor; and this could explain some of the confusion in the literature regarding which receptors are expressed by which cell types (5.1.2). These results illuminate the potentially significant effects of using different methods for the isolation, culture, differentiation and maintenance of airway cells at an ALI on the ability to model transduction by rHIV.F/HN. The similarly reduced transduction efficiency of rSIV.F/HN in MucilAir compared with B-ALI cultures (Figure 39) confirms the impact of culture type is not specific to rHIV vectors, although further investigation is required to determine whether this is specific to the F/HN pseudotype.

7.1.2.4. Receptor binding can predict transduction

The complexity of receptor distribution and availability observed in the different models studied here highlights the need to investigate the specific receptor/subtypes that may be used by a given pseudotyped LV. Modelling specific receptor use for transduction is complex, however, since only the binding of pseudotyped LV is measurable using purified molecules. The relationship between receptor binding and the subsequent transduction by a pseudotyped LV is likely dependent on many factors. For viruses, there is evidence that binding does not necessarily predict viral propagation; for example, Mazzetto et al. (2020) showed that despite a lack of influenza D virus *attachment* to sheep trachea (inactivated virus binding to fixed tissue (Van Riel et al., 2007)), the virus achieved efficient replication during explant infection.

Here, LV pseudotype binding to specific receptors was compared with transduction mediated by different receptor subtypes (Chapter 6). The use of free glycans (modelling specific receptors) to inhibit hemagglutination, allowed the relative binding affinity between glycans and

pseudotypes to be determined. The relative affinity between the pseudotyped LVs for the LSTd glycan (MAI, α 2,3 linkage, Figure 44) correlated with their relative transduction efficiency in SALL cultures, which were shown to express only the MAI stained neolacto α 2,3 sialylated receptor subtype (Figure 46 C) modelled by LSTd. This supports the potential to predict receptor use to mediate transduction by pseudotyped LV, via assaying binding interactions.

It could be argued that a binding assay would be more appropriate to predict LV transduction, rather than the replicative infectivity of a virus, with the multiple receptor-independent processes required to complete a virus life cycle. Using inhibition of hemagglutination to assay binding (6.2.3), a comparison could be made of pseudotype receptor binding/use which is independent of differences in the quality of LV preparations that could influence transduction efficiency (e.g. relative pseudotyping of particles). Differences in LV preparation quality might be implied by the contrast in relative HA unit concentrations compared with TU concentrations determined for the pseudotyped LV preparations used. Although the HA unit concentrations were the same, or differed only two-fold, for each RBC batch (Figure 47), the TU concentrations for these LV preparations differed by a minimum of 3.5-17 fold (Table 4 B; Wave 96, 193 & 191). A different relationship between HA unit and TU concentration for the F/HN versus influenza-based pseudotyped LV could be anticipated, given the alternative glycoproteins and mechanism involved in mediating both binding and transduction. However, a five-fold disparity in TU concentration, despite only 2-fold HA unit concentration difference between the Rostock and ShanghaiN9 pseudotyped LV preparations used (Wave 193 versus Wave 191; Table 4 B), suggests a qualitative difference between the LV preparations.

By using exogenous application of sialidase enzymes to manipulate the cellular receptors available for transduction (6.2.5-7), it was possible to confirm receptor use to mediate transduction both *in vitro* and *in vivo*. The use of broad-specificity sialidase in this manner, to determine receptor use, is reported previously (Bell et al., 2011; M. A.K. Markwell & Paulson,

1980), however here, the use of sialidase S (6.1.5) was also investigated for the potential to resolve subtype-specific use. It was apparent however that this enzyme could not be used to resolve the impact of α 2,3 sialylated receptor cleavage alone. The interpretation of studies using the sialidase S enzyme was complex since an unexpected relative increase in SNA staining was observed (MDCK-Siat1 cells, Figure 50 A; and *in vivo* mouse, Figure 52). Furthermore, enhanced transduction by rHIV.ShanghaiN9 (MDCK-Siat1 cells, Figure 50 C; and B-ALI cultures, Figure 55), could support greater access to (SNA stained) α 2,6 sialylated receptors. This result supports the complex effects of incomplete sialic acid residue cleavage upon the availability of receptors for use by viral glycoproteins (6.3.2 (Wassilewa, 1977)).

7.2. Performance of the pseudotypes

7.2.1. Evaluating the F/HN pseudotype

Transduction by the F/HN pseudotype was confirmed in all models investigated. In the mouse lung, efficient transduction of airway epithelia without the need for co-administration of adjuvant, was confirmed *in vivo* and *ex vivo*, and a greater tropism for airway versus parenchymal cells was observed. Further, following confirmed transduction of ciliated, club, goblet and ATII cells in mouse lung (Figure 19), a potential trend towards greater transduction of epithelium in secondary bronchi and smaller airways versus primary bronchi was apparent, correlating with a reduced abundance of ciliated cells (3.3.3, Figure 13 B & 20). Although transduction of mouse ciliated cells was identified by native EGFP colocalization using confocal microscopy, colocalization was infrequent given the abundance of both markers in the airways captured (3.2.3.1, Figure 19 A). Interestingly, although the expected transduction of basal cells was not confirmed, likely due to their infrequency (3.2.3.2), high levels of EGFP expression in the airways of mice was observed 1 year after transduction (Figure 22). This could indicate persistent expression from long-lived transduced cells, or expression resulting from transduced

progenitor cell mitosis. Progenitor cell transduction was further suggested by the appearance of clustered EGFP-expressing cells in these mice (Figure 23), as well as those transduced and then subjected to polidocanol-induced regeneration of primary bronchi epithelium (Figure 25). Although similarly clustered EGFP-expressing cells have been previously reported with the F/HN pseudotype following polidocanol stripping of transduced murine nasal epithelium (Mitomo et al., 2010), expression following airway epithelium regeneration had not been determined previously. Further work is required to confirm the clonality of these cell types. It may be possible to subject tissues from these, or longer-term, studies to amplification-free, integration site sequencing (Van Haasteren et al., 2020) to determine the presence of multiple cells with identical integration sites. Nevertheless, the ability of the F/HN pseudotype to mediate transduction of stem/progenitor cells upon sufficient access is indicated by the transduction observed following polidocanol induced injury (Figure 26).

The significantly lower reporter expression in murine primary bronchi versus secondary/smaller airways at 1 year after transduction (Figure 22) has not been reported previously. This possibly reflects a trend towards transduction of non-ciliated cells in more distal airway epithelia in this model (Figure 19 A & 20), as well as a greater rate of epithelial cell turnover in proximal airways with an abundance of terminally differentiated ciliated cells (Rawlins & Hogan, 2008). Potential tropism for transducing non-ciliated airway cells in the mouse lung was in contrast to a trend towards transducing ciliated cells by this pseudotype in human B-AI cultures (Figure 40). There are no published reports of the *human* airway epithelial cell types transduced by the F/HN pseudotype but, here, transduction of all four major airway cell types was observed. Transduction of terminally differentiated ciliated cells could explain the reduction in EGFP expression detected over the 20 weeks investigated (Figure 37), with turnover of cells possibly similar to that in the primary bronchi of mice. In contrast, some indication of stable expression from F/HN transduced PCLS of all species (a minimum of 45 days; Figure 28 &

30), might reflect the capture and transduction of smaller airways/parenchymal cells in PCLS with a lower rate of cell turnover.

The use of α 2,6 sialylated receptors by F/HN remains uncertain. However, transduction by the F/HN pseudotype observed across all models, as well as the relative distribution between airway and parenchymal cells in each, is consistent with the use of α 2,3 sialylated neolacto (MAI stained) receptors as indicated in studies throughout Chapter 6 (Figure 57). The presence of the neolacto (MAI stained) α 2,3 receptor subtype in the human lung, as well as the transduction of human parenchyma, pig airway within PLCS, and human airway cells in B-ALI cultures; strongly suggest that the F/HN pseudotype holds potential for efficient transduction of the human lung. The receptor binding and use indicated here is also in line with the binding preference/affinity characterised for Sendai virus (T. Suzuki et al., 2001) from which the F/HN pseudotype was derived. A similar affinity for α 2,3/ α 2,6 neolacto receptors is reported for human infective hPIV1/3 (1.5.1.1), thus, despite not being transmissible in humans, use of murine Sendai virus glycoproteins for targeting human airway cells appears most promising.

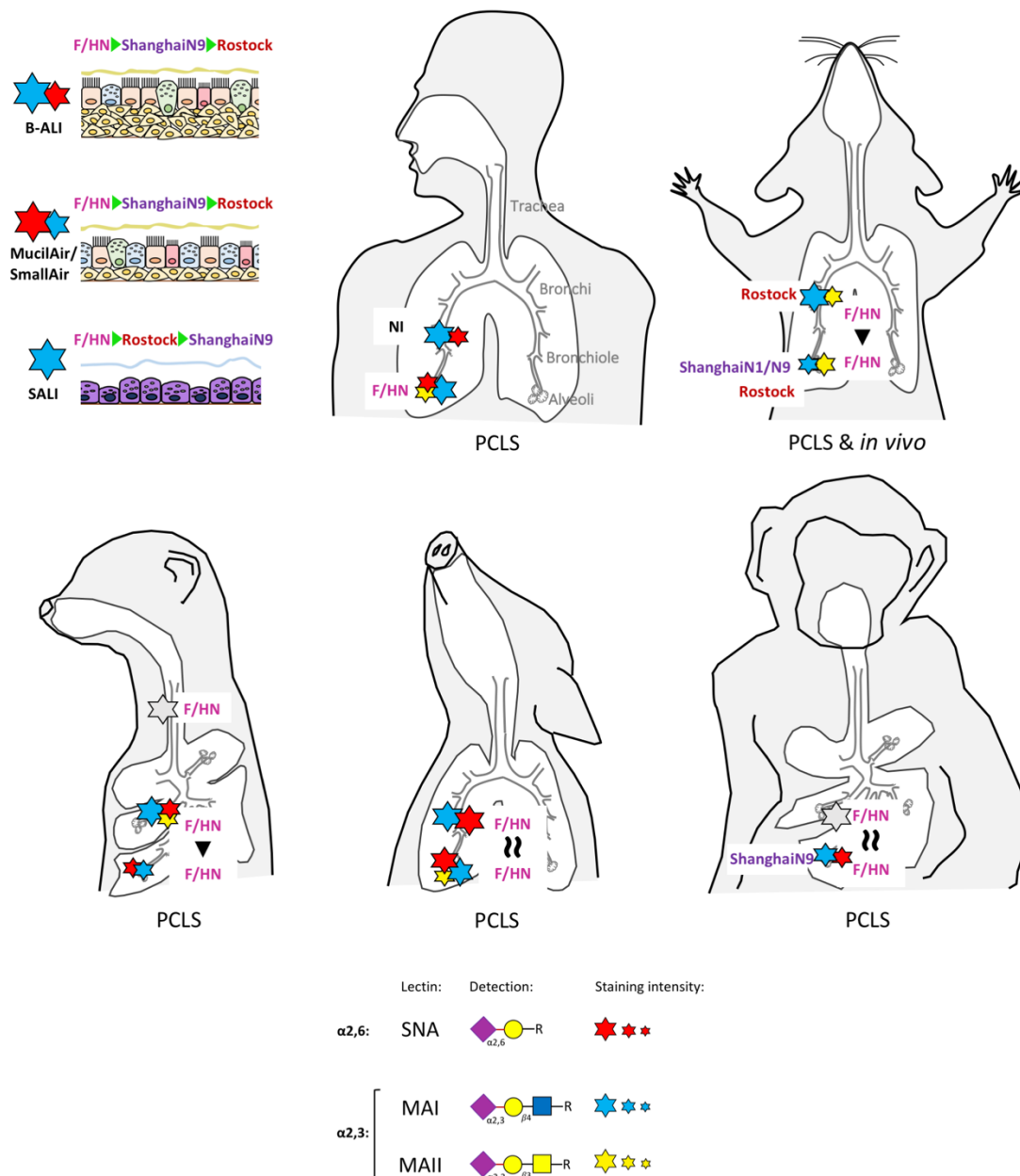


Figure 57- Summary of lectin staining for receptor subtypes across multiple models and transduction by pseudotyped LV. Distribution and relative abundance of sialylated receptors, as determined in by intensity of lectin staining (Figures 45 & 46), is shown by relative size of coloured stars. Red stars: SNA. Blue stars: MAI. Yellow stars: MAII. Grey stars: lectin staining not determined here. Pseudotype labeling indicates detection of transduction. NI: transduction of human airways in within PCLS by pseudotypes not investigated. Where pseudotype transduction of a model could be compared using equivalent TU, the relative efficiency is indicated (green arrow heads). Additionally, where a relative airway versus parenchymal cell transduction patterns were observed for the F/HN pseudotype in PCLS from each species (and mouse lung *in vivo*), relative tropism is indicated between tissues. Black arrow heads: airway cell tropism. Double tilde: similar relative distribution.

7.2.2. Evaluating Rostock and Shanghai influenza-based pseudotypes

Given the success of the F/HN pseudotype in utilising sialic acid to mediate transduction, the development of pseudotypes based on other sialic acid-targeting viruses, such as the intensively studied influenza A virus, remains of high interest. With an ever-expanding register of candidate strains extensively characterised, with particular focus on human infectivity, there is potential to develop pseudotypes with greater, or more cell type or tissue type-specific, targeting efficiency. Detecting transduction by Rostock and ShanghaiN1 or ShanghaiN9 influenza-based pseudotypes was challenging in the models used here, however, transduction of mouse (Figures 16 & 32), macaque (ShanghaiN9 only, Table 8) and all ALI cultures (Figure 41 & 43) was confirmed. Furthermore, similar to F/HN, both Rostock and ShanghaiN9 transduced all major human airway epithelial cells determined in the airway ALI cultures (no club cells modelled, 5.2.3.2). The *in vivo* mouse and PCLS models did not allow comparison of transduction efficiency at equivalent TU doses, with insufficient detection/ transduction efficiency even at maximal TU (Figure 16 & Table 8). Using the airway ALI and SALI cultures however, facilitated comparison of the different pseudotypes, as well as the use of specific receptors. The transduction efficiency of F/HN was superior to both Rostock and ShanghaiN9 pseudotypes across all ALI models (Figure 41 & 43). Furthermore, a greater binding affinity for the α 2,3 sialylated LacNAc receptor (LSTd, MAI stained) was observed for F/HN compared with Rostock and ShanghaiN9 (Figure 47); and considering the robust MAI staining detected across all ALI cultures (Figure 46), the different affinities for this receptor could explain the different transduction efficiencies with these pseudotypes. The impact of receptor availability upon transduction efficiency between the pseudotypes was also suggested by the 'reversed' order of transduction efficiency between Rostock and ShanghaiN9 in SALI cultures (MAI staining only) compared with airway ALI cultures (MAI & SNA staining). Relative transduction efficiency between all three pseudotypes in SALI cultures was in line with their relative affinity for the LSTd glycan (Figure 43 & 47). In contrast, in B-ALI and SmallAir cultures, the ShanghaiN9 pseudotype transduced with greater efficiency

than Rostock (Figure 41). This could be explained by the presence of $\alpha 2,6$ receptors in airway ALI cultures (figure 46), and an expectedly greater affinity of ShanghaiN9 for this subtype. Use of $\alpha 2,6$ receptors by ShanghaiN9 is consistent with the maintained, and possibly enhanced, transduction in models after sialidase S cleavage of $\alpha 2,3$ receptors (Figure 50 C, 53 & 55), however, binding could not be confirmed using the hemagglutination inhibition assay (Figure 47).

The large difference in transduction efficiency between F/HN and the influenza-based pseudotypes could be explained by receptor use, as supported by the observed differences in efficiency between ALI cultures with different receptor availabilities (6.3.1 & 6.3.2). However, it is likely that other factors for cell entry and transduction of ALI cultures are also involved. As described previously (Figure 7), the F/HN pseudotype should mediate fusion at the surface of the target cell, with no F glycoprotein dependency on pH, therefore delivering pseudotyped LV into cells in a similar way to lentivirus (Figure 2). In contrast, influenza HA is dependent on reduced pH to facilitate fusion (Figure 8), subjecting LV particles to endocytosis and an acidic endosomal environment. This could be inhibitory to effective transduction in a number of ways, by altering the intracellular pathway of LV post-entry and prolonging PAMP exposure of LV particles to the cells. A recent study identified reduced efficiency of gene delivery by influenza virus, as related to inefficient endosomal uncoating (low pH/high stability HA)(Singanayagam, Zambon, & Barclay, 2019). Therefore, a negative impact of endosomal processing upon transduction by pseudotyped LV could be hypothesised. Pseudotyping LV with influenza M2 could additionally reduce transduction efficiency. For influenza virus, the M2 glycoprotein plays an important role in particle assembly, stabilising the pH-sensitive HA conformation through the golgi (Takeuchi & Lamb, 1994), and in transduction, by lowering pH within influenza particles to achieve disassembly of M1 (Figure 8, 1.5.2). For Rostock pseudotyped LV, however, M2 incorporation results in enhanced yield of infectious LV (McKay et al., 2006), but for LV transduction, M2 is not thought to be required. Indeed, McKay *et al* report with data not shown,

that the use of the M2 channel-blocking drug amantadine following LV production, did not inhibit transduction efficiency. Following endocytosis, M2 could mediate exposure of LV capsid within particles to low pH and in turn disrupt LV capsid function. This could understandably impact transduction given the many restriction factors known to target LV capsid (1.2.3.1 and 1.2.3.2); implicating its important role for infection. Additionally, through equilibrating pH across the LV particle envelope within the endosome, it is possible that M2 actually delays reaching the required low-pH at the particle surface to trigger fusion and facilitate uncoating; this in turn could reduce efficiency in endosomal uncoating and potentially restrict transduction efficiency further.

7.3. Further development of pseudotypes

7.3.1. Improving yield and efficiency of LV pseudotyped with influenza

HA

Production of sufficient quantities of influenza-based pseudotyped LV was challenging (3.2.2). In contrast with the F/HN pseudotype, which uses a single glycoprotein to mediate the binding and cleavage of sialylated residues, influenza-based pseudotypes comprise separate HA and NA glycoproteins. Although supplementation with exogenous neuraminidase did not enhance production (3.2.2.1), optimising the incorporation of both glycoproteins into LV could, in principle, improve the yield of functional LV.

There is also potential to improve both the performance and yield of influenza-based pseudotyped LV through modification of the HA. By substituting the polybasic cleavage site for a monobasic site (1.5.2), HA cleavage and activation would no longer be facilitated by ubiquitous intracellular furin or furin-like proteases during production, and instead this would be achieved only after LV production using exogenously added trypsin-like proteases (as is necessary for F/HN pseudotyped LV). This could be advantageous for functional LV production; since the HA

is not vulnerable to irreversible inactivation (through pH-induced conformational change) prior to cleavage, the chance of incorporating inactivated HA is minimised. This also means that M2 should no longer be important to locally stabilise the pH in the golgi during HA processing. Along with this potential to improve yield of functional pseudotyped LV, pseudotyping without the M2 component could also improve transduction, as M2 may be inhibitory (discussed 7.2.2).

Such a modification may additionally resolve the particular challenge in production of the Shanghai pseudotypes versus Rostock (3.3.2). A minimum five-fold difference in final TU concentration achieved was observed between preparations that were produced and titrated using the same methods (Wave 191 versus Wave 193; Table 4 B), supporting a relative advantage for pseudotyping LV using HA from Rostock (3.3.2). Pseudotyping of HIV-like particles with human IAV HA (H3N2- Aichi/2/68) was shown to be dependent on neuraminidase to facilitate particle release, in contrast with HA from FPV/Rostock, which was efficiently incorporated independently (V. Bosch et al., 2001). This is speculated to be due to the occupation of the Rostock HA binding site with sialylated oligosaccharides attached to an asparagine residue on the Hemagglutinin glycoprotein (positions 123 and 149) (M Ohuchi, Ohuchi, Feldmann, & Klenk, 1997; Masanobu Ohuchi, Feldmann, Ohuchi, & Klenk, 1995). This quirk of the Rostock HA could be an essential adaptation to mediate efficient particle production/propagation during infection, particularly because the Rostock HA cleavage site is polybasic. Activated intracellularly, these HA are vulnerable to mediating superinfection of producer cells upon inefficient release, in contrast to non-activated HA, such as WT Shanghai HA. Given the Shanghai HA used to pseudotype LV here incorporates a synthetic polybasic cleavage site, this could constrain the efficient production of Shanghai HA pseudotyped LV, compared with Rostock HA. The requirement for this adaptation (to occupy the HA binding site) may be less pronounced in the production of LV pseudotyped with monobasic HA, with the non-activated particles unable to mediate superinfection of producer cells.

By pseudotyping particles with non-activated HA (monobasic and M2-independent), there is further opportunity to investigate the impact of HA-pH stability on the efficiency of gene delivery. For influenza virus infection, pH stability is important for dissociation of M1 within endosomes, as well as for maintaining particle infectivity for propagation in low pH environments. For mediating LV gene delivery, however, a less stable HA with a higher pH threshold to mediate fusion could be advantageous by enhancing efficiency of endosome uncoating and reducing the exposure of LV particles to low pH.

Should such modifications to influenza-based pseudotypes result in greater production yield and efficiency, such pseudotypes could be useful if neutralising antibodies against the F/HN pseudotype become limiting for re-administration. Additionally, it might be possible to identify a new HA pseudotype with a narrower receptor tropism, conferring a more cell-type specific transduction profile.

7.3.2. Investigating alternative paramyxovirus for LV pseudotyping

The robust success in achieving high concentrations of F/HN pseudotyped LV, capable of mediating efficient gene delivery across a range of models, supports the use of paramyxovirus-based glycoproteins for LV pseudotyping. The pH-independent fusion follows attachment via a separate HN glycoprotein, thereby achieving balanced receptor binding and cleaving activity independent of LV pseudotyping efficiency. It is likely that both aspects are advantageous to LV pseudotyping and efficient gene delivery. Despite this, relatively few LV pseudotypes based on paramyxovirus glycoproteins have been investigated (Joglekar & Sandoval, 2017). In particular, development of an alternative sialic-acid targeting pseudotype based on human respiroviruses hPIV1 and hPIV3 could offer a specific pulmonary cell-type tropism. With a receptor subtype specificity greater than Sendai virus (T. Suzuki et al., 2001)(1.5.1.1), and some evidence for human airway cell type-specific infection (e.g. with hPIV3 (L. Zhang et al., 2005)), development

of such pseudotypes could offer potential for clinical application, as well as a tool to characterise human receptor subtype availability.

7.3.3. Predicting pseudotype potential for transduction of human lung

The F/HN pseudotype appears well suited to applications that benefit from expression in many cell types in the lung, supported by a broad pulmonary cell tropism observed in multiple models, including those identified to best represent human (7.2.1). There may be some evidence to support a preference for targeting ciliated cells in models of human airway, in line with the indicated availability of (6.2.2), and affinity for (6.2.3), α 2,3 sialylated LacNAc receptors. Such an indication is in contrast with the mouse model, however, highlighting the flaws of over-reliance on this model (7.1.2.1). Further work is required to characterise the full extent of the observed preferences in models, as well as the reality of any pulmonary cell-type specific receptor expression in human lung, and thus potential for cell-type specific targeting of gene delivery.

The studies presented here emphasise the importance of modelling the appropriate *subtypes* of sialic acid receptor to better predict transduction preferences by a given LV pseudotype, using receptor binding interactions (7.1.2.4). This is specifically highlighted by the potential of the F/HN pseudotype for human lung gene delivery, despite being derived from a murine (Sendai) virus that is not adapted to human infection/propagation. It is recommended, therefore, that an assay of receptor binding would be useful to predict the potential of a specific viral glycoprotein for LV transduction. Further, investigating binding of the target tissue/cell types directly using an assay of virus pattern of attachment (7.1.2.4 (Van Riel et al., 2007)) could be particularly advantageous where knowledge of receptor availability in target cells/tissues is limited.

As discussed for the influenza-based pseudotypes (7.2.2 & 7.3.1) there may be many limitations for mediating efficient gene delivery independent of their potential for directing

transduction. This also advocates the use of binding assays, independent of the confounding requirements of efficient transduction, for identifying new candidate pseudotypes.

7.4. Concluding remarks

In this thesis, the aim was to directly compare the potential of the F/HN, Rostock and new Shanghai LV pseudotypes for gene delivery to the human lung. Detecting native EGFP expression, a method was identified to facilitate comparison of transduction efficiency, as well as distribution and types of cells transduced. It was discovered that in human airway cells, efficient transduction was restricted using rSIV vectors versus rHIV, and that the hCEF promoter mediates a lower level of transgene expression compared with CMV. Although the mouse model offers the potential to investigate transduction *in vivo*, as well as long-term expression in conjunction with polidocanol, an alternative availability of sialylated receptors to human lung, with only one subtype (MAI stained) in common, restricts the ability to model human transduction by these LV pseudotypes. The potential to model *in vivo* transduction using PCLS was indicated using mouse lung tissue, and using tissue from alternative species, transduction of pulmonary cells modelling human sialylated receptor availability could be investigated. Although PCLS were limited by airway availability, ALI cultures could model polarised human airway epithelia, including receptor availability. The efficiency of transduction by rHIV.F/HN, as well as the relative abundance of receptor subtypes, appears to be dependent on the method of HBEC isolation and culture at the ALI. Further, by assaying pseudotype binding of glycan receptor analogues, receptor use for transduction could be predicted. There was an hypothesised advantage of the Shanghai pseudotypes via enhanced binding of $\alpha 2,6$ receptors prevalent in the human lung and, in B-ALI and SmallAir cultures expressing this receptor subtype, a greater efficiency was observed compared with the Rostock pseudotype. Nevertheless, the F/HN pseudotype demonstrated superior efficiency and transduced all human airway cell types characterised. The potential of F/HN to mediate transduction of progenitor cells and long-term

expression was also indicated using the *in vivo* mouse model, with efficiency seemingly limited only by access to these cells.

Although there is potential to further improve the influenza-based pseudotypes, the greater affinity of F/HN for α 2,3 sialylated LacNAc receptors, also prevalent throughout the human lung, indicates that the F/HN pseudotype is likely to retain an efficacy advantage for human lung gene transfer. The success of F/HN to transduce a broad range of pulmonary cell-types in all models investigated also indicates a strong translational potential to treat diseases of the human lung.

8. References

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