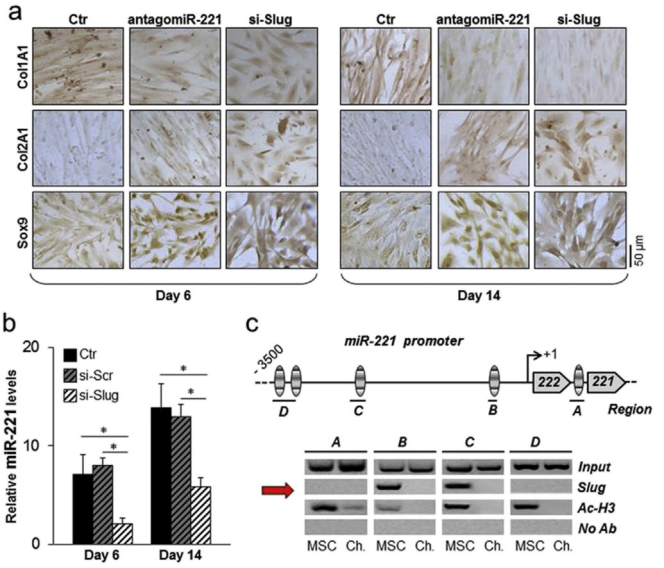
that the efficiency of gene silencing is well maintained for at least 28 days.

Conclusions: miR-221 or Slug silencing in hMSCs induces differentiation towards the chondrogenic lineage, in the absence of conventional chondrogenic inducers, such as TGF-β. Our data demonstrate that miR-221 expression is, in part, regulated by Slug, and support the hypothesis that a novel Slug/miR-221 circuit is crucial for the regulation of the chondrogenic program of hMSCs. We showed that the combination of the engineered hMSCs with 3D-culture conditions and biocompatible scaffolds preserves the efficiency of silencing, particularly for miR-221, demonstrating the feasibility of our approach for the generation of tissue engineering constructs. On-going experiments are aimed at 1. evaluating the ability of miR-221 depleted hMSCs to synthesize cartilage repair tissue in vivo, by using a specific model of osteochondral defect, and 2. determining specific miR-221 targets during the chondrogenic process.



53
UNRAVELING THE ROLE OF snoRNAs IN CHONDROGENIC DIFFERENTIATION

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Purpose: Ribosomes are universally responsible for translating mRNAs into protein. Ribosome synthesis is integrated with many cellular processes including growth, differentiation and oncogenic events. Chondrogenic differentiation and cartilage homeostasis requires a high cellular translational capacity to meet the demands of extracellular matrix production. However, how ribosome biogenesis may control chondrogenic differentiation remains to be determined. Within the nucleolus, snoRNAs regulate the post-transcriptional maturation of rRNAs, and therefore ribosome biogenesis. Two major classes of snoRNAs can be distinguished: box C/D and box H/ACA snoRNAs. By means of rRNA substrate sequence complementarity these snoRNAs post-transcriptionally methylate or pseudouridylate specific rRNA nucleotides, respectively. The precise effect of specific modifications on the function of the mature rRNAs is poorly documented, but they are thought to support RNA folding and interaction with ribosomal proteins. As chondrogenic differentiation is associated with a high demand of protein synthesis, we hypothesized that chondrocyte translation capacity is supported by snoRNA-mediated post-transcriptional fine-tuning of rRNAs.

Methods: ATDC5 cells were differentiated in the chondrogenic lineage. RT-qPCR was used to determine expression of fibrillarin, dyskerin, UBF-1, Sox9, Col2a1, Runx2, Col10a1 mRNAs and 18S, 5.8S and 28S rRNAs. Protein expression of fibrillarin, dyskerin and UBF-1 was determined by immunoblotting. Ribosomal RNA content per cell was addressed by calculating rRNA RT-qPCR signals relative to DNA content (SYBR Green assay). SnoRNA expression during ATDC5 differentiation was analyzed by RNA sequencing of samples acquired from day 0 (progenitor stage), day 7 (chondrogenic stage) and day 14 (hypertrophic stage). Total RNA was isolated and after library preparation (NEBNext library generation kit) inserts <200 nt were selected and libraries were sequenced (Illumina). After normalization, reads that mapped to snoRNAs were analyzed for differential expression and confirmed by RT-qPCR.

Results: UBF-1 (involved in pre-rRNA transcription), fibrillarin (methyltransferase associated with box C/D snoRNAs) and dyskerin (pseudouridylase associated with box H/ACA snoRNAs) expression increased as function of differentiation and displayed highest fold induction at day 5/6 in differentiation. Ribosomal RNA content per cell was significantly increased at day 7, but not at day 14 in differentiation. These data suggest that ribosome biogenesis adapts to the chondrocyte's differentiation status.

To determine whether specific snoRNAs are involved in the cellular adaptation to high translational capacity, RNA-Seq was performed. Expression of at least 224 individual snoRNAs was identified. As a result of initiation of chondrogenic differentiation (Δt0-t7), 21 snoRNAs were differentially expressed (DE; $p<0.05$, 14 box C/D, 7 box H/ACA). Hypertrophic differentiation (Δt7-t14) caused 23 snoRNAs to be DE (16 box C/D, 7 H/ACA). When comparing t0 with t14, 43 snoRNAs were found to be DE (33 box C/D, 10 box H/ACA). To understand the biological relevance of differential expression of snoRNAs, their rRNA target nucleotides were plotted in 2D structures of 18S, 5.8S and 28S rRNA. Plotting revealed that DE snoRNAs, amongst others, target nucleotide modifications in the 28S rRNA peptidyl transferase center and the 18S rRNA decoding center (DC). Snora40 was DE (target: helix 27 of 18S rRNA). Helix 27 partially controls DC function. Helix 68 of 28S rRNA is part of the ribosome's E-site, therefore, DE snord36c and snora31 (which both target helix 68) potentially fine-tune translation by targeting nucleotides in this region. As a final example we found snord46 to be DE (target: helix 69; 28S rRNA), mutations in which have been shown to severely affect cell viability.

Conclusions: Our data show that increased demand for translational capacity during chondrogenic differentiation is associated with differential expression of snoRNAs and potentially controls ribosome fidelity by regulating site-specific rRNA modifications in functionally important rRNA regions. Differentiation-phase specific expression of snoRNAs further suggests that specific snoRNAs may modulate the chondrocyte's phenotype on an rRNA nucleotide modification basis. These data enable us to determine the function of individual snoRNAs in tuning the chondrocyte's translational capacity/differentiation status and current efforts focus on confirming site-specific rRNA modification and study the impact thereof on chondrocyte phenotype.

54
VARIATION IN A SINGLE NUCLEOTIDE POLYMORPHISM IN THE 5'UTR OF GROWTH AND DIFFERENTIATION FACTOR-5 (GDF-5) PREDICTS CLINICAL OUTCOME AT 3 MONTHS FOLLOWING ACUTE KNEE JOINT INJURY

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Purpose: Growth and Differentiation Factor (GDF)-5 is a member of the TGF-beta superfamily, closely related to BMPs. A single nucleotide polymorphism (SNP) rs143383, a C-to-T transition in the 5'UTR of GDF-5, has been associated with osteoarthritis in several populations, and found to be functional (T allele associated with reduced function and increased OA risk)[1]. Joint injury is the major risk factor for osteoarthritis. Few studies have examined the influence of genetic variation on an individual's response to joint injury. We set out to document in a cohort with acute knee injury a) the genotype frequencies at this SNP, and a nearby SNP rs143384 (with which it lies in linkage disequilibrium), and b) whether polymorphism at these sites was associated with early clinical outcomes after injury.

Methods: DNA was extracted from 131/150 participants from the Knee Injury Cohort at the Kennedy (KICK) study who had consented to DNA testing. Primers flanking a 294bp sequence containing were designed, and PCR carried out. The product was checked by electrophoresis, and Sanger sequencing carried out, followed by sequence analysis and genotype allocation for rs143383 and rs143384. Knee Injury and Osteoarthritis Outcome Score (KOOS) was collected at baseline and 3 months.

Results: Participants were young (median age 25), mostly male, largely Caucasian and had substantial impairment and pain by KOOS at baseline (within 8 weeks of their injury, median time to baseline 17 days). For rs143383 genotype of 131 participants, 37 (28%) were TT, 81 (62%) were heterozygotes and 13 (10%) CC. There was no significant difference between the $KOOS_4$ of individuals with TT, TC or CC genotypes at baseline. There were similar increases in $KOOS_4$ in TT and TC individuals over the 3 month period (P<0.0001 for each). In contrast, $KOOS_4$ did not increase significantly over 3 months in CC homozygotes (P = 0.35). A very similar pattern was seen for the rs143384 genotype, in terms of genotype frequency and change in KOOS over time. In a linear regression model of change in $KOOS_4$ over the 3 month period, CC at rs143383 was significantly associated with a smaller improvement in $KOOS_4$ compared with TC/TT (unadjusted coeff. −12.58 (−24.15, −1.0), P = 0.033. When adjusted for other pre-defined explanatory variables (Age, Gender, extent of injury) a significant effect remained for the CC genotype (coeff. −11.89 (−23.48, −0.32), P = 0.044.
Conclusions: Possession of the CC genotype at SNP rs143383 of GDF-5 would appear to be an adverse prognostic factor for early clinical outcome after knee injury in this cohort.This finding should be tested in other joint injury cohorts, in larger numbers of individuals and in other populations. What effect this polymorphism has on subsequent incidence of post-traumatic osteoarthritis remains to be established.

1. Miyamoto, Y. *et al.* A functional polymorphism in the 5' UTR of GDF5 is associated with susceptibility to osteoarthritis. *Nat. Genet.* **39,** 529–533 (2007).

55
AGE-RELATED DNA METHYLATION CHANGES IN NORMAL AND OSTEOARTHRITIS CARTILAGE

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Purpose: Epigenetic alterations are one of the common cellular hallmarks of aging. Several studies have identified CpG sites whose methylation levels change with age in different tissues, and DNA methylation at 353 'clock' CpG sites can be used to calculate epigenetic age independently of tissue, cell type and disease status. Several human diseases are associated with accelerated epigenetic aging, including Down syndrome and obesity, and it has been hypothesised that the age-related loss of normal epigenetic control may be responsible for the late onset of common human diseases. Given that age is the strongest risk factor for OA, we decided to investigate if OA is associated with accelerated epigenetic aging in cartilage.
Methods: A meta-analysis was performed on three cartilage methylation datasets, which used identical sample types and were generated using the Infinium HumanMethylation450 BeadChip. The raw IDAT data files were normalised using the preprocessFunnorm function from the Minfi package. The following CpG probes were removed from the analysis: probes with a detection P value threshold of <0.01 in at least 50% of samples; those on the sex chromosomes and; probes containing SNPs with a MAF of greater than 5%. This resulted in a total of 422,069 CpG probes for the age analysis. After QC and normalisation, 179 cartilage samples with an age range of 45 to 95 years (mean age of 71.7 years) were included in the analysis; these were composed of non-OA hip cartilage, preserved and lesioned OA hip cartilage, and preserved and lesioned OA knee cartilage. Age regression tests were performed using the Limma package with linear models that controlled for the different origins of the datasets. A Benjamini-Hochberg adjusted P value cut-off of <0.05 was used to identify age-associated CpGs (age-CpGs). Gene ontology analysis was performed using Ingenuity Pathways Analysis.
Results: No correlation was observed between mean probe methylation of all probes included in the analysis and age. 716 and 345 age-CpGs were identified when all samples (n = 179) or all OA samples (n = 144) were analysed, respectively. The majority of age-CpGs were hypermethylated with age (91.3% of cartilage and 93.3% of OA-only age CpGs). Age-CpGs were enriched relative to non-age CpGs within CpG islands (2.0 fold and 2.15 fold, respectively) and depleted in the regions >2kb away from a CpG island (p<0.05, Pearson Chi2 test). Significantly fewer age-CpGs were located in genic regions than expected, but those within gene loci were enriched in the region spanning 200bp upstream of the transcription start site (1.6 fold and 1.3 fold for cartilage age-CpGs and OA age-CpGs, respectively) and depleted in gene body and 3′UTR. Age-CpG sites were significantly enriched for binding sites of the polycomb proteins EZH2, SUZ12 and the co-repressor protein CTBP2, and depleted for sites where the histone demethylase KDM5A transcription factor binds (p<0.001, Pearson Chi2 test). After removing age-CpG sites identified from other studies in other tissues, we identified 605 age-CpGs specific to cartilage and 126 specific to OA cartilage. The cartilage-specific age-CpGs were enriched for in genes involved in cancer, embryonic and organismal development, and neuronal migration and function. OA-specific age-CpGs were enriched for in genes involved in cancer, analgesia, synaptic transmission, and embryonic development; relevant canonical pathways included circadian rhythm signalling and neuropathic pain signalling in dorsal horn neurons. There was limited overlap in age-CpGs between the five cartilage subtypes when analysed separately, especially between hip and knee cartilage. We used the 353 'clock' CpGs to calculate DNA methylation age for each sample, and observed accelerated epigenetic aging in males relative to females (p = 0.019, Kolmogorov-Smirnov 2 sample t test), and in OA hip samples relative to OA knee samples independently of gender (p = 0.012). DNA methylation age was different between preserved and lesioned cartilage from the same joint, although the direction of this difference varied between individuals.
Conclusions: We have identified CpG sites whose methylation levels significantly correlate with chronological age in cartilage, over 90% of which are hypermethylated with age. The limited age-CpG overlap and accelerated epigenetic aging observed in OA hip cartilage relative to OA knee cartilage suggests that the aging processes may be different in cartilage from different joint sites, although cell communication and development pathways are common targets of age-related methylation changes for both joints.

56
IDENTIFICATION OF SERUM SNORNAS AS BIOMARKERS OF MUSCULOSKELETAL AGEING AND DISEASE

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Purpose: The development of effective treatments for age-related musculoskeletal diseases and the ability to predict disease progression has been hampered by the lack of substantive biomarkers able to demonstrate tissue pathology preceding identifiable tissue alterations. We have previously identified age-related dysregulation of a defined set of small nucleolar RNAs (snoRNAs) in cartilage and tendon ageing as well as the age-related disease osteoarthritis (OA). SnoRNAs are a class of evolutionary conserved non-coding small guide RNAs of which the majority directs the chemical modification of other RNA substrates, including ribosomal RNAs and spliceosomal RNAs. Profiling the expression patterns of snoRNAs in disease may help comprehend their functional significance in the development and progression of age-related musculoskeletal diseases, and provide diagnostic biomarkers and therapeutic targets.
Methods: C57BL6/J male mice were used for the study. RNA was isolated from joints and serum of old (n = 6; 18 months old), young (n = 6; 8 months old) and old OA (n = 6 destabilisation of medial meniscus (DMM), n = 3 sham; 24 months old) mice. Samples were submitted for library preparation using NEB small RNA library kit. To reduce bias in our workflow we used tobacco acid pyrophosphatase to remove 5'CAP found on some snoRNAs. Samples were amplified for 15 cycles, mixed into 3 pools, and size selected using a range 120-300bp. The size selected material was purified with Ampure beads. Small RNA sequencing was undertaken on the Illumina HiSeq 2000 platform using 100 base paired-end reads. The reference genome used for alignment was the mouse reference genome assembly GRCh38. The snoRNA count data were used to perform a data variation assessment. Differential expression analysis was conducted using edgeR. Significantly differentially expressed (DE) snoRNAs were defined with a FDR-adjusted P-values<5%. Counts per million (CPM) were used to present expression values.
Results: Reads mapping percentages for joint libraries were between 12 ~ 29%, and for serum libraries 0.03 ~ 0.9%. Between 42 and 53% of the