INVOSSA™ – a cell and gene therapy for osteoarthritis

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INTRODUCTION: Osteoarthritis (OA) is a debilitating and painful disease that is growing in incidence due to the aging population. Current treatment modalities attempt to address pain and improve function, but no treatment is curative. Until recently, OA was seen as the result of joint trauma, but is now recognized as a chronic, inflammatory process. OA's inflammatory process is an imbalance between pro-inflammatory and anti-inflammatory cytokines.[1,2] This process can be interrupted by TGF-β1. Treatment with Invossa™, provides genetically-modified chondrocytes that produce TGF-β1 directly to the joint space, thereby mitigating the inflammatory process and improving joint structure. This keynote will briefly describe how Invossa™ progressed from the laboratory and preclinical studies to late clinical development and regulatory approval.

Kolon TissueGene Inc. (KTG) has conducted numerous nonclinical pharmacology studies on Invossa that has included the use of monosodium iodoacetate (MIA)-induced rat models of OA. These studies showed that the M2 macrophage was the main mechanism through which inflammation can be improved. These findings were mirrored in improved pain (epidemiological studies) and through immunohistopathology and immunohistology studies.

Korean Phase III Study: A Phase III study in 163 subjects with OA was conducted in South Korea using Invossa™. A total of 159 subjects with Kellgren & Lawrence (K&L) Grade 3 OA received a single injection of either Invossa™ (n=78) or placebo (n=81). Our primary evaluation endpoint was 12 months with interim evaluations occurring at Months 1, 3, 6, and 9. Each subject provided informed consent prior to undergoing any study evaluations.

Primary efficacy endpoints included International Knee Documentation Committee (IKDC) and Visual Analogue Scale (VAS) from Baseline to 12 Months. Secondary efficacy endpoints included WOMAC, KOOS, X-Ray, MRI, and biomarkers comparison at the same time points.

US Phase II and III Studies: KTG conducted a Phase II study in 102 subjects with OA at 5 institutions in the United States (US) using Invossa™; 67 subjects received the study drug and 35 subjects received placebo. All subjects had OA of K&L Grade 3. The primary evaluations were the same (IKDC and VAS); however, the evaluation time period was 24 months.

DISCUSSION & CONCLUSIONS: In the Korean Phase III study, 34.6% (n=27) in the Invossa treatment group and 12.6% (n=10) in the placebo group experienced drug-related AEs. All of the related AEs were expected and disappeared within 10 days of drug administration. At 12 months, subjects in the Invossa™ treatment group experienced a mean 15.1-point improvement in IKDC scores from baseline and a mean 24.5-point reduction in VAS score. Both of these changes were statistically significantly different ($P<.05$) from mean changes seen in the placebo treatment group (mean change in IKDC from baseline = 5.0 and mean change in VAS from baseline = 10.3). At 3-years follow-up, Invossa™ treated subjects had experienced a delay in OA disease progression indicating the DMOAD capability of Invossa™. KTG has ongoing follow-up of these patients with no safety signals indicated to date.

In the US Phase II study at 24 months, subjects in the Invossa™ treatment group experienced a mean 22.0-point improvement in IKDC scores from baseline and a mean 34.4-point reduction in VAS score. Both of these changes were statistically significantly different ($P<.05$) from mean changes seen in the placebo treatment group.

In July 2017, the South Korean Ministry of Food and Drug approved for Invossa-K Inj for the treatment of K&L Grade 3 OA of the knee.

The Korean Phase III and US Phase II demonstrated pain relief, increased joint mobility, and indicators towards improved joint structure. KTG has planned two large Phase III studies in the US to evaluate joint pain and function with statistical power to prove structural improvement for classification as a disease modifying osteoarthritis drug (DMOAD).

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Allogeneic MSCs and autologous chondrons: Clinical application

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INTRODUCTION: Cartilage defects are a known cause of often disabling knee pain. It is generally treated by autologous chondrocyte implantation (ACI), an effective but costly procedure requiring two surgeries and expansion of autologous cells. The aim of this study was to demonstrate safety, feasibility and initial efficacy of the investigator-driven Instant MSC Product accompanying Autologous Chondron Transplantation (IMPACT) regenerative therapy that combines allogeneic mesenchymal stromal cells (MSCs) with autologous chondrons implanted in focal cartilage defects in a one-stage procedure [1,2].

METHODS: A total of 35 patients were treated in this first-in-man phase I/II prospective monocenter study (NCT02037204). All patients (age 18-45 years) had a focal defect on the femoral condyle or trochlea (mean size 3.6 ± 0.7 cm^2). Cartilage defects were treated with a combination of freshly enzymatically released autologous chondrons, rapidly isolated from cartilage taken from the rim of the defect, combined with allogeneic cryopreserved bone marrow MSCs (ratio 1:9 or 2:8), which were implanted in the knee defect using fibrin glue within two hours. Clinical outcome was measured by the Visual Analogue Scale (VAS) for pain and the Knee injury and Osteoarthritis Outcome score (KOOS) at 3,6,12 and 18 months. Magnetic Resonance Imaging (MRI) and a second-look arthroscopy measured structural outcome at 12 months. Full- thickness biopsies taken from the centre of the repair tissue were used for histological analysis for proteoglycans (Safranin-O staining) and type I and II collagen deposition (immunostainings) and DNA tracking with short tandem repeat (STR) analysis.

RESULTS: No serious adverse events were found. All patients showed statistically significant clinical improvement in the VAS and KOOS scores after both 6 and 18 months (all $p<0.001$). MRI scans and second-look arthroscopies indicated complete filling of the defects and integration with native tissue and attachment to the subchondral bone. Histological analysis of the biopsies showed hyaline cartilage-like repair for most patients (Fig. 1). No DNA of the allogeneic MSCs could be detected in the repair tissue.

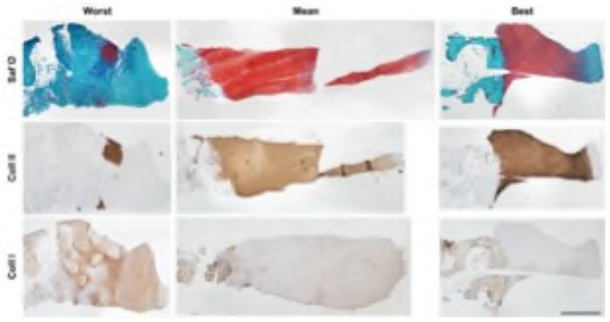

Fig. 1: Safranin-O staining (Saf O), and type II and I collagen immunostaining (Coll II and I, best, mean, worst) on biopsies from the center of the repair tissue twelve months after surgery. Best (n=5), mean (n=26) and worst (n=2) samples were selected based on the ICRS II histological outcome scores. Scale bar indicates 1 mm.

DISCUSSION & CONCLUSIONS: This is the first study to show preliminary safety of allogeneic MSCs, which together with autologous chondrons stimulate cartilage regeneration. All MSCs were disappeared 12 months after surgery, while the defects were restored with functional hyaline cartilage-like tissue. This suggests regenerative properties through trophic factors by the allogeneic MSCs.

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Nucleus pulposus profiling in development, ageing and degeneration

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During early development the nucleus pulposus (NP) of the intervertebral disc is populated by large, vacuolated notochordal (NC) cells. In most animal species these NC cells are retained throughout the majority of their lifespan. However, in humans these cells are lost with skeletal maturity and replaced by smaller, round, non-vacuolated NP cells. The age at which these NC cells are lost coincides with the initiation of degenerate changes that can ultimately lead to IVD degeneration and back pain. Interestingly, animals which retain their NC cells tend not to demonstrate signs of degeneration, suggesting an important role for NC cells in NP homeostasis. This is further supported by evidence that NC cells derived from animals produce more proteoglycans than NP cells, stimulate NP cells to produce a healthier extracellular matrix and prevent interleukin-1 (IL-1)-induced cell death in NP cells. Such findings highlight the importance of elucidating the phenotype and ultimately function of human NC and NP cells to both to establish their therapeutic potential and to inform development of other biologic and cell-based regenerative therapies.

We[1], and others[2], have previously elucidated the transcriptomic profile of adult human NP cells and demonstrated clear differences from other chondrocytic cells (i.e. articular chondrocytes). This molecular profiling has led to the publication which provides recommendations on the definition of a young, healthy NP cell[3]. Interestingly, such studies have revealed evidence of expression of NC cell markers in adult NP [cells1,4], suggesting that at least a sub-population of adult NP cells may be derived from the embryonic notochord.

To further elucidate the ontogeny of human NP cells we have recently undertaken transcriptomic profiling of foetal human NC cells and identified a phenotypic profile of these cells. Expression analysis in adult human samples has further identified expression of these markers throughout ageing and degeneration, adding further weight to the argument of a sub-population of adult NP cells being notochordally-derived.

Using this detailed transcriptomic profile it should be possible to undertake further investigation into the function of NC cells and to develop methods to generate phenotypically correct NC cells from stem cells. Further investigation into these cells may also enable development of strategies to prevent, retard, or treat intervertebral disc degeneration in the future.

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Lgr5 and Col22a1 mark a committed progenitor lineage to juvenile articular chondrocytes

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INTRODUCTION: The synovial joint forms from a pool of progenitor cells in the interzone region of the future joint. Expression of *Gdf5* and *Wnt9a* has been used to mark the earliest cellular processes in the formation of the interzone and the progenitor cells. However, progression and lineage specification toward the different tissues of the joint is not well understood. Here, by lineage tracing studies we identify a population of *Lgr5+* interzone cells that contribute to the formation of cruciate ligaments, synovial membrane and articular chondrocytes of the joint. Furthermore, we showed that *Col22a1*, a marker of early articular chondrocytes, co-expresses with Lgr5+ cells prior to cavitation as an important lineage marker specifying the progression towards articular chondrocytes. Characterization of the specific cell pools in this specific lineage will provide insights into the molecular controls, and prospect of using these cells for repair of joint tissues.

Importance of endogenous non-canonical WNT5A and WNT11 for hypertrophy of mesenchymal progenitor-derived chondrocytes

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INTRODUCTION: Bone marrow-derived multipotent progenitor cells (MPCs) are attractive for cartilage regeneration. However, during in vitro chondrogenesis MPCs upregulate hypertrophic markers like COL10A1, IHH, and PTH1R as well as osteogenic markers like IBSP, ALPL, and SPP1. This program is believed to predetermine subsequent mineralization and bone formation, which is highly undesired for cartilage regeneration. The signals driving MPC hypertrophy in vitro are only partially defined. Consequently, there is currently no means to suppress all hypertrophic and osteogenic markers to the low levels found in re-differentiating articular chondrocytes (ACs) that do not mineralize and do not form ectopic bone. WNT signals drive osteogenesis and bone development, but their role during MPC in vitro chondrogenesis is unknown. WNT signalling is mediated via the canonical β-catenin-dependent and several non-canonical pathways that are independent of β-catenin. We here aimed to unravel the contribution of canonical and non-canonical WNT pathways to MPC hypertrophy. Specifically, we wanted to know whether β-catenin levels differ and which WNT network genes are differentially expressed between chondral AC re-differentiation and endochondral MPC development.

METHODS: β-catenin levels during MPC in vitro chondrogenesis and AC re-differentiation were determined via Western blotting. Gene expression of 71 WNT network components were extracted from a whole-genome microarray of MPCs and ACs at days 0 and 28 of re-/differentiation. Expression of the most strongly differentially regulated WNT network genes was studied in detail via qPCR in independent time course experiments of MPCs and ACs at days 0, 7, 14, 21, 35, and 42.

RESULTS: A similar downregulation of active β-catenin occurred during MPC chondrogenesis and AC re-differentiation, indicating that the canonical WNT pathway is unlikely to drive MPC hypertrophy.
On gene expression level, 19 WNT genes changed their expression from day 0 to day 28 of in vitro differentiation at least 1.5-fold in MPCs or ACs. This change was more than 2-fold different in MPCs vs. ACs for only seven molecules (*FZD9, PTK7, LEF1, SFRP1, FRZB, WNT5A, WNT11*).

Time course experiments revealed that expression of the receptor *FZD9*, the co-receptor *PTK7*, and the transcription factor *LEF1* was very low in ACs and remained unregulated during re-differentiation. In contrast, these genes were significantly upregulated during MPC chondrogenesis.
In turn, the inhibitor *FRZB* was strongly expressed in ACs throughout re-differentiation and was significantly lower in MPCs at day 0 as well as after 42 days of chondrogenesis. These data suggested higher WNT activity during MPC chondrogenesis than in AC re-differentiation.

The two non-canonical WNT ligands *WNT5A* and *WNT11*, remaining at low levels in ACs, showed an interesting inverse regulation in MPCs. *WNT5A* became downregulated during MPC chondrogenesis but still remained 4-fold higher than in ACs. *WNT11* expression on the other hand was strongly upregulated from day 21 on.

DISCUSSION & CONCLUSIONS: MPC-specific regulation of non-canonical ligands WNT5A and WNT11 suggests them as candidate drivers of hypertrophic markers during MPC chondrogenesis. Manipulation of WNT5A and WNT11 seems attractive for future specific interventions to suppress the undesired MPC hypertrophy.

Gene expression comparison of intervertebral discs from diffuse idiopathic skeletal hyperostosis (DISH) patients and traumatic/degenerative intervertebral discs

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INTRODUCTION: Diffuse idiopathic skeletal hyperostosis (DISH) affects mostly mid-aged and elderly people. DISH is characterized by the formation of bone along the anterior spine. Abnormalities like the formation of osteophytes, following an ossification anterior to the vertebral bodies and the outer intervertebral discs (IVD) are frequently observed in DISH patients [1]. However, the nucleus pulposus remains unaffected by this disease. We have recently shown, that the IVD cells are expressing BMP antagonists, which could lead to inhibition of bone formation for spinal fusion. In this study, we investigate the transcriptome of discs of the BMP pathway from DISH patients with traumatic or degenerative discs.

METHODS: Fresh DISH-IVDs and trauma or degenerated IVDs were obtained from patients undergoing spinal surgery (approved by the Ethics Committee of the Canton of Bern, CH). IVD cells were released from their native extracellular matrix by digestion of the tissue with pronase (1.9 mg/mL Roche, Basel, CH) and subsequent collagenase II digestion (129 U/mL, Worthington, London, UK). Cells were then filter-strained (100 µm) and cultured up to passage two and then lysed in TRI reagent for total RNA extraction. RNA integrity and quantity were monitored using Experion™ RNA electrophoresis prior qPCR (Bio-Rad). PrimePCR™ (TGF β BMP Signaling Pathway Plus H96, 90 genes, Bio-Rad, US) was run on CFX96 machine (Bio-Rad). Gene expression of three DISH patients was tested against three control patients.

RESULTS: In all DISH-IVDs an up-regulation of *Interleukin 6* (*IL-6*) was detected (mean ± SEM of all five comparisons) 122.32 ± 110.67-fold. Furthermore, were *Early Growth Response* 2 (*EGR2*) and *Insulin-like Growth Factor 1* (IGF1) up-regulated in two of the DISH-IVD donors (26.62 ± 17.11-fold and 27.20 ± 27.17-fold, respectively). Whereas, the two *Growth and Differentiation Factors 5 and 6* (*GDF5* and *6*) were down-regulated in two of the three DISH-IVDs (-29.67 ± 22.47-fold and -8.28 ± 5.80-fold, respectively).

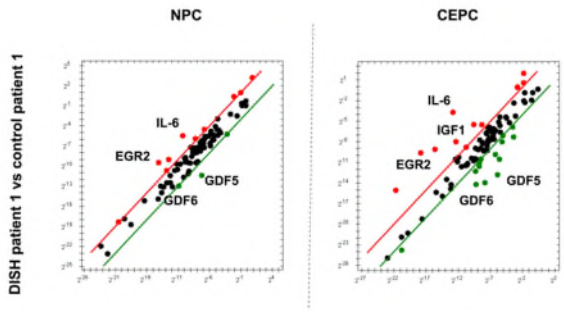

Fig. 1: TGFβ pathway transcriptome analysis of one donor with the according trauma control. RNA transcripts that differed more than four times are indicated in red (up-regulated in DISH patients) or in green (down-regulated in DISH patients). Investigated were nucleus pulposus (NPC) and cartilaginous endplate cells (CEPC).

DISCUSSION & CONCLUSIONS: In this study, we aimed to shed light into the phenotype of disc cells from patients suffering from DISH. Most interestingly, the DISH-IVD cells showed a considerable change in *IGF1* and *IL-6*. The increased production of these two growth factors was also hypothesized before [2,3]. IGF-1 was already determined as a serum marker for rheumatic diseases, such as DISH. *GDF5* and *GDF6* showed a minimum of 8-fold down-regulation in DISH-IVDs. However, the exact role of these two members of the TGFβ pathway in DISH disease is yet unknown. These results are unexpected considering the fact that the ossification occurs in the neighboring ligaments and enthesis leaving the inner part of the IVD macroscopically unaffected.

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Hypertrophy-associated regulation of RUNX3 and MEF2C during chondrogenesis of human mesenchymal progenitor cells

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INTRODUCTION: Bone marrow-derived mesenchymal progenitor cells (MPC) are an attractive cell source for cartilage regeneration strategies. Unfortunately, MPC undergo hypertrophy during in vitro chondrogenesis building transient cartilage, which develops into bone at ectopic sites in vivo. Instead, articular chondrocytes (AC) redifferentiate into non-hypertrophic cartilage tissue in vitro which is resistant to mineralization in vivo [1]. An inherent mineralization tendency of implants is undesired for therapy of focal cartilage defects in clinical applications. During mouse embryonic development, balancing of SOX9 and RUNX2 transcription factors determines endochondral development, while MEF2C expression is associated with chondrocyte hypertrophy. RUNX3 rather is expressed in prehypertrophic than hypertrophic chondrocytes [2]. Surprisingly little is so far known on how these key fate determining factors are regulated during chondrogenesis of human MPC versus redifferentiation of human AC. Better knowledge may enable optimal guiding of MPC between chondral versus endochondral pathways to improve cell-based cartilage and bone regeneration strategies for clinical application. Aim was to uncover expression and regulation of SOX9, RUNX2, RUNX3 and MEF2C in MPC versus AC under chondrogenic (re-)differentiation conditions.

METHODS: Human MPC (n=4 donors) and human AC (n=3 donors) were isolated after informed consent, expanded and subjected to chondrogenic conditions for up to 6 weeks. Gene expression (qRT-PCR), protein levels (Western-Blotting), ALP activity and histology were performed at the end of expansion and weekly during (re-)differentiation.

RESULTS: Expanded MPC expressed less SOX9 mRNA and protein but showed higher RUNX2 and RUNX3 mRNA levels than expanded AC. During redifferentiation, AC strongly upregulated SOX9 and remained negative for RUNX2 and RUNX3 protein throughout. Interestingly, RUNX2 protein was undetectable during MPC chondrogenesis in spite of hypertrophic development [Fig.1]. However RUNX3 and MEF2C mRNA and protein levels were significantly upregulated together with hypertrophic markers COL10A1, IHH, IBSP and ALP activity.

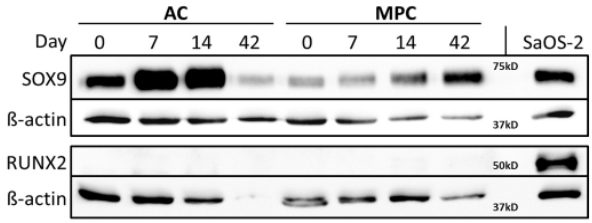

Fig. 1: Western-Blotting for SOX9 and RUNX2 protein levels in (re-)differentiating AC and MPC with ß-actin as reference.

DISCUSSION & CONCLUSIONS: Although RUNX2 is considered the essential master regulator of endochondral development in mouse studies, hypertrophy of human MPC was not driven by RUNX2 regulation during chondrogenesis. Selective RUNX3 and MEF2C upregulation along with hypertrophic markers during human MPC but not AC differentiation suggests to set the future focus on these two transcription factors. Manipulation of relevant pathways driving RUNX3 and MEF2C transcription factor levels in MPC may be promising for guiding MPC differentiation towards an articular chondrocyte phenotype.

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The role of TGF on cartilage homeostasis

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Transforming growth factor-β (TGFβ) is a pleiotropic cytokine that regulates joint homeostasis and disease. TGFβ signalling is induced by loading and maintains the differentiated phenotype of articular chondrocytes in young, healthy joints.
However, concentrations of active TGFβ differ greatly between healthy and osteoarthritic (OA) joints. In healthy joint low concentrations of active TGFβ are present while these concentrations are greatly elevated in OA joints, leading amongst others to the activation of different signalling pathways in cells in OA joints than in healthy joints. These high active TGFβ levels stimulate specific pathological characteristics of OA joints, such as cartilage damage, osteophyte formation and synovial fibrosis. Furthermore, subchondral bone changes are induced by high active TGFβ concentrations that appear to be causally-related to OA progression.
Consequently, TGFβ has a changing role in health and disease, being protective against pathological changes in young healthy joints but a driving force of pathology in osteoarthritic joints.

Osteoarthritis as an organ disease

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INTRODUCTION: Considered for many decades as a cartilage disease, recent studies of osteoarthritis (OA) have taken us back to the concepts considered at the naming of the disorder in 1890 as 'bone-joint-inflammation'. While some forms of OA follow trauma and affect a single joint, most is still classed as idiopathic – unknown cause - and is polyarticular. If the joint is considered to be an organ, can we describe OA as an organ disease similar to the way in which we describe heart disease? Is there a systemic (which system?) involvement? Does this help with diagnosis or therapy?

METHODS: We have used a variety of approaches to study the osteoarthritic joint *in vivo* and *in vitro*. MRI, DXA and plain radiography have enabled us to measure joint shape and intra-medullary fat distributions. Linking these measures with GWAS, across joints and with life-course factors is beginning to uncover factors that may act over long periods to predispose to joint disease in later life. Applying cyclic and impact loading to cartilage explants in vitro has shown how cartilage responds to such loads and led to the tentative identification of possible mediating factors. The physical properties of cartilage and bone have been measured using mechanical testing, x-ray and neutron diffraction, thermal analysis and Raman spectroscopy.

RESULTS: Hyperplasia of all the joint tissues is one of the most notable features of early disease. Articular cartilage thickens, chondrocytes proliferate and increase matrix biosynthesis, although not incorporation. Subsequent cartilage degeneration, we have hypothesized, is due to an inability of the tissue to accommodate the new matrix being produced. There is an increase in the thickness of the subchondral plate and in the amount of the underlying cancellous bone. This bone, however, is hypomineralized and shows evidence of increased turnover. Despite the increase in bone volume there is also an increase in fat content and a doubling of the fraction of arachidonic acid, a precursor to pro-inflammatory prostaglandins. In the Chingford study,[1] an analysis of metabolic factors, such as hypertension, hypercholesterolemia and blood glucose, showed these to be associated with OA independent of obesity, leading to the conclusion that the aetiology of OA has important systemic and metabolic components. Early studies recognized a thickening of the fibrous capsule and ligaments crossing the joint. The source of pain is still unknown but there is evidence for peripheral and central sensitization. Neurogenic effects on musculoskeletal tissues could provide a feedback loop through the numerous neuropeptides and receptors now identified[2].

Joint deformity may be a result of OA, *e.g.* osteophytosis, or a causative factor, *e.g.* developmental dysplasia. Quantifying joint shape is challenging but statistical shape modelling has given us a tool to use as an imaging biomarker. Radiographic hip and knee shapes show an association with OA severity and are a sensitive marker of progression. A recent GWAS meta-analysis has identified novel genes associated with hip shape and, interestingly, many of these genes are related to tissue growth and development. In a birth cohort at age 60-64 years there is an association between specific hip shapes and obesity through adulthood, and the hip shapes of those who walked early in childhood have features resembling those found in OA hips.

DISCUSSION & CONCLUSIONS: Many insights into the structures and behaviours of joint tissues have been learnt along the way, but the overriding impression in OA is of a dysregulated musculoskeletal system expressed as a disorder of the whole joint. Biomarkers, then, may not be specific tissue components, as cholesterol is not a 'heart molecule', and treating the cartilage may have limited therapeutic benefit as it is only one component tissue interdependent with many others.

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The systemic impact of bone injury in an animal model of rheumatoid arthritis

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INTRODUCTION: Rheumatoid arthritis (RA) is an autoimmune disease characterized by chronic joint inflammation associated with disability and skeletal complications [1]. RA patients are at risk of osteoporosis, and are two times more likely to suffer bone fractures. These correlate with high disease activity, and are more prevalent in hip and spine [2]. Here, we aimed to investigate the systemic impact of bone injury in an RA animal model and establish a new *in vivo* combined model to study bone repair in inflammatory conditions.

METHODS: A Collagen-induced arthritis (CIA) model was performed in Wistar rats by immunization with type II collagen and incomplete Freund's adjuvant emulsion. Disease progression was monitored, and paw inflammation scored. At day 21, CIA animals were divided in two groups and one was submitted to a femoral defect surgery (CIA+FD). Healthy animals were used as controls. Animals were euthanized 3 days after surgery. Animal limbs were fixed and decalcified for histology analysis. Cells from blood, draining lymph nodes and spleen were counted, immunostained and analyzed by multicolor flow cytometry for T, B and myeloid cells, as well as activation markers CD40 and CD86.

RESULTS: The arthritis induction was effective in all animals, with evident signs of swelling in both hind paws, compared to control animals. This was accompanied by synovial membrane hyperplasia and infiltration of inflammatory cells (Fig.1). Arthritis severity score increased along monitoring until a plateau between 14 and 17 days. At day 24, CIA+FD animals showed a slight decrease of swelling in both hind paws, compared to CIA animals. Both CIA and CIA+FD animals had increased cell proliferation in spleen and lymph nodes, relative to control animals. CIA group showed increased proportions of B cells in blood, and increased B and decreased T cells in lymph nodes, relative to controls. However, CIA+FD, but not CIA alone, showed increased proportions of myeloid cells, particularly in spleen. Moreover, myeloid cell activation marker CD40 in the spleen of CIA animals was similar to control animals, while in CIA+FD animals CD40 was significantly increased.

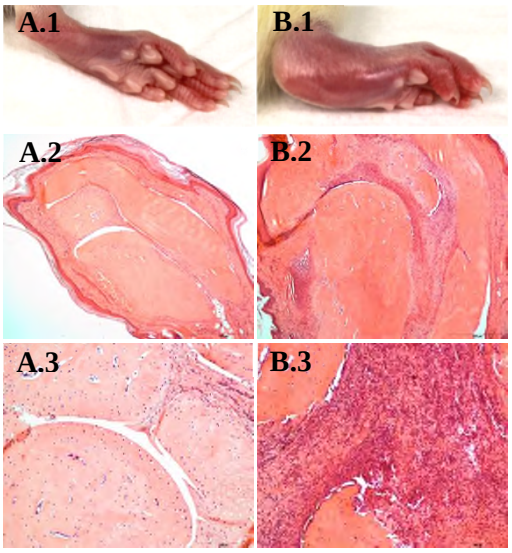

Fig. 1: Morphology and histology of collagen-induced arthritis. A. Control paw without swelling (A.1) with healthy joint histology (A.2-3); B. CIA paw with visible swelling (B.1), histologically visible synovial hyperplasia (B.2), with infiltration of inflammatory cells (B.3). A.2 and B.2, scale 500 µm; A.3 and B.3, scale 200 µm.

DISCUSSION & CONCLUSIONS: The combination of CIA with FD did not compromise animal welfare. Despite the chronic inflammation, CIA animals responded to bone injury, with significant changes in systemic myeloid cells proportions and activation status. Thus, the CIA model can be used to study bone repair in chronic inflammatory conditions that mimic human RA.

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Biologic canine and human intervertebral disc repair by notochordal cell-derived matrix: from bench towards bedside

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INTRODUCTION: The socioeconomic burden of chronic back pain related to intervertebral disc (IVD) disease is high and current treatments are only symptomatic. Minimally invasive strategies that promote biological IVD repair should address this unmet need. Notochordal cells (NCs) are replaced by chondrocyte-like cells (CLCs) during IVD ageing. The regenerative potential of NC-secreted substances on CLCs and mesenchymal stromal cells (MSCs) has been demonstrated, but identification of these substances remains elusive.

METHODS: This study exploits the regenerative NC potential by using healthy porcine NC-derived matrix (NCM) and employs the dog as a clinically relevant translational model. After *in vitro* experiments on 3D canine and human CLC and MSC micro-aggregates, NCM (1x and 2x injected – the latter reinjected after 3 months), MSCs and NCM+MSCs were injected in mildly (spontaneously) and moderately (induced) degenerated canine IVDs *in vivo* and, after six months of treatment, were analysed.

RESULTS: NCM increased the glycosaminoglycan, collagen type II and DNA content of human and canine CLC aggregates (Fig. 1) and facilitated chondrogenic differentiation of canine MSCs *in vitro*.

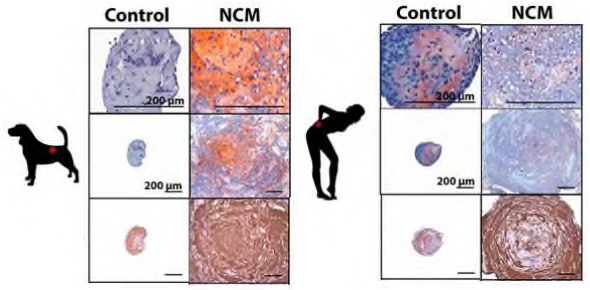

Fig. 1: In vitro regenerative effects of NCM on canine and human CLCs. Safranin O staining (upper panels) and collagen type II IHC (lower panel). n = 6 per species, in duplicates.

NCM injected in moderately degenerated canine IVDs exerted beneficial effects at the macroscopic and MRI level (Fig. 2), induced collagen type II-rich extracellular matrix production, improved the disc height, and ameliorated local inflammation. MSCs exerted no (additive) effects.

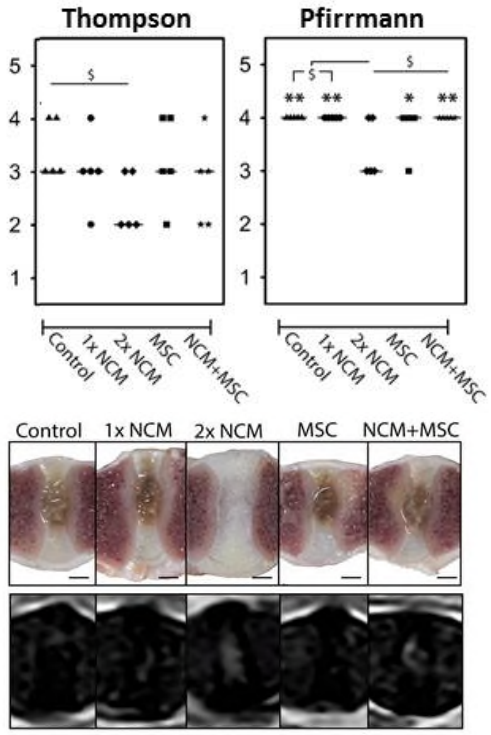

Fig.2: In vivo regenerative effects of NCM on canine IVDs. Reinjection of NCM (2xNCM; reinjected after 3 months) improved Thompson and Pfirrmann scores of canine IVDs in which degeneration was induced by partial NP removal.

DISCUSSION & CONCLUSIONS: NCM facilitated chondrogenic MSC differentiation and induced *in vitro* and *in vivo* regenerative effects on degenerated canine IVDs. NCM may, comparable to demineralized bone matrix in bone regeneration, serve as 'instructive matrix', by locally releasing growth factors and facilitating tissue repair. Therefore, intradiscal NCM injection could be a promising regenerative treatment for IVD disease, circumventing the cumbersome identification of bioactive NC-secreted substances.

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Link N retards disease progression in a rabbit model of osteoarthritis

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INTRODUCTION: Osteoarthritis (OA) is a degenerative joint disease characterized by a slowly progressive degradation of articular cartilage, Increased density or erosion of the subchondral bone, formation of osteophytes, inflammation of the synovium, degeneration of ligaments and the menisci, and hypertrophy of the joint capsule. Currently, there is no medical treatment to reverse or even retard OA. The purpose of the present study was to determine if Short Link N (sLN), a recently discovered fragment of the Link N peptide, could retard disease progression in a rabbit model of OA.

METHODS: Skeletally mature New Zealand white rabbits underwent unilateral anterior cruciate ligament transection (ACLT) of their left femorotibial joints to induce joint degeneration [1]. Beginning 3 weeks post-operatively, and every three weeks thereafter for 12 weeks, either saline (1 mL) or sLN (100 µg in 1 mL saline) was injected intraarticularly in the operated knee. Additional rabbits underwent Sham surgery but without ACLT. The effects on gross morphology and histologic changes were evaluated [2].

RESULTS: In all joint compartments of the Sham group, the articular cartilage had normal macroscopic appearance with no sign of lesion. In the Saline group, fibrillation of articular cartilage occurred in all joint surfaces, and prominent erosion occurred in both femoral condyle compartments and the lateral compartment of the tibial plateau. sLN treatment reduced the severity of the cartilage damage in the 3 compartments of the knee showing erosion. In the lateral tibial plateau and medial femoral condyle sLN prevents the erosion, and reduces erosion in the lateral femoral condyle compartment. In the medial tibial plateau compartment, only minor fibrillation is observed with saline or sLN. Overall there is a trend in reducing the severity of joint damage, reaching statistical significance in the MFC compartment. Furthermore, when all histologic parameters were combined from each site to obtain a total joint score, statistically significant differences were detected between the Saline treated joints and the sLN group (Fig 1, p=0.04).

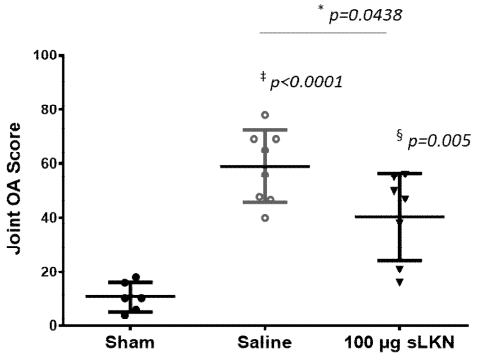

Fig. 1: OA lesion severity throughout the joint. Combined histologic scores comparisons between Sham, Saline and sLN groups.

DISCUSSION & CONCLUSIONS: Overall sLN reduces the severity of joint damage and joint lesion severity in a statistically significant manner. Thus, pharmacologically, sLink N supplementation could be a novel therapeutic approach for treating OA by retarding disease progression.

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How articular chondrocytes versus MSC-derived chondrocytes respond to mechanostimulation

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During osteoarthritis (OA)-development extracellular matrix (ECM) molecules are lost from cartilage, thus changing gene-expression, matrix synthesis and biomechanical competence of the tissue. Mechanical loading is important for the maintenance of articular cartilage; however, the influence of an altered ECM content and composition on the response of articular chondrocytes (AC) to loading is not well understood. Hypertrophic marker expression is elevated in OA versus normal cartilage and chondrogenic differentiation of bone-marrow derived mesenchymal stroma cells (MSC) can serve as an in vitro OA model due to a similar expression of typical endochondral markers in the developing tissue. This opens the possibility to address whether the articular versus endochondral phenotype of chondrocytes changes the response to mechanical loading and whether this relates to ECM content. Better knowledge on the role of ECM-content and composition of engineered cartilage for the chondrocyte response to compressive loading may provide important insights into mechanisms underlying OA development as well as supplying new therapies for OA. This talk will summarize how a changing ECM-content of engineered cartilage affects the molecular loading response and which differences occur when cartilage is grown from articular chondrocytes versus MSC.

Stem cells and biomaterials for the regenerative medicine of intervertebral disc: "when tissue engineers meet developmental biologists"

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Intervertebral disc (IVD) plays a pivotal role in spine kinematics ensuring the function of "shock absorber" against the constraints that the spine undergoes throughout life. IVD is a fibrocartilaginous tissue that grossly consists of a peripheral network of type 1 collagen fibers (namely *Annulus fibrosus,* AF), which surrounds a highly hydrated gel (namely *Nucleus pulposus*, NP) rich in type 2 collagen and proteoglycans.

IVD degeneration is one of the major causes of low back pain (LBP). Currently, LBP is primarily managed by pharmacological treatments and if unsuccessful by surgical procedures (spine fusion or arthroplasty) that are reserved for severe debilitating LBP. To clinically address LBP earlier in the degenerative cascade of IVD, biology-inspired regenerative strategies could offer less invasive and etiological alternatives to spinal reconstructive surgery[1]. Whilst AF mostly contains fibroblastic cells, NP contains two different cell types: the notochordal cells (NTC) and the nucleopulpocytes (NPCytes). NTC are embryonic notochord derived cells that are considered the resident progenitor/stem cells of the NP tissue. NPCytes are considered the mature cells of NP niche responsible for the production and maintenance of a mechanically competent extracellular matrix (ECM). To maintain tissue homeostasis, NTC secrete several trophic factors that ensure the proliferation, survival and ECM secretory activities of NPCytes. During the growth and maturation of IVD some still unknown cues drive the disappearance of NTC (starting around age 10 in human). This progressive loss of NTC compromises the molecular dialog between NTC and NPCytes and thereby triggers NPCytes apoptosis and inability to produce and maintain NP niche. The subsequent loss of ECM in turn results in NP dehydration, loss of IVD height and formation of tears and cracks. Viewing these data, NP regeneration by re-establishing the NTC/NPCytes molecular dialog has recently been considered as a promising strategy to clinically address LBP. We will first share our view of the mesenchymal stromal cells (MSC)-based therapeutic approaches that have been preclinically developed and, for some of them, clinically transferred in patients with discogenic LBP. Then, we will comment on the recent biomaterial-assisted MSC therapies that recently enter the preclinical and clinical scene of IVD regeneration. Finally, we will share with you our REMEDIV project that aims at developing an injectable NP substitute containing bioactive stem cells-derived NPCytes and NTC-like cells within a hydrated biomaterial that could be percutaneously transplanted into degenerated IVDs. We will present our data regarding the generation of NPCytes from adipose-derived MSC[2]. We will also share our recent unpublished evidences that human induced pluripotent stem cells (iPS) can be differentiated into notochord-like cells. Finally, we will consider our ability to transplant stem cells-derived derived NPCytes and NTC using a self-setting hydrogel in various animal models from mice and rabbit to sheep[3]. Whether this concept could open new therapeutic windows in the management of discogenic low back pain will finally be discussed.

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BMP9/GDF2 rescues chondrogenesis in cartilage-derived progenitors from mature synovial joints

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INTRODUCTION: Articular cartilage-derived chondroprogenitors are classical mesenchymal stromal-like colony forming cells residing in synovial joints[1]. Manipulation of progenitors is important for two reasons, first in tissue engineering, where culture-expanded cells can be used to repair focal lesions in damaged joints, and secondly, in regenerative medicine where progenitors in joint cartilage are targets for intervention in order to reverse chronic degenerative conditions[2].

The target progenitor population is in adult tissue, however, much of the research conducted has used cells derived from immature, pre-pubertal cartilage, we do not know if corresponding cells from mature post-pubertal cartilage are equivalent. Therefore, we tested how immature and mature chondroprogenitor populations responded to chondrogenic stimuli.

METHODS: We isolated colony forming progenitors and full-depth chondrocytes from skeletally immature and mature bovine metacarapalphalangeal joints and which were then cultured as high-density pellets. Pellets were cultured for 21 days in the presence or absence of growth factors then processed for histology and papain-digested to quantify proteoglycan and collagen content, normalized to DNA content.

RESULTS: Results show that immature full-depth chondrocytes and progenitors are primed to differentiate and addition of growth factor TGFβ1 does not increase the deposition of proteoglycan in pellets compared to the basal medium, Figure 1. In fact, TGFβ1 addition reduces proteoglycan deposition in immature progenitors, whereas addition of BMP9 significantly increased proteoglycan content leading to larger pellet size. Full-depth chondrocytes from mature cartilage required growth factor supplementation in order to efficiently differentiate, however, mature chondroprogenitors required GDF2/BMP9 to stimulate chondrogenic differentiation, see Figure 1.

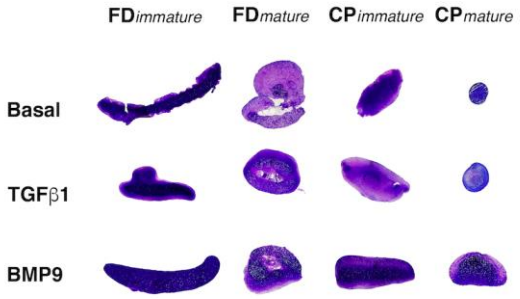

Fig. 1: Pellet cultures stained with toluidine blue show the response of immature and mature derived full-depth chondrocytes (FD) and chondroprogenitors (CP) to basal and growth factor (TGFβ1 and BMP9) supplemented medium.

DISCUSSION & CONCLUSIONS: Experiments show that there are fundamental quantitative differences in the reactions of chondrocytes from immature and mature cartilage to differentiation stimuli. The most important finding was the requirement of mature chondroprogenitors for GDF2/BMP9 to induce differentiation to levels comparable to freshly isolated full-depth chondrocytes. GDF2/BMP9 signals through the ALK1 receptor and activates SMAD1/5/8 phosphorylation and downstream signalling, but this pathway in chondrocytes can generate an epiphyseal phenotype. However, GDF2/BMP9 is known to activate SMAD2/3 in some tissues and also non-canonical signalling through map kinases. Delineating the downstream signalling mechanisms will be important not only for understanding chondrocyte differentiation but also the role of chondroprogenitors in development and disease.

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IL-1β effects on Mesenchymal stem cell (MSC) chondrogenesis are modulated by physioxia

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INTRODUCTION: Osteoarthritis (OA) is a degenerative condition affecting a wide range of the adult population. Autologous Chondrocyte Implantation (ACI) has been used to treat focal OA defects, although there are negative long-term outcomes due to poor graft integration and the presence of inflammatory factors, e.g. interleukin-1 beta (IL-1β)[1]. Bone marrow derived Mesenchymal Stem Cells (MSCs) are an alternative cell type that can be used for cell based treatments due to their chondrogenic capacity. In vivo, cartilage and bone marrow reside under a low oxygen tension, between 2-7% oxygen or physioxia[2]. Recent studies have described enhanced MSC chondrogenesis under this condition[3]. However, the presence of IL-1β inhibits MSC chondrogenesis[4]. The present investigation sought to understand the effect of physioxia on MSC chondrogenesis and hypothesized that physioxia reduces IL-1β inhibited chondrogenesis.

METHODS: Human MSCs (Male donors; Mean age, 32 years; n = 9) were split equally for expansion under either 2% (physioxia) or 20% (hyperoxia) oxygen. Chondrogenic pellets (2 x 10^5 MSCs/pellet) were formed and cultured in the presence of 10 ng/ml TGF-β_1 and in combination with either 0.1 or 0.5 ng/ml IL-1β under their respective expansion conditions. Pellet GAG and wet weight were measured on day 7, 14 and 21. Pellets on day 21 were analysed for collagen II content and evaluated histology and immunohistochemistry (DMMB (GAG), collagen II and X, TGF-β receptor II and MMP-13). Statistical analysis was performed using a Two-way ANOVA with Tukey post-hoc test, significant differences are stated when $p < 0.05$.

RESULTS: A significant dose-dependant IL-1β inhibition in MSC chondrogenesis was observed with respect to pellet wet weight under hyperoxia ($p < 0.05$). Pellet GAG content was also inhibited in the presence of IL-1β, with significant differences observed at 0.5 ng/ml IL-1β ($p < 0.05$). Physioxia alone significantly increased wet weight, GAG and collagen II content ($p < 0.05$) compared to hyperoxia. A donor-dependant physioxia response was observed, whereby 80% of donors positively responded to low oxygen and 20% were unresponsive. Analysis of only responsive donors demonstrated a significant increase in wet weight and GAG content (Fig 1) in the presence of 0.1 and 0.5ng/ml IL-1β under physioxia ($p < 0.05$). Reduced protein expression of hypertrophy markers (collagen X and MMP-13) and increased TGF-β receptor II expressing cells were observed under physioxia, especially in the presence of 0.1ng/ml IL-1β.

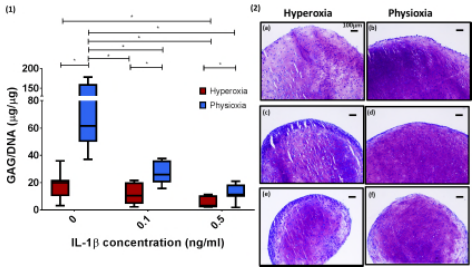

*Fig. (1) GAG/DNA analysis for responsive chondrogenic donors (*p < 0.05) and (2) DMMB stained chondrogenic pellets under hyperoxia (a,c,e) and physioxia (b,d,f) at 0 (a,b), 0.1 (c,d) and 0.5ng/ml IL-1β (e,f)*

DISCUSSION & CONCLUSIONS: Physioxia enhanced MSC chondrogenesis compared with hyperoxia, whilst alleviating IL-1β inhibited chondrogenesis. Specifically, physioxia significantly increased pellet wet weight and GAG content at 0.1 and 0.5 ng/ml IL-1β MSC chondrogenesis compared to hyperoxic culture. The molecular signalling mechanisms controlling this response are to be investigated.

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Amniotic Epithelial Cells shown chondrogenic phenotype after *in vitro* prolonged Estradiol and Progesterone treatments

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INTRODUCTION: Cartilage tissue engineering is rapidly emerging as a promising potential cure for cartilage lesions and this has intensified the screening of many different types of stem cells. Amniotic-derived Epithelial Cells (AECs) [1] a new stem cell source of fetal origin, have recently attracted the attention of researchers for their great regenerative potential for mesenchymal tissues [2] as cartilage [3]. However, the mechanisms inducing AEC chondrogenic commitment remains still unexplored. Because steroids have an important role in regulating osteochondral metabolism [4], this study has been designed to clarify the Estradiol (E_2) and Progesterone (P_4) *in vitro* effects on ovine AECs (oAECs) amplification, epithelial mesenchymal transition (EMT) and osteochondral differentiation attitude.

METHODS: oAECs were cultured as previous report [5] and treated with 25µM of E_2 or P_4, alone or in combination, until Passage 3. The effect of steroids on amplification as well as on EMT transition process were evaluated as previously report [6]. Pretreated steroid cells at Passage 4 were then exposed to an osteogenic inducing protocol [7] for 21 days in absence of steroids. Osteo and chondrogenic *in vitro* differentiation were evaluated as following: 1) Real Time PCR for chondrogenic (*SOX9*, *COL2A1* and *Aggrecan*) and osteogenic (*OCN*) genes; 2) Alizarin Red and Alcian-Blue stain. One-way ANOVA followed by Tukey's test were performed, *P< 0.05*.

RESULTS: Steroids did not affect oAECs proliferation. P_4 and E_2 treated cells maintained their epithelial native shape and did not undergo to EMT compared to CTR and E_2+P_4 treated cells that instead acquired the elongated mesenchymal morphology. Moreover, when cells were pretreated with P4 alone or in combination with E_2, they were induced to enhance osteogenic differentiation as confirmed by OCN gene overexpression (Fig. 1 and Alizarin staining). By contrast, E_2 pretreated cells, even if exposed to the osteogenic differentiation protocol, acquired a chondro-like morphology by increasing *SOX9*, *COL2A1* and *Aggrecan* chondrogenic gene expression (Fig. 1) as well as Alcian-Blue stain.

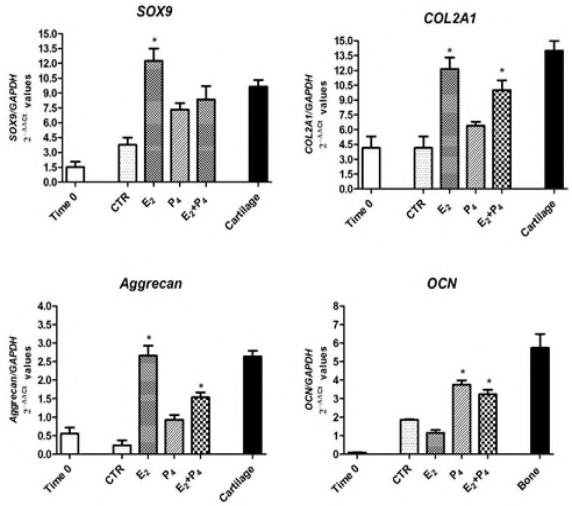

Fig.1: Chondrogenic and Osteogenic gene expression in CTR and pretreated oAECs at Passage 4 of culture in osteogenic media. P<0.05 vs CTR.*

DISCUSSION & CONCLUSIONS: Steroid treatments were able to modify oAECs plasticity. P_4 is essential to commit cells towards osteogenic differentiation, while E_2 pretreated cells enhance their chondrogenic attitude. E_2 treatments may represent a new strategy to improve the ability of an emerging source of stem cells, AECs, to support chondrogenic regenetration.

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Immunomodulation in osteoarthritis and cartilage repair

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INTRODUCTION: Macrophages play a role in the development of osteoarthritis. They are one of the cell types residing in synovium and intra-articular fat tissues. During osteoarthritis, new macrophages can infiltrate the synovium or the fat, attracted from the peripheral blood. Macrophages can acquire a spectrum of phenotypes with the pro-inflammatory or M1 macrophages and anti-inflammatory/repair or M2 macrophages on both ends of this spectrum, seeming very plastic. Stimuli from the environment can direct cells to these different phenotypes. Cytokines and growth factors are the usual suspects here, but extracellular matrix, immune complexes, bacterial fragments, and implanted biomaterials can also alter the phenotype of macrophages.

We have demonstrated that the secretome of explants of osteoarthritic synovium or infra-patellar fat pad tissue can influence cartilage, synovium or bone marrow derived stromal cells (MSCs). Macrophages from fat and synovium can stimulate inflammatory and degenerative processes in cartilage and fibrosis in synovium. They can also inhibit chondrogenesis of MSCs, all depending on the phenotype. When comparing the reaction of isolated tissue macrophages from osteoarthritic synovium or infra-patellar fat pad with the effect of peripheral blood monocytes that were differentiated to M1 and M2 macrophages in vitro, the effect of macrophages from osteoarthritic joint tissues was mostly comparable to the effect of M1 macrophages.

We are investigating the ability to modulate the macrophage phenotype in the tissue, aiming to modulate osteoarthritis or to create an environment that fosters tissue regeneration. First attempts are made in our lab to modulate macrophages residing in the synovium or infrapatellar fat pad using commonly used drugs. The results of these experiments are encouraging; Triamcinolone is capable of modulating macrophage phenotype in the tissue from pro-inflammatory to anti-inflammatory. Treatment of osteoarthritic synovium and infrapatellar fat pad explants with triamcinolone reduced the negative effects of these tissues on cartilage degeneration or MSC chondrogenesis.

Taken together, targeting macrophages seems a good strategy when aiming for regenerative therapies and possibilities seem to exist to improve this regeneration via the macrophage.

Immunomodulatory therapy for the intervertebral disc

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INTRODUCTION: Intervertebral disc (IVD) degeneration and aging evidence changes at the cellular level and un unbalanced matrix remodelling, that ultimately culminates in disc herniation. Although not always evident, an exacerbated inflammatory response has been strongly associated with IVD degeneration (Molinos et al. 2015).

Human IVD degeneration is often studied using 2D cultures of IVD cells or animal models which lack the biped position and have an increased number of notochordal cells. 3D cultures are more physiological and reliable systems than traditional 2D cultures and are essential to study nucleus pulposus cells behaviour since these cells lose their capacity of produce native extracellular matrix (ECM) in 2D.

A pro-inflammatory/degenerative IVD organ culture model from bovine origin was previously established by our group in collaboration with the Institute of Orthopedic Research and Biomechanics, University of Ulm, Germany (Teixeira et al, 2015). This model has been posteriorly validated, and anti-inflammatory nanoparticles developed by our group were able to reduce inflammation in IVD while promote cartilaginous ECM (Teixeira et al, 2016).

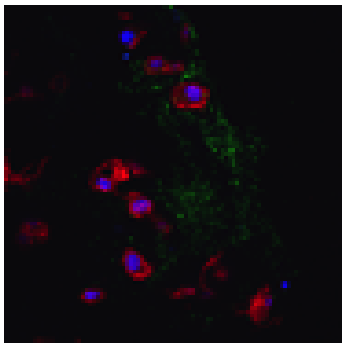

Figure 1: Chitosan-FITC/Poly-γ-glutamic acid nanoparticles (green) in a pro-inflammatory/degenerative IVD organ culture of bovine origin. IVD cells cytoskeleton is stained with Phalloidin (red) and nuclei with DAPI (blue) (image from Teixeira G et al, Acta Biomat, 2016).

In the last years, the immunomodulatory role of mesenchymal stem/stromal cells (MSCs) has been emerging in numerous clinical contexts, and particularly in the context of LBP and IVD degeneration. Recently, we have also shown that MSCs in the presence of the pro-inflammatory/ degenerative microenvironment of the IVD do not recover the unbalanced ECM, but instead present a strong immunomodulatory activity on IVD cells (Teixeira et al, 2017). This effect seems to related with an increasing pro-inflammatory profile of MSCs and seem to be via a paracrine mechanism. Moreover, MSCs systemic administration (intravenous) in a rat model of punctured IVD, reduced IVD herniation while up-regulated anti-inflammatory cytokines in the plasma, also suggesting an immunomodulatory potential of MSCs in this context (Cunha et al, 2017).

Still, a huge number of challenges in the field remain to be addressed. This presentation will focus on an immunomodulatory nanotechnology-based therapy for degenerated IVD and on the immunomodulatory role of MSCs in this context. Furthermore, the presentation will also bring new insights on the development of more advanced models of human IVD degeneration and associated inflammatory response and also on the deeper knowledge of IVD cell subsets that could contribute to regulate the inflammatory response associated with IVD degeneration.

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Influence of Substance P and αCGRP on articular chondrocytes from osteoarthritic patients

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INTRODUCTION: Osteoarthritis (OA) is the leading cause of disability and chronic pain in the elderly. To date pharmacological interventions can only relieve pain, whilst cell- and compound based therapies have limited success in the regeneration of damaged tissues (1). Thus, there is a need for identification of novel targets for diagnostic and therapeutic approaches. The joints are innervated by calcitonin gene-related peptide (αCGRP) - and substance P (SP) positive sensory nerve fibers which are a potential source of tibial-femoral pain during OA pathogenesis. Alteration of sensory joint innervation might be partly responsible for degenerative changes which contribute to development of OA (2). We aim to analyze the effects of SP and αCGRP on the metabolism of articular OA-chondrocytes.

METHODS: Human chondrocytes from OA patients were expanded for 1 passage and stimulated with SP or αCGRP (10^{-8}M and 10^{-10}M) in 2D- and 3D-cell culture systems. Subsequently, proliferation was analyzed via BrdU assay, apoptosis via Caspase 3/7 activity assay and flow cytometric Annexin V measurement, senescence via ß-galactosidase assay, adhesion ability with crystal violet staining, gene expression of marker genes with qPCR and activation of signaling pathways with western blot and ELISA. GAG concentration was evaluated via DMMB assay.

RESULTS: Stimulation with SP (10^{-8}M) for 1 day resulted in a significant increase in apoptosis, in senescence and in gene expression of the TNFα, IL-6 and for IL-1 by trend. Stimulation with a lower SP concentration (10^{-10}M) increased apoptosis after 3 days stimulation, but accelerated also proliferation. αCGRP in both concentrations (10^{-8}M and 10^{-10}M) decreased adhesion ability on cell culture plastic and increased senescence. Dose-dependently αCGRP (10^{-8}M) induced proliferation rate, whereas αCGRP (10^{-10}M) increased apoptosis. Chondrocytes had increased intracellular cAMP-levels after stimulation with αCGRP. Phosphorylation of ERK1/2 was induced after stimulation with both neuropeptides in both concentrations. Cells cultured in fibringels for 21 days showed decreased GAG production after 7 and 21 days, when treated with αCGRP (10^{-10}M).

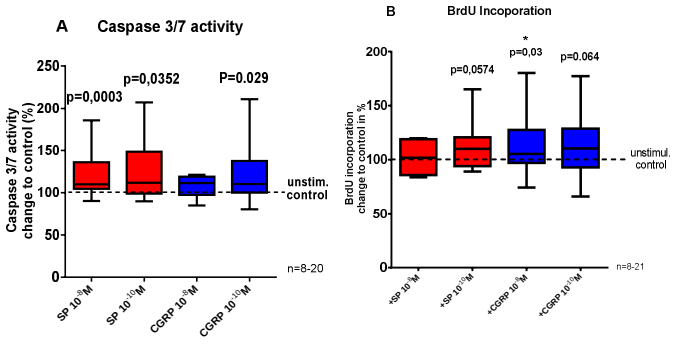

Fig. 1:Metabolic parameters of chondrocytes
A) Caspase 3/7 activity after 1 day stimulation of chondrocytes with SP and aCGRP
B) BrdU incorporation into chondrocytes after 3 days stimulation with SP and aCGRP.

DISCUSSION & CONCLUSIONS: We observed for both neuropeptides dose-dependent effects on chondrocytes. SP exerted a rather catabolic influence on the cells by inducing the expression of pro-inflammatory genes, apoptosis and senescence, whereas αCGRP affected the cells in an ambivalent manner by enhancing proliferation and decreasing adhesion, but also by increasing apoptosis and senescence. These different effects of SP and αCGRP seemed to be at least in part, mediated via the same signalling pathways (ERK1/2), whereby only αCGRP increased cAMP concentration. Determination of involved signalling pathways and there interactions are a prominent focus of this study.

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ACKNOWLEDGEMENTS: This template was modified with kind permission from eCM Journal.

Role of TLR2 in degenerating articular cartilage from adolescent idiopathic scoliosis patients

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INTRODUCTION: Adolescent idiopathic scoliosis is a 3-dimensional deformity of the spine which affects 2-4% of children aged 10 to 16[1]. It has been shown that abnormal biomechanics can drive articular cartilage degeneration and osteoarthritis but it has not been well characterized in the context of scoliotic facet joints. In this study, we characterize scoliotic facet joint cartilage for signs of osteoarthritis (OA) and evaluate the role of Toll-like receptors (TLR) in cartilage degeneration.

METHODS: Facet joints from scoliotic and non-scoliotic organ donors were collected with consent and ethical approval. Cartilage tissue was dissected from the bone and either 1) cultured in media and fixed for histology 2) digested with collagenase type II for chondrocyte isolation or 3) extracted using guanidium hydrochloride. Proteoglycan content, cell density and secreted degenerative factors were assessed by Safranin O, Hematoxylin and MMP3, MMP13, IL-1B and IL-6 IHC respectively. Extracellular matrix (ECM) fragmentation was evaluated by Western blot. Gene expression analysis was used to assess TLR expression and degenerative factor expression after TLR activation using a synthetic agonist Pam2Csk4 (Invivogen).

RESULTS: Histological analysis of scoliotic and non-scoliotic cartilage revealed significantly lower proteoglycan content with significant increase in OARSI score in scoliotic tissue (fig 1). Scoliotic cartilage also displayed higher cell density and increased expression of MMP3, MMP13, IL-1B, IL-6, IL-8. Furthermore, the presence of ECM protein fragmentation of CHAD, Decorin, fribromodulin, biglycan was evident, especially in patients with spinal curvatures above 70°. TLR2 gene expression was significantly increased in scoliotic samples and the expression was directly correlated to MMP3, MMP13, IL-1B, IL-6, IL-8 expression. When activated with a TLR2 agonist, scoliotic chondrocytes responded stronger than non-scoliotic chondrocytes with significantly higher MMP3, MMP13, IL-1B, IL-6, IL-8 as determined by gene expression and ELISA analysis. Finally, *ex vivo* cartilage biopsies confirmed an enhanced response to TLR2 agonist exposure.

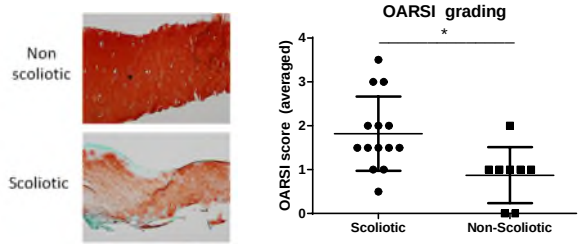

Fig. 1: A) Safranin O histology of both facet joint cartilage from one vertebra. B) Averaged OARSI scoring of lumbar facet joints

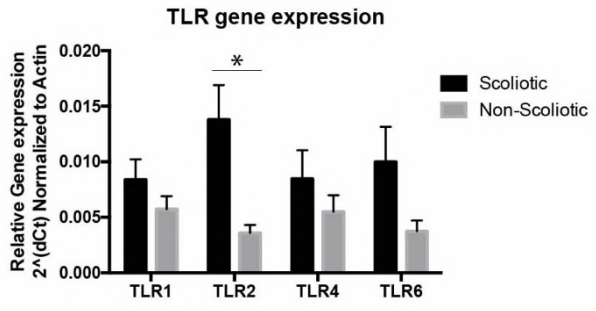

Fig. 2: Gene expression analysis of select TLRs in scoliotic and non-scoliotic chondrocytes.

DISCUSSION & CONCLUSIONS: Facet joint cartilage form young individuals with AIS show clear signs of OA with higher OARSI score compared to healthy non-scoliotic tissues. The cartilage also displayed higher and side to side variation in cell density. The finding of elevated TLR2 levels and direct correlation with proteases and pro-inflammatory factors makes TLR2 an interesting potential therapeutic target not only in scoliosis but also relevant in other articular cartilage exposed to abnormal loading.

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Mechanical loading inhibits inflammatory signaling via HDAC6-dependent modulation of tubulin and primary cilia elongation

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INTRODUCTION: Physiological mechanical loading reduces inflammatory signaling in a variety of cell types but the underpinning mechanism remains unclear. Studies by the authors have shown that primary cilia and/or the associated intraflagellar transport (IFT) genes, are required for inflammatory signaling in response to the cytokine IL-1β[1]. This study investigates the mechanism through which mechanical loading inhibit inflammatory signaling in articular chondrocytes and the role of the primary cilium.

METHODS: Primary articular chondrocytes were treated with and without IL-1β (1ng/ml) in unloaded conditions and with 0-10% tensile strain (0.33Hz, 24h) in the presence or absence of HDAC6 specific inhibitor, tubacin (0.5μM). The Griess assay was used to measure nitric oxide release. Primary cilia were labelled for acetylated α-tubulin and Arl13b and visualized by confocal and super resolution microscopy. HDAC6 activation and tubulin expression were measured by a commercial kit and western blot, respectively. Immortalised chondrocytes from Tg737^{ORPK} mice with hypomorphic mutation of IFT88 (ORPK cells) and wild types (WT) were used to further assess the role of IFT and primary cilia.

RESULTS: In isolated chondrocytes, mechanical loading blocked the normal inflammatory response to IL-1β characterized by the release of nitric oxide (Fig. 1A). Loading also blocked release of PGE_2 and had a similar anti-inflammatory response in cartilage explants (data not shown). This was associated with inhibition of elongation of primary cilia induced by IL-1β (Fig. 1B). Loading activated HDAC6 thereby preventing IL-1β-induced tubulin acetylation and reducing the pool of non-polymerized tubulin required for cilia elongation. HDAC6 inhibition with tubacin blocked the anti-inflammatory effects of loading and restored primary cilia elongation in the presence of IL-1β (Fig. 1A and B). Hypomorphic mutation of IFT88 disrupted ciliogenesis, reducing the inflammatory response to IL-1β and blocking the anti-inflammatory effect of loading.

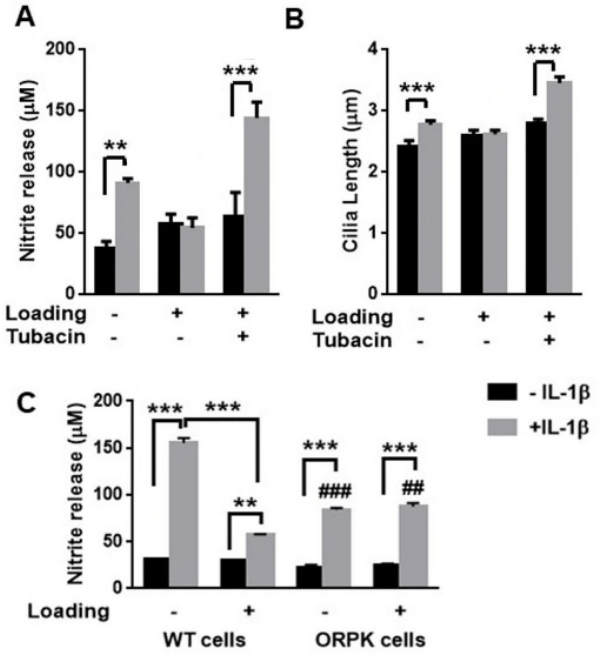

Fig. 1: Mechanical loading blocks the response to IL-1β in terms of (A) pro-inflammatory nitrite release and (B) primary cilia elongation. These effects of loading are dependent on HDAC6. (C) Hypomorphic mutation of IFT88 supresses blocks mechanoregulation of IL-1β signaling.

DISCUSSION & CONCLUSIONS: We demonstrate that loading reduces the inflammatory response to IL-1β via an IFT-dependent pathway regulated by mechanical activation of HDAC6. Associated post-transcriptional modulation of tubulin inhibited primary cilia elongation, potentially influencing ciliary IFT via the differential cargo-loading model. We thereby reveal a novel mechanism through which loading regulates inflammatory signaling associated with modulation of primary cilia elongation.

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Additive Manufacturing of Cartilage Tissues: Promises and Challenges

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INTRODUCTION: Bioprinting's great promise lies in delivering living tissue replacements which are personalized in terms of geometry, material properties and the patient's own cells. Current bioprinted constructs are fabricated from relatively inert and soft biomaterials, limiting their performance in *in vivo* weight-bearing applications. Further these materials undergo an uncontrolled amount of shape deformation upon crosslinking and culture.

New generations of bioinks are needed to increase the biological, printing and mechanical properties of bioprinted tissues. Our laboratory has followed several directions, including incorporation of extracellular matrix signals into the bioink and use of nanoparticle-containing composite bioinks to enhance mechanical properties and printing fidelity (1, 2, 3). Other strategies use of thermoresponsive polymers (4) to increase the viability of resident cells. We are also developing the extruder hardware and shape retention algorithms to increase reliability of the printing process. These approaches take us steps closer to fabricating bioprinted tissue analogues which could be one day used in patients.

METHODS:. Two classes of bioinks are described. The first is an enzymatically crosslinked bioink based on high molecular weight hyaluronan modified by peptide subtrates for transglutaminase. Cellulose and poly-(N-acetyl glucosamine) nanofibrils were added to obtain zero shear yield points and shear thinning properties. In the second example, bioinks based on anionic biopolymers were converted to nanocomposites through the addition of amine functionalized nanoparticles which could participate in physical crosslinking. Rheological measurements of all bioinks were performed using an Anton Paar MCR 301 rheometer with a PP20 probe. Crosslinking kinetics were characterized by measuring the storage and loss moduli of the gels in 1% shear strain and 1 Hz.

To study the reliability and reproducibility of the printing process, a progressive cavity pump was incorporated into the bioprinting workflow. The volume of material deposition was compared to the performance of the same bioink extruded with a pneumatic time-pressure systems.

RESULTS: The bioinks were fully biocompatible and allowed cell proliferation and matrix production to the extent that the *in vitro* properties matured to that approaching native cartilage after 2 months. Also, shape retention algorithms showed a good fidelity of the bioprinted structures compared to that of the original CAD model. Finally, comparing the extruded weight of printed structures, we found that extrusion using progressive cavity pumps was far more accurate compared to time-pressure systems.

DISCUSSION & CONCLUSIONS: The use of commercialized bioprinted products in the clinics is still a long way off. We present a developmental and regulatory roadmap showing the necessary steps to achieve successful translation of this technology.

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Regeneration of the IVD- Priming and conditioning of cells to function in the disc microenvironment

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INTRODUCTION: Degeneration of the intervertebral disc (IVD) occurs over many years and is influenced to an extent by genetic, environmental and physicochemical effects. To reverse or delay the onset of degeneration, injectable cell-based therapies may offer significant potential to repair or regenerate damaged tissues of the IVD. However, for normal cellular function and tissue regeneration, the local physicochemical microenvironment cells will experience once implanted is critical and an important factor that may limit the potential and success of any cell-based regeneration strategy.

The microenvironment of the human IVD is characterized by reduced oxygen, reduced glucose, matrix acidity, elevated levels of pro-inflammatory cytokines as well as being subjected to mechanical loads presenting a challenging microenvironment for normal cell function. As the IVD is avascular, the surrounding blood vessels in the cartilage end plates (CEP) and vertebral bodies supply vital nutrients to the disc primarily through diffusion. Balance between nutrient transport and cellular consumption rates establishes concentration gradients throughout the disc of these nutrients and metabolites which in turn markedly affects viability, proliferation and function of cells, Unsurprisingly, different cell types respond differentially to varying environmental conditions due to altered metabolic activity.

Understanding the demands and requirements of transplanted cell populations depending on the severity of degeneration will be a critical step to enable strategic decisions for determining effective clinical translation concomitant with the identification of an appropriate target patient cohort. For example, for an injectable cell therapy, it is unclear how many cells are required or could sustain and function in the compromised physicochemical microenvironment of an individual patient. Indeed, based on the nutritional concentration gradients in the disc and differences in metabolic profiles of various cell types, this may need to be tailored for specific patient cohorts.

In addition, in vivo challenges remain with respect to providing injected cells the necessary biochemical and exogenous growth factor stimuli to regulate inflammation and activate or promote in situ matrix formation. Given the regulatory challenges in the translational use of growth factors in vivo, a potential strategy to enhance regeneration may involve priming or conditioning of cells to attain the desired phenotype prior to transplantation thereby overcoming such limitations.

With these critical translational challenges in mind, this talk will outline emerging strategies that include cellular microencapsulation, in vitro priming and conditioning of microencapsulated cells, measurement of metabolic profiles and integration of in silico predictive models. It is envisaged combining these approaches will enhance our understanding and elucidate how the delivery of exogenous cells may alter the nutrient-metabolite milieu that exists in degeneration and assist in the design of patient specific or personalized therapies to suit unique and distinct microenvironments as part of a targeted approach for IVD tissue regeneration.

ACKNOWLEDGEMENTS: Work supported by Science Foundation Ireland Career Development Award (15/CDA/3476) and Irish Research Council Postgraduate Scholarship (2014-2018), Grant No: 202860/13027.

In vivo tracking of mesenchymal stem cells after intra-articular delivery

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INTRODUCTION: Articular cartilage degeneration remains a major clinical challenge in our society, because cartilage tissue has only a poor self-renewal capacity. Mesenchymal stem cells (MSCs) represent a potential therapy for treating cartilage diseases due to their capability to differentiate into several cell lineages and to secrete site-specific bioactive factors. Intra-articular (IA) injection of MSCs appears to be an effective therapy, promoting articular cartilage repair. Nevertheless, due to the lack of appropriate cell tracking models, the fate of MSCs after implantation is still unclear. This study aims at evaluating the biodistribution of MSCs at several time points after IA injection, employing a novel immunocompetent transgenic rat model[1].

METHODS: The animal model is based on human placental alkaline phosphatase (ALPP)[2]. This protein is a heat-stable enzyme that can be easily tracked by histochemical staining. Bone marrow-derived MSCs were isolated from transgenic rats that ubiquitously express the genetic marker ALPP. A focal cartilage defect was surgically created in immunocompetent transgenic recipient rats, which express a heat-labile form of the protein ($ALPP^m$). ALPP-labelled MSCs were intra-articularly injected into the synovial cavity of $ALPP^m$ recipient rats, 2 weeks post-surgery. Transgenic recipient animals are tolerant to cells carrying the genetic marker ALPP since the two protein forms differ in only a single amino acid. ALPP-labelled MSCs were histochemically tracked in the knee joint and in other, distant organs.

RESULTS: Intra-articularly injected MSCs migrated to the cartilage lesion and engrafted within the injured tissue. ALPP-labelled MSCs were detected at the defect site, suprapatellar bursa and synovial cavity, 1 day after cell injection. Injected MSCs were also found in the synovial cavity and the cartilage defect, 1 week and 1 month after cell administration, although the number of MSCs decreased over time. A few ALPP-labelled MSCs were observed in pulmonary blood vessels, 1 day after injection, but only in one of the studied animals. We failed to detect MSCs in the heart, spleen or kidney, 1 day, 1 week and 1 month after cell delivery.

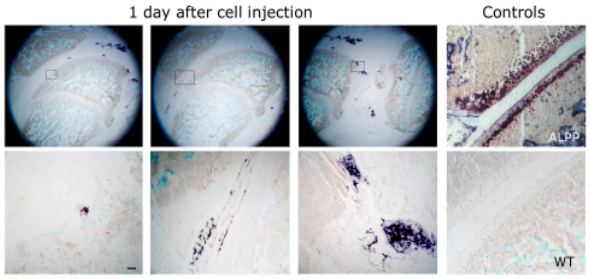

Fig. 1: ALPP staining of cryosections from $ALPP^m$ knee joints cryosections with a focal cartilage defect, 1 day post-injection, and ALPP and WT controls. Scale bar = 50 μm.

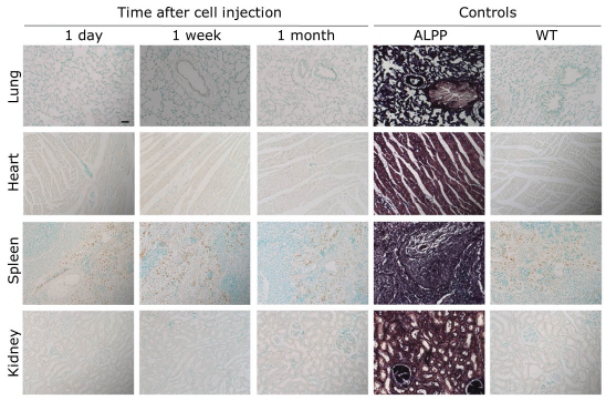

Fig. 2: ALPP staining of paraffin sections from various organs of $ALPP^m$ recipients injected with ALPP-labelled MSC, as well as ALPP and WT controls. Scale bar = 50 μm.

DISCUSSION & CONCLUSIONS: Most of the intra-articularly-injected MSCs were retained in the synovial cavity. However, the number of cells attached to the cartilage lesion was low, indicating poor engraftment efficiency. Only very few MSCs were detected in the lung, 1 day after injection, whilst no cells were found in any studied distant tissue at later time points. Taken together, our study supports the safety of using MSCs for cartilage repair via IA delivery.

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Evaluation of the stability and biocompatibility of tissue-engineered cartilage in a humanized mouse model

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INTRODUCTION: Autologous chondrocyte implantation and matrix-associated chondrocyte implantation are widely used for articular cartilage repair. However, these techniques create morbidity at the donor site that could be overcome by the use of allogenic chondrocytes. It has long been thought that the lack of vasculature in articular cartilage contributes to an immunologically privileged transplantation site that would allow implantation of cells from various sources without the risk of rejection. However, this view remains a matter of debate since the immune responses of tissue-engineered constructs with allogenic chondrocytes have been evaluated only in animal models, with conflicting results. Here we investigated the reaction of the human immune system against cartilaginous implants grafted to so-called humanized mice (hu-mice).

METHODS: NSG (NOD SCID gamma -/-) immunodeficient newborn mice were sub-lethaly irradiated and intra-hepathically injected with human HLA-A2+ CD34+ cells isolated from human cord blood. This cell transplantation results in development of a human adaptive immune system in mouse [l]. A prerequisite of the study was to identify the scaffold that would preserve the reconstructed cartilage from the infiltration of immune cells. Therefore, we first compared by immunohistochemistry (IHC) the responsiveness of two biomaterials used in clinics (collagen sponges or agarose hydrogels) that were grafted in hu-mice for 4 weeks. Then, in a second set of experiments, we used agarose hydrogels (a non-porous scaffold) combined with human articular chondrocytes (HACs) to reconstruct cartilage *in vitro* with a well-established protocol from our lab [2]. Particular attention was paid to HLA-A2 compatibility between donor (HACs) and recipient. Implantations were performed with HLA-A2+ chondrocytes implanted in HLA-A2+ mice or with HLA-A2- chondrocytes implanted in HLA-A2+ mice. Four weeks post-implantation, mice were sacrificed and the cartilaginous constructs were harvested for western-blotting and immunohistochemistry analysis. Spleen and blood samples were also collected for flow cytometry analysis.

RESULTS: Four weeks after implantation in hu-mice, acellular collagen sponges, but not acellular agarose hydrogels, showed positive staining for CD3 (T lymphocytes) and CD68 (macrophages), suggesting that this biomaterial elicits weak inflammatory response. These data led us to select agarose hydrogel for cartilage reconstruction. Four weeks post-implantation of HACs-agarose constructs in HLA-A2+ mice, the constructs maintained their structural integrity and no sign of local infiltration of T lymphocytes or macrophages was evidenced. In addition, there was no significant variation in subpopulations of T lymphocytes and monocytes present in peripheral blood and spleen. These results were obtained with both HLA-A2+ and HLA-A2- chondrocytes.

DISCUSSION & CONCLUSIONS: We have developed an original model system to evaluate acceptance of allogenic cartilage construct by the human immune system. Our results demonstrate that the use of allogenic chondrocytes is a reliable cell source to be combined with clinical-graded biomaterial for tissue engineering of cartilage.

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Effects of multi-axial loading on the intervertebral disc

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INTRODUCTION: The intervertebral disc is a fascinating structure. It connects two adjacent vertebrae while allowing complex motion between them. Along with the facet joints, they accommodate the variety of complex force and moment combinations, acting on the spine. Unfortunately discs can fail due to mechanical overload or degeneration changing their biomechanical properties and behaviour. Failure can be caused by short duration overloading, which can cause irreparable structural damage. Sustained loading as well as cyclic loading may initiate the degeneration process or cause steady accumulation of micro injuries leading to internal derangements.

Finite element studies indicate that the risk of prolapses is highest in the posterior and posterolateral annulus, especially in non- and mildly degenerated discs. High shear load could be calculated at the endplate (Schmidt et al. 2007). Moderate or strongly degenerated discs have a lower risk of prolapse. The biomechanical mechanism of lumbar intervertebral disc herniation is still not fully understood. It has been shown recently (Rajasekaran et al. 2013) that in vivo herniations result from anular failure (AF, 35%) or endplate junction failure (EPJF, 65%). Both cases have been reproduced in vitro (Wilke et al. 2015) with complex loading in a new dynamic disc loading apparatus.

METHODS: Several experiments have been performed using lumbar spinal segments from mature ewes. They were subjected to different complex loading conditions. Prior and following testing the discs were scanned with ultra-high field MRI (11.7 T) to identify the effects of testing on disc structure. They were then fixed and decalcified to enable cryosectioning into 30 µm sagittal sections, which were then analyzed microstructurally with light microscopy.

RESULTS: Some discs suffered herniation, other annular failure, involving subligamentous herniation of inner disc material or just disruption of the annular wall as judged by examination of the high resolution MRI images. Most interestingly, many discs that herniated contained minor irregularities in the posterior annular wall that were visible in the pretest MRI images as can be seen in the example in the Figure below.

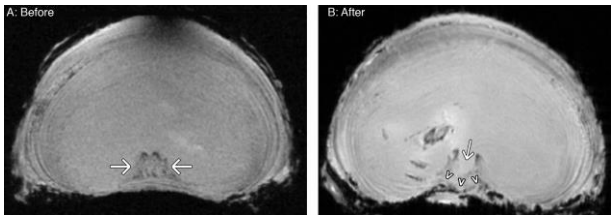

Fig. 1: Transverse 11.7T MRI section through an ovine disc prior to testing (A) with the irregularities in the posterior annulus (arrows). Following testing (B) inner disc material can be seen to have been extruded through this region (arrow) and to have been largely contained by the posterior ligament as indicated by the arrowheads.

The results indicate that irregularities in the lamellar structure are required for annular failure to occur under the loading conditions used in this study. Further, if such defects are present, herniation will initiate at the site of these irregularities when the disc is overloaded, likely because of the hydrostatic loading of the relatively healthy nucleus present in the tested discs.

DISCUSSION & CONCLUSIONS: Our dynamic six-degree-of-freedom disc loading device allows provocation of lesions, protrusions, herniations, and endplate avulsions. Different mechanisms for disc failure are possible. The failure mode is influenced by load combinations and velocity. Large deformations cause endplate junction failure. Additional high axial load cause disruptions which may result in herniations. Pre-existing defects increase the risk of disc failure. Velocity may have an effect on disc failure mechanism.

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Inducing chondrogenesis by multiaxial load: Cell differentiation and biomarker discovery

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INTRODUCTION: The emerging field of regenerative medicine aims to enhance regenerative medicine approaches by harnessing and enhancing the body's endogenous healing response by way of rehabilitation protocols after treatment. It has been defined as "Regenerative rehabilitation integrates regenerative technologies with rehabilitation clinical practices to restore function and quality of life in individuals with disabilities due to otherwise irreparable tissues or organs damaged by disease or trauma" [1]. It is widely accepted that the musculoskeletal system is strongly regulated by mechanical load, yet most new cartilage repair therapies are developed using static culture. However, it is clear that due to the critical role mechanics plays *in vivo*, a more physiological loading regime *in vitro* would be more appropriate, and this can be achieved by the use of bioreactors.

During kinematic motion, cells experience several different mechanical stimuli, hydrostatic pressure, tension, shear, compression or a combination of these. Maintenance of tissue homeostasis requires a basal level of stimulation to maintain the health of the tissue, with long term unloaded conditions such as bed rest known to have a detrimental effect. Regenerative medicine studies are frequently performed in the absence of these stimuli potentially making clinically relevant conclusions difficult. Incorporation of a bioreactor system into the study allows the composite effect of physical and soluble stimuli to be established. *In vitro* studies frequently rely on the application of exogenous growth factors. While this is a highly successful approach for tissue engineering and mechanistic studies, it does not investigate the endogenous source of these factors during normal healing.

Multiaxial load bioreactors can be utilized to this end, by mimicking the articulating joint, thus acting as an *in vitro* test bed for novel cartilage repair strategies. Using bioreactors it has been demonstrated that multiaxial load leads to an increase in the production, and activation, of endogenous TGF-β by mechanically stimulated cells [2]. The production and activation of TGF-β is a valuable output measure when studying the functionality of materials under loading conditions. This is providing further insights into novel material design for cartilage regeneration. We have also demonstrated that asymmetrical seeding of the construct, with a greater percent of the total cells being deposited in the superficial zone, leads to increased cartilage matrix deposition when using the same number of total cells. Deposition of both glycosaminoglycan and collagen II are increased in asymmetrically seeded scaffolds when compared to homogenously seeded scaffolds [3]. The applied load enables the production of growth factor gradients, and this induced anisotropy is an interesting example of naturally induced changes induced by physical loads.

In addition to the effect of load on direct differentiation, it is known that biomechanical stimulation can modulate the secretome of the cells. Investigating these changes may lead to new potential clinical targets, that may be presentation during articulation, to be identified. This offers new avenues for potential clinical therapies. One such marker is nitric oxide. Normally absent during static culture, it is found after the application of complex load. Whether this has a positive or negative influence, is still under investigation.

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Microarray analysis of the chondrocyte mechanome: The role of TRPV4

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INTRODUCTION: Transient receptor potential vanilloid 4 (TRPV4) is a mechanosensitive Ca^{2+}-permeable ion channel that regulates anabolic gene expression and increases mechanical properties and extracellular matrix biosynthesis in chondrocytes[1]. However, the gene pathways downstream of mechanical loading and TRPV4 activation are not well understood. The goal of this study was to identify the gene expression profiles of articular chondrocytes in response to mechanical loading or to chemical activation of TRPV4.

METHODS: Chondrocytes were isolated from porcine femoral cartilage and embedded in 2% agarose disks (4x2.25mm, 20 M cells/mL)[1]. After a 14 day preculture, disks were stimulated for 3 hr/day for 3d at 37°C/5%CO_2 with: (1) control, (2) 10% dynamic mechanical compression at 1 Hz, or (3) 1nM GSK1016790A, a TRPV4 agonist[1]. Disks were snap frozen in liquid N_2 at 0, 3, 6, 12, 20, 24, and 72 hours. Total RNA was extracted and analyzed using the Porcine Gene 1.0 ST Array (Affymetrix). Data were normalized using *'affy'* and *'oligo'* package, and differentially expressed genes (DEGs) were identified and analyzed using one regression model in R with package *'limma'*, *'RUV'*, *'splines'*, *'gplots'* and *'plotly'*. Then the DEGs were imported into IPA to perform the pathway enrichment analysis.

RESULTS: After 1 bout of loading or GSK101 stimulation, 43 genes were significantly regulated in both groups (Fig 1). The most highly upregulated common genes included transcription factors *NR4A2*, *NOR-1*, *C-JUN*, *KLF4*, and *ATF3* and TGF-β family members *FOS*, *INHBA* and *BMP2*. In both groups, expression was generally upregulated immediately after treatment and decreased to control levels over time. GSK101 stimulation yielded larger relative changes in gene expression and more significantly regulated genes (215 genes) than mechanical loading (54 genes). After repeated stimulation (at 0, 24, and 72 hours) 41 genes were activated by mechanical loading, 112 genes were activated by GSK101 stimulation, and 15 of these genes were common between both treatments.

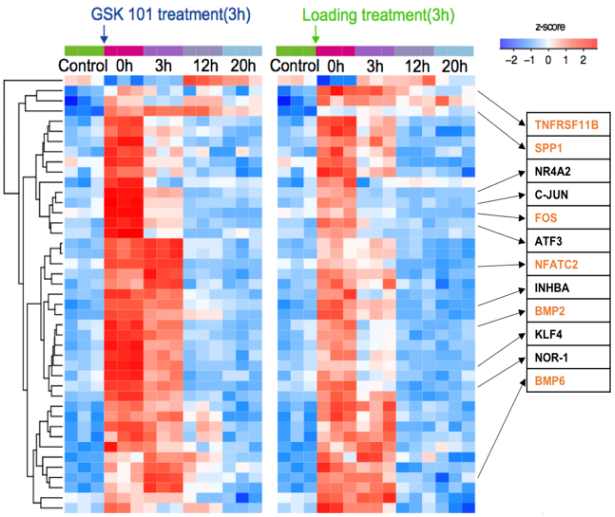

Fig. 1: Heat map showing gene expression changes (relative to group mean over time).

DISCUSSION & CONCLUSIONS: The finding of common genes upregulated by both mechanical loading and GSK101 treatment begins to reveal the pathways responsible for transducing mechanical signals through TRPV4 and may provide therapeutic targets to modulate chondrocyte mechanotransduction. In particular, we identified Ca^{2+}-regulated transcription factors (*FOS, ATF3, NR4A2,* and *NOR-1*)[2] and growth factors (*BMP-2,* and *BMP-6*)[3,4], likely involved in TRPV4-mediated mechanotransduction. Genes were also identified that have been shown to inhibit matrix metalloproteinases (*NR4A2*)[2,] and enhance chondrogenesis (*BMP-2, BMP-6*)[3,4]. The stronger responsiveness to GSK101 than mechanical loading is not unexpected given that GSK101 is a potent small molecule activator of TRPV4. Several genes were differentially regulated by mechanical loading compared to GSK101 stimulation, suggesting that TRPV4 activation may have regulatory effects beyond mechanotransduction.

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Mechanosensitive miR clusters regulated after loading of human engineered cartilage

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INTRODUCTION: Mechanical loading plays an important role in regulating the development and maintenance of cartilage. Dynamic compressive loading can support extracellular matrix (ECM) synthesis of cartilage, whereas permanent improper loading disrupts the ECM, softens the tissue and may lead to osteoarthritis (OA). MicroRNAs (miRs) are post-transcriptional regulators of gene expression which represent promising molecules to quickly adjust the cellular transcriptome in a stimulus-dependent manner. Regulation of several miR clusters was related to skeletal development, joint homeostasis and OA pathophysiology but whether some miRs are mechanosensitive in cartilage and involved in shaping the response to cyclic loading is so far unknown. [1]. We aimed to identify potential mechanosensitive miR clusters which may be characteristic for beneficial versus non-beneficial loading regimes and may serve as future diagnostic tools or targets for therapeutic intervention in joint diseases like OA.

METHODS: Human chondrocyte-seeded ($5x10^5$ cells) collagen scaffolds were connected to ß-tricalcium-phosphate to produce engineered cartilage under chondrogenic culture conditions. Cyclic unconfined compression protocols were established leading to an increase or decrease of proteoglycan synthesis after loading. Global miR microarray profiling was performed after exposure to the anabolic or catabolic loading protocol and selected miRs were re-evaluated in independently generated samples via qRT-PCR.

RESULTS: miR microarray profiling revealed only few consistent changes in miR expression (7 significantly upregulated miRs) in response to the anabolic loading protocol. In contrast, a significant regulation of 80 miRs occurred after non-beneficial stimulation. 25% of the miRs (n=20) were significantly upregulated, 10 of them > 2-fold. Hierarchical clustering of samples based on all tested miRs fully separated control from compressed samples indicating a robust and consistent miR-response after non-beneficial loading. Regulation of 8/14 miR was confirmed by qRT-PCR with mean amplitudes of up to 2.5-fold for catabolic loading. Cross-testing showed that 2 miRs were upregulated by both loading conditions while 6 were specifically elevated by the non-beneficial loading regime.

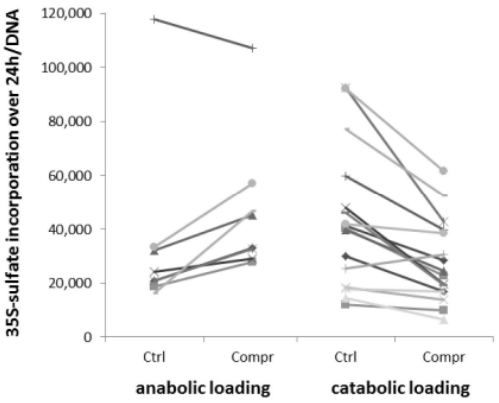

Fig. 1: GAG synthesis over 24 hours defining the anabolic versus catabolic loading conditions.

DISCUSSION & CONCLUSIONS: Conclusively, this study defines the first mechanosensitive miR cluster regulated in response to non-beneficial compressive cyclic loading of human engineered cartilage. Next step is to test whether these miRs are differentially expressed in healthy versus OA-affected human cartilage tissue.

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ACKNOWLEDGEMENTS: We thank the patients for providing primary sample tissue for isolation of human chondrocytes, the RMS Foundation for providing the β-TCP constructs and I. Heschel from Matricel for donation of the Optimaix scaffolds. Part of this study was supported by DFG-grant RI707/12-1 as part of the Excarbon Research Group FOR2407.

Importance of mechanical forces for intervertebral disc development: implications for engineered disc replacements

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INTRODUCTION: Disc degeneration is strongly implicated as a cause of low back pain [1]. Tissue engineered disc replacement is a promising potential therapy, but one of the major challenges is to engineer tissues with sufficient mechanical integrity to restore their function [2]. In this context, an understanding of the mechanisms underlying disc development might influence regenerative strategies. The current study investigates the effects of abnormal fetal movements on the developing discs and vertebrae. We hypothesise that fetal movements contribute to normal vertebral shape and disc development.

METHODS: Embryonic mice from a genetically modified line in which Pax3 gene was modified were harvested at Theiler stage TS27 (roughly 18.5 embryonic days). Pax3 mutation leads to abnormal musculature. In particular, several muscles that play a synergistic role in extension and lateral flexion of the spine are reduced or absent [3]. After dissection, whole spines were stained with Alcian blue (cartilage) and scanned in 3D using optical projection microscopy. Sagittal curvature and vertebral shape were compared between mutant and control mice. Disc development was characterised using histology. Cryosections were stained with Alcian blue (cartilage) and Picrosirius red (collagen), and the nucleus pulposus (NP), inner annulus fibrosus (IA), and outer annulus fibrosus (OA) were compared between mutant and control mice.

RESULTS: Control spines displayed regular vertebral shape and clear separation between vertebrae using optical projection microscopy (Figure 1A), while mutant spines displayed abnormal vertebral shape in some locations, resulting in vertebral fusion (Figure 1B). Several specimens showed abnormal sagittal spinal curvature in thoracic and lumbar regions (data not shown). Histological analysis revealed abnormal disc segmentation in the developing mutant spine. In controls, the NP was surrounded by the annulus fibrosus, with IA and OA exhibiting differences in structure (Figure 1C). When vertebral segmentation failed to occur in the mutant, annulus fibrosus formation was disrupted with a thinner IA and lacked its OA on the fused side (anteriorly in the example shown in Figure 1D).

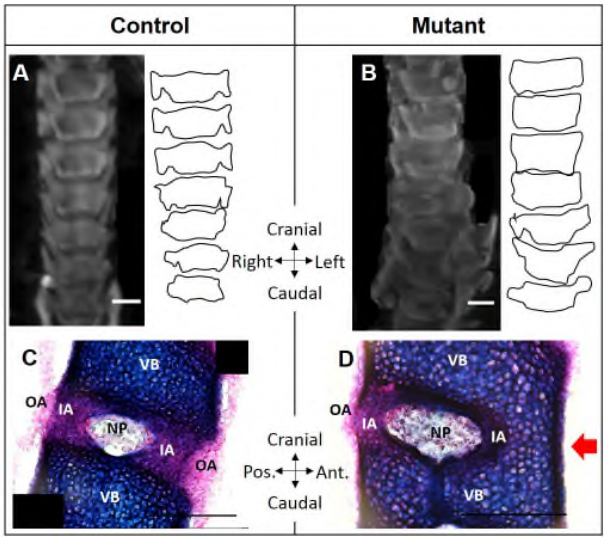

Fig. 1: 3D representations of lumbar spines and vertebral outlines in the frontal plane of control (A) and mutant (B) mice. Sagittal stained sections of cervical vertebrae and disc in control (C) and mutant (D) mice. Red arrow indicates vertebral fusion. Ant.: anterior; Pos.: posterior; VB: vertebral body. Scale bars: 500um.

DISCUSSION & CONCLUSIONS: This study indicates that abnormal muscle can have major consequences on intervertebral disc development, vertebral shape and segmentation, and spinal curvature. These findings emphasise the need for biomechanical stimuli in spinal development and future work will investigate underlying mechanisms and how abnormal muscle forces affect the disc over multiple developmental stages. These findings could provide clues for the refinement of tissue engineering therapies.

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Regulation of chondrogenesis by the long non coding RNA, *lncRNA-HIT*

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INTRODUCTION:

It is estimated that by 2040 nearly 78 million Americans 18 years of age and older will be diagnosed with osteoarthritis (OA), a degenerative disease of the articular cartilage (AC)[1] At the cellular level, AC is composed of highly stable populations of articular chondrocytes and extracellular matrix proteins. To date, the molecular mechanisms that regulate chondrocyte phenotypes within the AC are poorly understood, yet emerging evidence indicates that an epigenetic component, mediated by long non coding RNAs (lncRNAs), may play an essential role[2-3]. Our analysis of the programming events controlling chondrogenesis in the embryonic limb led to the identification of *lncRNA-HIT* a long noncoding RNA that is essential for the *in vitro* differentiation of the limb mesenchyme into cartilage. Analysis of *lncRNA-HIT* function indicates that it is retained in the nucleus where it recruits p100 and CBP to mediate H3K27ac at its chromosomal binding sites which genome wide analyses revealed to be multiple chondrogenic loci. Functional analysis using siRNA-mediated reductions in *lncRNA-HIT* or p100 transcripts revealed a significant decrease in H3K27ac modifications proximal to these chondrogenic loci, affecting gene expression and the *in vitro* differentiation of cartilage.

We now have data indicating that *lncRNA-HIT* may also be playing a role in the homeostasis of mature AC, as conditional loss of *lncRNA-HIT* in adult limbs results in decreased animal mobility and grip strength, two functional hallmarks of distal interphalangeal OA. Histological analysis of the phalangeal joints revealed irregular joint morphology and reduced Safranin O staining, suggesting that the proteoglycan composition of the AC was affected by the loss of *lncRNA-HIT*. qRTPCR and immunohistochemistry confirmed reduced levels of ACAN mRNA and protein in affected AC, suggesting that additional loci may be regulated by this lncRNA in the articular chondrocytes.

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Epigenetic regulation of cartilage

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INTRODUCTION: Chondrogenic differentiation of mesenchymal stem cells (MSC) is in part regulated by epigenetic mechanisms such as histone modifications and DNA methylation, along with gene regulation by long and short non-coding RNAs. Using a combination of ChiP-seq and global DNA methylation analysis of human MSC differentiation to 'chondrocytes' revealed a general demethylation, which is enriched at chromatin-state defined enhancer regions. Although less pronounced a similar finding occurred in osteoblastogenesis, but adipogenesis appears less dependent upon such epigenetic mechanisms. One of the most hypomethylated loci during chondrogenesis encompasses the microRNA miR-140; with our analysis of chondrogenesis indicating this chondrocyte-selective microRNA is the most critical for the process. We also find miR-140 to be differentially expressed in osteoarthritic compared to normal cartilage, and through small RNA-seq have identified various 'isomers' of the microRNA which are functional. Using CRISPR-Cas9 we have deleted miR-140, and a number of other microRNAs, in mice as a tool to further define their function and targets in vivo.

These data underline the importance of epigenetic regulation to chondrogenesis and such epigenetic changes have also been implicated in cartilage diseases such as osteoarthritis. Overall, we hope a greater understanding of the chondrocyte epigenome may have potential therapeutic value.

Endoplasmic reticulum stress: A possible contributor to the pathogenesis of degenerative disc disease?

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INTRODUCTION: Degenerative disc disease (DDD) is associated with increased expression of pro-inflammatory cytokines in degenerated intervertebral disc (IVD). However, it is not completely clear how inflammation arises in the IVD and which cellular compartments are involved in this process. Recently, endoplasmic reticulum (ER) stress has been associated with the harsh microenvironment of degenerated IVDs [l-2]. Therefore, the aim of this study was to analyse the role of ER stress in inflammatory responses of degenerated human IVDs and to identify related molecular mechanisms.

METHODS: IVD tissue was obtained from patients suffering from DDD. Gene expression of the ER stress marker GRP78 and the pro-inflammatory cytokines IL-6, IL-8, IL-1β and TNF-α was analysed in human surgical IVD samples (n = 50). Another set of human surgical IVD samples (n = 25) was used to prepare primary cell cultures. Primary cells were treated with ER stress inducer Thapsigargin (Thaps) and gene and protein expression of ER stress markers as well as IL-6, IL-8, and COX-2 were tested. Calcium depletion was measured by Fura-2 QBT Calcium Kit and cytotoxicity by LDH assay. The ability of IL-1β and TNF-α to induce ER stress was also analysed (gene, protein, calcium depletion).

RESULTS: The ER stress marker GRP78 was expressed in surgical IVD tissue and its expression positively correlated with IL-6, but not with IL-1β and TNF-α. The ER stress inducer Thaps (100 and 500 nM) activated gene and protein expression of IL-6 in primary IVD cells and induced phosphorylation of p38 MAPK. Both inhibition of p38 MAPK by SB203580 (SB, 10 μM) (*Fig. 1*) and knockdown of ER stress effector CHOP (siRNA 5 nM) partially reduced gene and protein expression of IL-6 in Thaps-treated IVD cells. IL-1β (5 and 10 ng/mL) activated gene and protein expression of GRP78, but did not influence $[Ca^{2+}]_i$ flux and expression of CHOP. TNF-α (5 and 10 ng/mL) did not induce ER stress. This could explain no correlation between GRP78 and IL-1β/TNF-α in human surgical tissues.

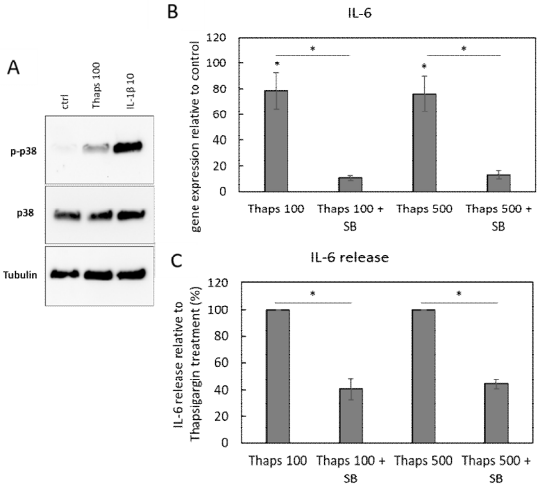

*Fig. 1: (A) ER stress inducer Thapsigargin (Thaps) activated p38 MAPK in primary human IVD cells; (B) Thaps induced gene expression of IL-6, while addition of p38 inhibitor SB203580 (SB) reduced it (shown relative to control set at 1); (C) SB also reduced secretion of IL-6 protein (shown relative to Thaps-treated cells set at 100). Data is presented as mean ± SEM, *indicates $p < 0.05$ (n = 4-7, one-way ANOVA with Tukey post-hoc test).*

DISCUSSION & CONCLUSIONS: Previous studies showed that ER stress can be activated in IVD cells by e.g. low pH or glucose. However, IL-1β and TNF-α alone may not be able to induce ER stress responses. This study provides evidence that once activated, ER stress can initiate IL-6 release in human IVD through p38 MAPK and CHOP. Therefore, therapeutic inhibition of ER stress could possibly be used in the future to counteract the consequences of the harsh microenvironment in degenerated IVD.

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Inhibiting Paracrine Senescence Improves Expansion and Chondrogenic Differentiation of human MSCs

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INTRODUCTION: Human bone marrow derived mesenchymal stem cells (MSCs) rapidly are a very promising cell source in regenerative medicine and cartilage repair. However, MSCs have limited expansion capacity since they rapidly undergo senescence and, in parallel, they lose their differentiation capacity[1,2]. Since senescence hampers the use of MSCs for cartilage repair, we investigated why senescence increases in vitro and ways to inhibit it.

METHODS: We compared senescence in MSC over time by determining cell cycle regulator expression, senescence-associated beta-galactosidase activity, telomerase activity and proliferation. We have previously shown that WNT3A maintains MSC expansion and chondrogenic differentiation potential[2]. Here we evaluated the effect of WNT3A (250ng/ml) on senescence and correlated senescence with the capacity to differentiate into cartilage. To distinguish cell-autonomous from paracrine effects, we analysed by proteomic and transcript analysis how the secretome of senescent MSCs affected the expansion of non-senescent MSCs. For transcript analysis we focused on the detection of genes known to be involved in senescence[3], such as *IGFBP4*, *IGFBP7*, *CCL2* and *IL6*. Since NF-KB inhibitors have been shown to repress a broad spectrum of SASP genes[4], we tested the effect of BAY11-7082 on senescence in MSCs.

RESULTS: In vitro MSCs became senescent at donor-dependent rates, but WNT3A treatment significantly slowed entry into senescence. However, no effect on telomerase activity or in cell cycle length was detected comparing the cells treated with or without WNT3A. However, all the senescent associated genes (SASPs) tested resulted to be 1.5 to 10 fold downregulated in MSCs treated with WNT3A compared to vehicle control (Fig 1A). Remarkably, a response to WNT3A only occurred when senescent cells were present in the culture (Fig.1B). Moreover, we found that WNT3A did not inhibit senescence cell-autonomously, but by modulating paracrine signalling of senescent cells. Medium conditioned by senescent MSCs induced senescence in recipient MSCs, abrogating proliferation. Medium conditioned by non-senescent MSCs did not induce senescence, indicating these secreted factors are senescent cell specific. After treating senescent MSCs with WNT signalling agonists, their secretome did not induce senescence. Interestingly, when we added the NF-KB inhibitor BAY11-7082 to the cultures we also obtain detected a reduction of paracrine senescence albeit too less extend than with WNT agonists.

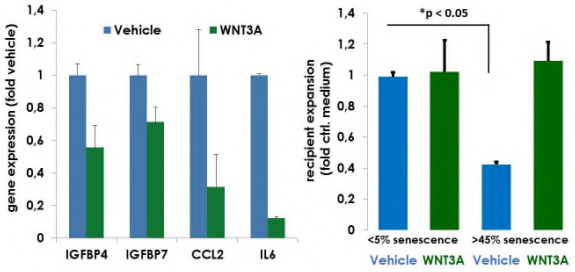

Fig. 1: (A, left panel) Gene expression analysis of senescent associated genes (IGFBP4, IGFBP7, CCL2 and IL6) in MSCs treated with or without WNT3A for 6 days. (B, right panel) Expansion rate of MSCs with different fractions of senescent cells cultured with WNT3A. SEM, n=3

DISCUSSION & CONCLUSIONS: Senescent MSCs secrete factors inducing senescence in surrounding cells, triggering a chain reaction that renders the entire culture senescent, thus unable the differentiation process. Preventing secretion of those senescence inducers halts the spread of senescence and therefore allows MSC expansion for cartilage repair.

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Profiling serum cytokines in patients with disc disease: Immune dysregulation and Disease Biomarkers.

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INTRODUCTION: Low back pain (LBP), the leading cause of disability worldwide is a symptom diseases, including intervertebral disc diseases. Inflammation contributes to the pathological processes of disc degeneration as well as discogenic pain mechanisms. Local responses of disc cells during degeneration lead to increased expression of pro-inflammatory cytokines. However, whether local disc changes are measurable in the periphery (i.e. in the circulation) is largely unknown. In clinical cross sectional studies, we discovered that patients with painful disc diseases have systemic inflammation, and that profiles of circulating serum cytokines, chemokines and growth factors vary by diagnosis. Since patient responsiveness to treatments of LBP vary widely, we investigated if serum cytokines change in response to treatment with epidural steroid injection (ESI). We also evaluated the potential for serum cytokines to be used as a predictive biomarker of treatment response.

METHODS: Subjects were consented and enrolled in this IRB approved study. Subjects were recruited from those receiving ESI treatment for a single level lumbar disc disease (N=55). Prior to treatment, blood samples and patient reported outcomes (PROMs) were evaluated for pain intensity (VAS), and disability (ODI) using questionnaires. Blood samples and PROMS were also collected after treatment at first follow-up. Control subjects, with no history of LBP, were also recruited as a control group (N=57). Study 1: Cytokine profiles (29 mediators) were measured in samples from patients with disc disease and was compared to levels measured in control subjects. Complete cytokine profiles were compared using principal component analysis (PCA). Study 2: Serum cytokines were compared in subjects receiving ESI, for change in cytokines from pre and post treatment. Cytokine changes were analysed pre to post treatment, and compared to VAS change. In addition, a predictive model was developed to predict response to treatment using baseline cytokine levels.

RESULTS: Study 1: PCA of serum cytokine profiles measured in control subjects vs. disc disease patients demonstrates independent clusters, indicative of unique circulating cytokine profiles. Top variables in each eigenvector are: PC1: T-cell cytokines, PC2: chemokines and DAMPs, PC3: MMPs, PDGFbb. Study 2: Pain outcome post-treatment significantly decreased vs. pre-treatment (9.5±0.9 to 5.5±2.9; p=0.0078), while ODI did not significantly change. A heterogeneity in VAS responses was observed, as expected, with 23 of 55 subjects reporting a positive clinical response (i.e. >50% decrease in VAS). In order to determine if VAS changes (% VAS difference) post treatment can be predicted by pre-treatment cytokine levels, each of the 54 cytokine levels measured pre-treatment were evaluated numerically for their contribution to a regression model. This approach yielded a highly predictive model (Adjusted R^2 = 0.87) using 12 independent variables (only 1 was related to patient factors (BMI) and the remaining 11 variables were cytokine levels at pre-treatment).

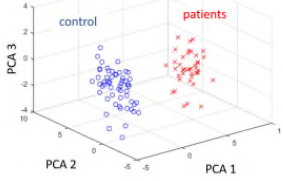

Fig. 1: PCA of serum cytokines measured in control (blue 'o') vs. LBP subjects (red 'x').

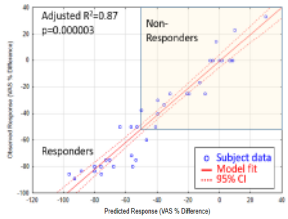

Fig.2: Predictive model of VAS response based on baseline serum cytokine levels.

DISCUSSION & CONCLUSIONS: The circulating immune system of patients with disc disease is highly dysregulated compared to control subjects. Using baseline cytokine levels, we are able to predict pain response to treatment with ESI with high predictive power. These findings support the approach and utility of serum biomarkers for improved treatment of LBP.

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Magnetic Resonance Elastography - A clinical tool for in-vivo characterization of the intervertebral disc's mechanical function

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INTRODUCTION: Low back pain (LBP) is the leading cause of disability worldwide and is strongly associated with degeneration of the intervertebral disc (IVD) [1]. Biologic based regenerative therapies are being developed to restore the function of the IVD as an alternative to surgical interventions [2]. However, determining when to intervene and assessing treatment efficacy in-vivo remain significant hurdles to widespread clinical implementation. In-vivo quantification of the IVD tissue's mechanical properties may allow earlier diagnosis of injury and disease and allow evaluation of treatment efficacy of therapies aiming to restore the tissue's mechanical function. Magnetic resonance elastography (MRE) is a non-invasive imaging technique that tracks propagating shear waves as they travel through soft tissues and allows measurement of the material's shear properties and the degree of internal damage. The development of MRE as a tool to quantify in-vivo mechanical function of the IVD is described.

METHODS: 80Hz mechanical vibrations were applied and tracked via a commercially available MRE driver and a custom gradient-recalled-echo MRE sequence. Single 2D transverse slices were acquired through the lumbar levels of 52 subjects of which 47 had no prior history of LBP [3] and 5 had previously had surgery. All protocols were approved by the OSU investigational review board. The shear stiffness was assessed within the nucleus pulposus and annulus fibrosus regions via a principle frequency analysis (PFA) in asymptomatic subjects and the internal tissue integrity was assessed in the surgical subjects via an octahedral shear strain (OSS) analysis. Pfirrmann degeneration grade was assessed via a sagittal T2 weighted sequence.

RESULTS: MRE-derived shear stiffness of healthy subjects without LBP significantly increased with IVD degeneration (Table 1). OSS maps were generated for operated and non-operated adjacent control IVDs. 'Injured' IVDs had significantly increased average OSS values compared to controls (Control OSS: 1±0.22 / Injured OSS: 1.64±0.64) and had wave discontinuities consistent with a potential surgical path (Fig 1).

Table 1. Tissue stiffness increases with IVD degeneration, adapted from [3]

Pfirrmann Degeneration Grade	Number of IVDs (N)	Nucleus Pulposus (NP)		Annulus Fibrosus (AF)	
		Stiffness (kPa)	Stats*	Stiffness (kPa)	Stats*
1	11	12.50 ± 1.30	A	90 39 ± 9.26	A
2	132	13.93 ± 2.12	B	98.89 ± 12.57	B
3	53	14.25 ± 2.17	BC	99.69 ± 14.07	B
4	31	15.07 ± 2.51		106.02 ± 17.88	C
5	3	16.46 ± 2.06		120.13 ± 15.36	C

*Significant dill. btw different letters: one-way ANOVA tukey post-hoc

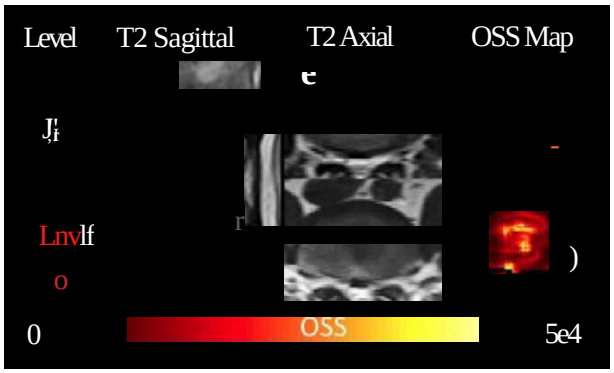

Fig. 1: OSS maps showing wave discontinuities in operated IVDs and not in adjacent un-operated controls.

DISCUSSION & CONCLUSIONS: MRE-derived shear stiffness values were able to differentiate the two earliest stages of degeneration, important for stratifying disease. The combined assessment of region specific shear stiffness and internal wave discontinuities as an analogue for tissue damage suggests that MRE may be a useful clinical tool for quantifying IVD mechanical function.

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Deriving nucleus pulposus-like progenitors from MAP Kinase Interference Coupled Chondrogenic Induction in mesenchymal stem cells

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INTRODUCTION: Depletion of extracellular matrix producing nucleus pulposus (NP) cells in intervertebral disc (IVD) degeneration limits the self-repair ability *per se*. Currently, tyrosine kinase receptor 2 (Tie2) and disialoganglioside 2 (GD2) expressing progenitor cells were identified in human adult NP tissue based on *in vitro* clonogenicity and *in vivo* self-renewal ability. [1] By microarray analysis of the transcriptome in rat primitive NP cells (PNPCs) and chondrocytes, we identified preferential expressions of *Cdh2*, *Krt19* and *Car3* in PNPCs. Further *in silico* analysis of signaling pathways suggested PNPCs exhibiting low TGF-β and MAP kinase activities. Mesenchymal stem cells (MSCs) were demonstrated to differentiate into NP-like cells via TGF-β stimulation. Here, we hypothesized that MAP kinase interference coupled chondrogenic induction (MICCI) of MSCs could differentiate into NP progenitor-like cells. To this end, we characterized protein expressions of defined molecular markers in Colonies Formation Units-Spherical (CFU-S) from human NP cells. Thereafter, we evaluated gene expressions of CFU-S molecular markers in MICCI of MSC micro-pellets.

METHODS: CFU-S assay was accessed by seeding a single-cell suspension of 1 x 10^3 human NP cells in 1ml of methylcellulose medium (*Stem Cell Technologies*) for 14 days. Immunostaining of cyto-spun CFU-S were performed with rabbit anti-Tie2 (*Santa Cruz*); mouse anti-GD2 (*BD biosciences*); rabbit anti-Cdh2 (*abcam*); goat anti-Krt19 (*Santa Cruz*); goat anti-Car3 (*Santa Cruz*); mouse anti-CD24 (*abcam*); rabbit anti-type II collagen (*abcam*) and rabbit anti-aggrecan (*abcam*) antibodies. TGF-β1-based chondrogenic induction of human bone marrow MSC micro-pellet culture (2 x 10^5 cells) were treated with or without 10 ng/ml inhibitors of MEK1/2 (PD98059) for 14 days. Quantitative PCR (QPCR) was performed using Taqman probes (*Thermo Fisher scientific*): *TEK (*HS00945746_m1); *CDH2* (HS00983056_m1); *AGC1* (HS00153936_m1); and normalized by *GAPDH* (HS02758991_g1).

RESULTS: By immunostaining, previously reported markers: Tie2, GD2, aggrecan (AGC1) and type II collagen (COL2) were detected in human NP derived CFU-S. Cadherin 2 (CDH2), but not for keratin 19 (KRT19), carbonic anhydrase 3 (CAR3) and CD24, were detected in CFU-S. QPCR showed a strong upregulation of *TEK1*, *CDH2* and *AGC1* in MEK1/2 inhibited MSC micro-pellets after Day 7 and onwards (Fig. 1A). Enhanced proteoglycan deposition was also detected in MEK1/2 inhibited MSC micro-pellets (Fig.1B).

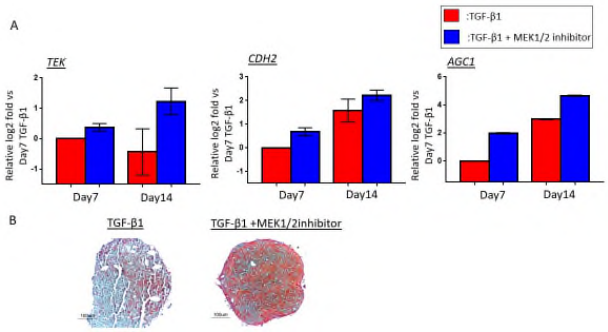

Fig 1. MICCI of MSC micro-pellets expressed NP progenitor-like phenotypes. (A) QPCR data shown an induction of *TEK*, *CDH2* and *AGC1* in MEK1/2 inhibited MSC micro-pellets. (data were shown as mean ± SEM) (B) Safranin O staining of MSC micro-pellet at Day 14. Red stains indicated proteoglycan deposition.

DISCUSSION & CONCLUSIONS: Tie2, GD2, COL2, AGC1 and CDH2 were characterized as putative molecular markers for human NP derived CFU-S. MICCI of MSC micro-pellets could strongly induce CFU-S molecular markers with robust proteoglycan deposition. This study provides a simple, yet effective protocol in the generation of NP progenitors from MSCs for IVD engineering.

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Hypoxia in degenerative intervertebral disc promotes neurite outgrowth

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INTRODUCTION: Chronic low back pain is one of the most common problems worldwide and is frequently correlated with pathologies of the intervertebral disc (IVD). Deeper neural ingrowth of IVD from dorsal root ganglia (DRG) was found for painful IVDs [l]. However, how the IVD microenvironment influences neurite outgrowth remains largely unknown. Previous studies suggest decreased oxygen, glucose and pH concentrations for the degenerative IVD. Oxygen is a key player in the homeostasis of tissues [2]. In this study, we investigated how the neurite outgrowth was influenced by the oxygen level.

METHODS: DRG neuronal cell line ND7/23 was cultured under both 2% (hypoxia) and 20% (normoxia) of oxygen for 48 hours. The viability was evaluated by both Live/dead and Hoechst nuclei staining. Another culture under hypoxia/normoxia was imaged to evaluate neurite outgrowth, and only those protrusions longer than 30 μm were defined as neurites. Proportion of cells that showed outgrowth and median length of outgrowth were assessed. In addition, rabbit spinal DRG explants were cultured under hypoxia and normoxia for 4 days. The two groups had DRGs from the same segment and were of similar size. Immunofluorescence was used to evaluate the number and length of neurite outgrowth and Hoechst staining for evaluating the glial migration from the DRG. Images were analysed using ImageJ. Statistical analyses were performed with SPSS (level of significance: $p<0.05$).

RESULTS: Hypoxia significantly reduced cell death (4.84 ± 0.59%) compared to normoxia (7.32 ± 1.17%). The proportion of cells that showed outgrowth was significantly lower for hypoxia than normoxia (37.71% and 46.54%, respectively, chi-square); however, the median length of outgrowth was significantly higher under hypoxia than normoxia (46.23 ± 1.49 μm and 43.21 ± 0.71 μm, respectively). For the DRG organ culture, the number of outgrowth for hypoxia was significantly lower than for normoxia (median number of 5.00 ± 3.04 versus 55.00 ± 9.92, respectively), but the median length of outgrowth under hypoxia was significantly larger than normoxia (228.63 ± 18.32 μm and 177.82 ± 5.58 μm, respectively).

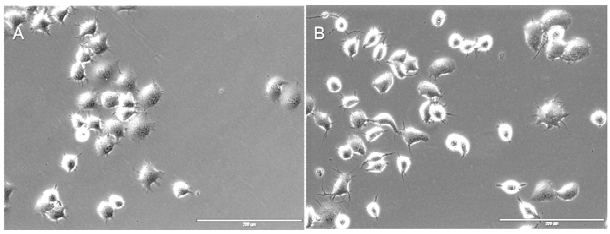

Fig 1: Phase contract showing outgrowth of ND7/23 under (A) 2% of oxygen and (B) 20% of oxygen. Scale bar = 200 μm.

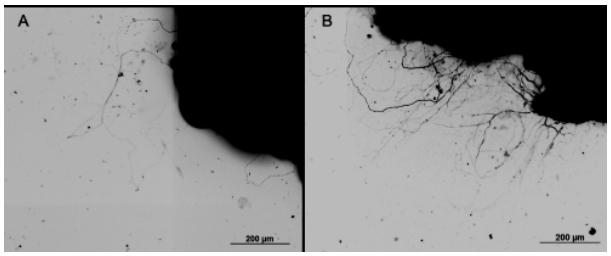

Fig 2: Immunofluorescent (anti-neurofilament 200) image of DRG organ cultures under 2% (A) and (B) 20% of oxygen. Scale bar = 200 μm.

DISCUSSION & CONCLUSIONS: Hypoxia in the centre of healthy IVD helps to maintain the phenotype of nucleus pulpous cells [3]. However, hypoxia caused by degeneration can lead to pathology of tissues outside IVD including the neural compartment. In this study, hypoxia reduced the number but increased the length of outgrowth of DRG neurons both in vitro and ex-vivo, which could explain the deeper innervation observed in clinical samples of degenerative and painful IVD.

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Early changes in the extracellular matrix of the degenerating intervertebral disc, assessed by Fourier Transform Infrared spectroscopy

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INTRODUCTION: Mechanical overloading induces a degenerative cell response in the intervertebral disc. However, it is difficult to assess early changes in the extracellular matrix (ECM) with conventional techniques. Fourier Transform Infrared (FTIR) spectroscopy allows visualization and quantification of the ECM. We aim to identify markers for disc degeneration identified and apply these to investigate early degenerative changes due to overloading and catabolic cell activity.

METHODS: *In vivo*, one disc in each of seven goats was injected with chondroitinase ABC (mild degeneration) and compared to the adjacent disc (control) after 24 weeks. *Ex vivo*, caprine discs received physiological loading (50-150N) (n=9) or overloading (50-400N) (n=10) in a bioreactor. In an additional group (n=18), cell activity was diminished in advance by freeze-thaw cycles. In both experiments, FTIR images (spectral region: 1000-1300 cm^{-1}) of mid-sagittal slices were analyzed using multivariate curve resolution.

RESULTS: *In vivo*, FTIR was more sensitive than biochemical and histological analysis in identifying reduced proteoglycan content ($p=0.046$) and increased collagen content in degenerated discs ($p<0.01$). Notably, FTIR analysis additionally showed disorganization of the ECM, indicated by an increased collagen entropy ($p=0.011$). In *Fig. 1*, the in collagen and proteoglycan distributions over the sagittal slices are shown of both groups.
Ex vivo, the proteoglycan/collagen ratio was decreased due to overloading ($p=0.047$) compared to control loading. Furthermore, there was an increase in collagen entropy ($p=0.047$). An interaction between loading and vitality was found only in the amount of collagen ($p=0.044$).

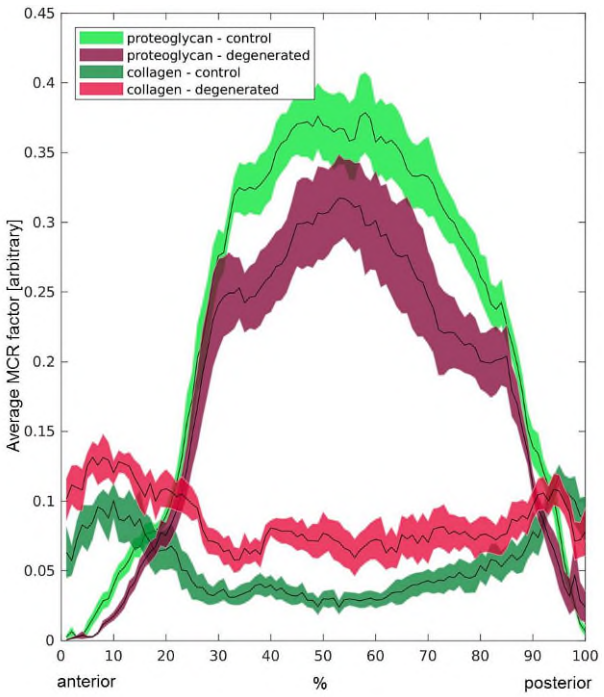

Fig. 1: Average distribution of the proteoglycan factor (top) and collagen factor (bottom) over percentage of width of the sagittal slices (SEM in color).

DISCUSSION & CONCLUSIONS: FTIR imaging provides quantitative, sensitive measures to study early changes in extracellular matrix with intervertebral disc degeneration. This is an important step forward, as such measures are necessary to be able to study the etiology of intervertebral disc or cartilage degeneration. Reduction in proteoglycan factor, increase in collagen factor, decrease of the proteoglycan/collagen ratio and increase in the collagen entropy were shown to be measures for early intervertebral disc degeneration.

Characterization and development of an in vitro disease model of the human cartilage end plate

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INTRODUCTION: Low back pain (LBP) is second only to cancer in terms of socioeconomic burden (1) and intervertebral disc (IVD) degeneration has been implicated its progression, but current treatments (aimed at purely relieving pain) are highly invasive and fail to target the underlying cellular mechanisms (2). In disease, the normally avascular aneural cartilaginous endplate (CEP) calcifies which is linked to the onset and progression of IVD degeneration (3). Although structural and biochemical changes to the CEP are associated with disc disease, the CEP itself remains uncharacterized. Furthermore, there are currently no in vitro models of CEP degeneration available to help elucidate biological mechanisms and evaluate potential therapies. **Aim 1**: Characterize the CEP on a histological and phenotypic gene level. **Aim 2:** Develop an in vitro model of CEP degeneration and related process.

METHODS: Aim 1: Picrosirius red/alcian blue (PR/AB) and haematoxylin and eosin (H&E) stains were done on a sagittal sections of an L3/L4 human IVD (n=1). 4X magnification stitched images and 20X images were taken of the whole IVD. QRT-PCR was completed on human lumbar (n=8) and bovine caudal (n=7) nucleus pulposus (NP) and CEP cells. **Aim 2:** CEP cells isolated from human lumbar discs (n=6) were cultured in pellet culture for 21 days in chondrogenic or degenerate (10% FBS, (4)) media in 5% oxygen. After 21 days, samples were assessed for cell viability, histology, qRT-PCR, and DMMB proteoglycan assay. Wilcoxon tests were performed with $p<0.05$ considered significant.

RESULTS: Aim 1: The PR/AB and H&E stains of the human IVD revealed differences in matrix composition and cell morphology along the CEP-IVD interface (Fig. 1A). QRT-PCR showed significant up-regulation of ACAN and COL1 gene expression in the human CEP compared to the NP (Fig. 1A) and significant down-regulation of COL2 and T gene expression in the bovine CEP compared to the NP. **Aim 2:** QRT-PCR performed on the human CEP pellets (n=5) demonstrated a significant down-regulation in COL2 and significant up-regulation of MMP-13, RUNX2, VEGF and NGF in the degenerate group compared to the chondrogenic control at 21 days (Fig. 1B). PR/AB staining of cell pellets demonstrated a reduction in proteoglycan in the degenerate group compared to the chondrogenic control. Similarly, the DMMB assay (n=5) demonstrated a significant decrease in proteoglycan (GAG) content in the degenerate group compared to chondrogenic control (Fig. 1B)

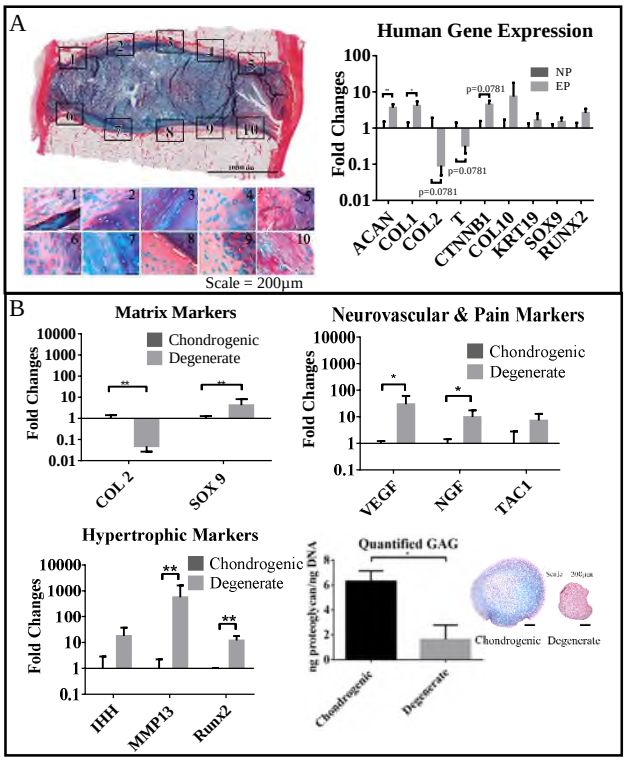

Fig 1: A) PR/AB histology on the human IVD and human NP and CEP gene expression. B) Matrix, hypertrophic, neurovascular and pain marker gene expression, DMMB proteoglycan assay, and PR/AB histology on chondrogenic and degenerate CEP pellets.

DISCUSSION & CONCLUSIONS: Aim 1: Regional histological differences in the CEP and differences in gene expression between the NP and CEP cells highlight a unique CEP phenotype distinct from the NP that needs further evaluation and validation. **Aim 2:** Decreases in healthy matrix and increases in hypertrophic, catabolic and pain markers indicate that we were able to induce a degenerate phenotype in human CEP cells in vitro and that this disease model may be used to further study CEP degeneration and potential therapeutics.

REFERENCES: (1) Vos T. et al, 2012 (2) Freemont et al, 2009 (3) Grant et al, 2016 (4) Pesesse, L. et al, 2013

Enhancing cartilage drug delivery using electrostatic interactions

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INTRODUCTION: There are no efficacious disease modifying osteoarthritis drugs (DMOADS) available today, i.e., drugs which halt or reverse the progression of OA. Current therapies provide only short term relief of pain and inflammation, but afford no protection against further degeneration of cartilage, the hallmark of OA. A critical problem is the inability to deliver drugs directly into cartilage tissue, and drugs delivered by intra-articular injection are rapidly cleared from the joint space [1]. We have developed experimental methods and theoretical models that reveal the utility of using cationic nano-carriers to deliver functionalized bioactive drugs directly into cartilage tissue.

METHODS: Experiments focused initially on the effects of nano-carrier size and charge on the penetration and transport into bovine and human cartilage explants in vitro, using a range of fluorescently tagged molecules, peptides and proteins. Recently we also quantified the effects of surface charge distribution of several ~4nm diameter carriers zero net charge and having otherwise the same size and shape. For all these experiments, we measured both the equilibrium uptake of carriers into human and bovine cartilage as well as non-equilibrium diffusive transport of carriers into and through the tissue. We have also pursued translation of these concepts in a study of delivery of dexamethasone functionalized to the cationic nano-carrier, Avidin, to rabbit knees using the ACLT model for post-traumatic OA.

RESULTS and DISCUSSION: Our approach takes advantage of electrostatic interactions between the positively charged nano-carriers and cartilage's negatively charged matrix to accelerate transport into the tissue, while increasing retention (half-life) within cartilage for sustained delivery to cartilage and other joint tissues [2]. We developed a model for uptake and transport of charged nano-carriers that includes Donnan partitioning at the synovial fluid-cartilage interface, diffusion within cartilage ECM, and electrostatically mediated reversible binding of nano-carriers to cartilage negative ECM. Model predictions were fit to experimental results and gave reasonable values of parameters including effective binding constants

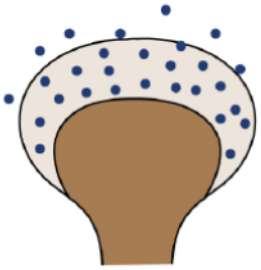
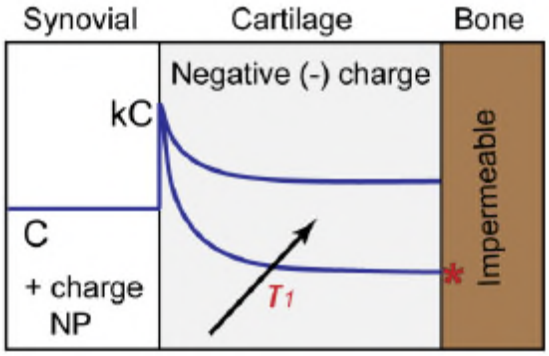


Figure 1. Cationic nano-particles rapidly enter cartilage: Transport is enhanced by upward Donnan partitioning factor (k), increasing concentration gradient at cartilage surface.



and diffusivities. The critically important role of Donnan partitioning, is shown schematically in Figure 1 [3]: upward partitioning (kC) leads to a dramatic increase in the concentration gradient at the cartilage surface, accelerating diffusion (***T***) of nanoparticles (NP) into the tissue. Weak binding enables more rapid penetration, but still enables extended lifetime for sustained delivery inside the cartilage tissue. Cartilage then becomes a depot for delivery to chondrocytes other joint tissues.

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ACKNOWLEDGEMENTS: NSF (MRSEC) grant DMR-1419807, NIH Grant AR060331 and DoD (CDMRP) Grant W81XWH-14-1-0544.

A novel nano-ghosts based system for targeted delivery of antimir-221 and stimulation of cartilage repair

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INTRODUCTION: Osteoarthritis (OA) is a leading cause of joint disability worldwide, characterized by articular cartilage degradation, with no disease-modifying treatments available. In recent years, RNA interference (RNAi) therapy has showed great potential, but the clinical translation is largely hindered by the lack of safe delivery systems. Recently, our lab has developed an innovative drug delivery platform, termed Nano-Ghosts (NGs), consisting of nano-vesicles reconstructed from the plasma membrane of mesenchymal stem cells (MSCs). The NGs retain MSCs' surface features and targeting capabilities towards tumor and inflammation sites[1]. Moreover, our group demonstrated the efficacy of NGs delivery system for gene therapy[2]. MicroRNA(miR)-221 is a negative regulator of chondrogenesis, and its silencing in MSCs in vitro leads to chondrogenic differentiation and enhances cartilage repair in vivo[3]. Our aim was to develop a novel NGs-based delivery system of antimiR-221 for targeted and efficient miRNA silencing in MSCs.

METHODS: Electroporation was optimised as encapsulation method for NG loading of antimiR-221. The active targeting and uptake of the loaded NGs were assessed using confocal microscopy and FACS analysis. NG intracellular fate was studied by High Content Analysis of co-stained target cells. The NG effect on cell viability and miR-221 silencing was investigated *in vitro* with cultured human MSCs using the Alamar Blue assay and qPCR, respectively.

RESULTS: Our data showed that antimiR-221 can be efficiently loaded in the NGs using electroporation, achieving high encapsulation efficiency (up to 45%), as confirmed by fluorescent quantification and cryo-TEM imaging. Confocal microscopy and flow cytometry analyses demonstrated selective targeting of NGs towards MSCs within 30 minutes. MSCs viability was not affected by the treatment, as confirmed by Alamar Blue assay. Imaging flow cytometry showed that MSCs were efficiently transfected with antimiR-221-loaded NGs within 30 minutes, with a cellular internalization of NGs >90%, and a cytosolic presence of antimiR-221 >80%. Moreover, high-content screening and image stream kinetic studies showed that NGs underwent only minimal endosomal and lysosomal recycling, with less than 5% and 9% co-localization, respectively. Finally, Figure 1 shows qRT-PCR analysis of a strong silencing of miR-221 at 24 and 72 hours (75% and 83%, respectively) after treatment of MSCs with 5 ug/mL antimiR-221-loaded NGs.

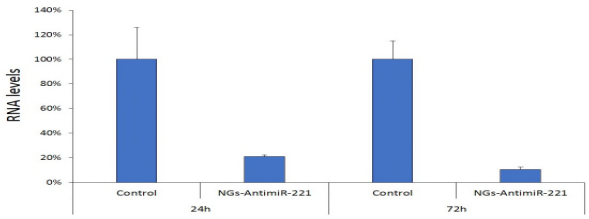

Fig. 1: miR-221 silencing using microRNA 221 inhibitor delivered by NGs, compared to positive control.

DISCUSSION & CONCLUSIONS: In conclusion, we developed a novel and effective NGs-based system for delivering RNAi therapeutic molecules such as antimiRNAs to MSCs. AntimiR-221-loaded NGs were shown to efficiently transfect MSCs, leading to rapid and strong silencing of the anti-chondrogenic miR-221. Our delivery system may be employed for targeting endogenous MSCs in a context of trauma or OA, thus stimulating and improving cartilage repair.

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3D non-invasive bioimaging of cartilage tissue engineered constructs using contrast enhanced computed tomography

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INTRODUCTION: Acquiring 3D information of musculoskeletal tissues using contrast agents has been used to measure concentration and distribution of tissue constituents [1,2]. In particular, contrast enhanced computed tomography (CECT) enables quantification of glycosaminoglycans (GAGs) in tissues such as cartilage [3]. In cartilage tissue engineering, assessment of GAG production is limited to endpoint destructive methods such as 1,9-Dimethyl-Methylene Blue (DMMB) assay on digested tissue or Safranin-O staining. Here, we propose a systematic CECT-based approach using positively charged contrast agent (CA4+) for non-destructive and real-time quantification of GAGs in cartilage constructs. Additionally, this technique allows for 3D assessment of GAGs distribution.

METHODS: Human chondrocytes and mesenchymal stromal cells (MSCs) were harvested from articular cartilage and bone marrow, respectively. Four pellets per group were subsequently cultured for two weeks in the following conditions: chondrocytes were cultured with i) no Transforming Growth Factor β (TGF-β), ii) 1 ng/mL TGF-β; iii) 10 ng/mL TGF-β. MSCs were only cultured without TGF-β. At the endpoint, pellets were incubated for 3 or 24 hr in medium containing 4 or 8 mg/ml of CA4+ (MW = 1354 g/mol). Subsequently, µCT was performed at voxel size of 20 μm^3 in four different protocols, namely 90 kV voltage with i) 3-minute and ii) 26-second scan time, and 70 kV voltage with i) 3-minute and ii) 26-second scan time, all under 200 µA current. Phantoms (0-40 mg/ml CA4+) were used to relate µCT grey values to CA4+ concentration. After scanning, DMMB was performed to quantify GAG content. To assess 3D distribution of GAGs, chondrocyte and MSCs pellets were cultured under different conditions (Fig. 1), incubated for 24 hr with 8 mg/ml of CA4+ and subsequently µCT scanned (voxel size = 10 μm^3) as described before. Safranin-O was performed on the same pellets.

RESULTS: At both 3 and 24 hr incubation times, high correlation was found between CA4+ concentration and GAG content (Fig. 1a and b). Furthermore, CA4+ distribution by µCT correlated with GAG distribution by Safranin-O staining (Fig. 1c).

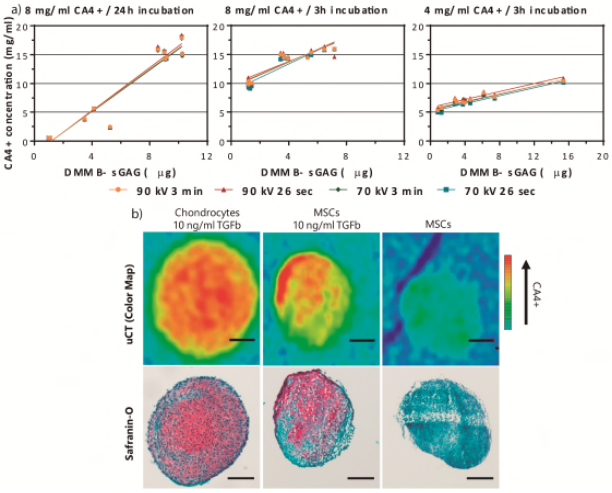

Fig. 1: a) CA4+/GAG correlation at different incubation conditions. y axis: CA4+ concentration (mg/mL). x axis: GAG content (µg). b) Correlation between color maps and safranin-O staining. Scale bars:200 µm.

DISCUSSION & CONCLUSIONS: Electric charge-assisted-CECT-based determination of GAG content proved to be an efficacious technique for quantification and assessment of 3D distribution of GAGs. This allows for other readouts as the proposed technique is non-invasive and non-destructive as opposed to DMMB and histology. Optimizing imaging parameters (i.e. voltage, CA4+ concentration, scan time) would potentially offer an unprecedented technique for spatiotemporal monitoring of GAG production in cartilage tissue engineered constructs.

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