DIVERSITY IN THE DEVELOPMENT AND EVOLUTION OF CARTILAGES

A.G. Cole[1]

[1]BioGem s.c.a.r.l. Istituto per Ricerche Genetiche G. Salvatore

INTRODUCTION and DISCUSSION: Textbook definitions of cartilage generally identify three classes of histologically distinguishable cartilage: hyaline, fibro- and elastic- cartilage. However a survey of cartilage histology throughout vertebrate lineages illustrates that cartilage as a tissue type exhibits an entire spectrum of histological diversity[1]. Indeed, the notochord remains ambiguous as to whether or not it can be considered a type of cartilage despite expression of various cartilage-specific markers (see below) and its persistence into adulthood in various lineages. In addition, examples of cellular cartilages are found in a number of invertebrate lineages, including arthropods, annelids, and molluscs[2]. Although the existence of these tissues outside of the vertebrate lineages is known, the relationship between invertebrate and vertebrate cartilage is at best unclear.[2,3] A strong vertebrate fossil record gives much information about the evolution of mineralized tissues, but has little to say on the origin of cartilage as this tissue rarely fossilizes. Thus, the question remains open: when did cellular cartilage arise during metazoan evolution? Or equally important: what are the necessary and sufficient characters required to call a tissue *cartilage*? One approach involves the characterization of a molecular fingerprint for cartilage: a suite of transcription factors and structural molecules specific for all types of cartilage. Often the presence of the transcription factor Sox9 is used to identify cartilage; in mammals this gene is necessary for cartilage specification[4], however it also plays important roles in testis development[5], neural differentiation[6], and carcinomas[7] among others.[8,9] Type II collagen is another important matrix component, often cited as being cartilage specific, however this molecule is also expressed in many non-cartilaginous tissues derived from all three germ layers.[10] Thus an indisputable molecular signature for cartilage as a cell type has yet to be well-defined.

Of special interest within the spectrum of invertebrate tissues are those cartilages found within cephalopod mollusks, which to the un-indoctrinated are indistinguishable from typical vertebrate hyaline cartilage at a histological level. Two important differences emerge upon close analysis of these tissues: cephalopod chondrocytes retain thin filopodial extensions that connect the cells[11], and cephalopod cartilage appears to possess regenerative capacities that are otherwise limited in most vertebrate lineages (unpublished observations). Analysis of the development of these tissues reveals that analogous to vertebrate cartilage development, many cephalopod cartilages pass through a cellular condensation phase similar to primary vertebrate cartilage.[12] Whereas other elements develop *di novo* from mesenchymal cells in a manner complementary to vertebrate secondary cartilage.[12] To date the molecular mechanisms underlying cephalopod cartilage specification remains unknown and thus whether these tissues represent a case of parallel or convergent evolution with analogous vertebrate cartilages remains elusive. Regardless, further studies on the development and mechanical properties of non-vertebrate cartilages holds promise for increased understanding of the various ways to build a tissue with the structural properties of cartilage.

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THE VARIOUS FUNCTIONS OF WNT-SIGNALING IN CHONDROGENESIS

C. Hartmann

Research Institute of Molecular Pathology, Vienna, Austria

INTRODUCTION: Wnt-signaling plays important and diverse roles in development and disease of invertebrates and vertebrates [1]. Historically Wnt-signaling has been subdivided in the canonical pathway, which is mediated via the intracellular molecule β-catenin, and the so-called non-canonical pathways, which are more diverse (Fig. 1). Their various functions in vertebrate chondrogenesis will be discussed.

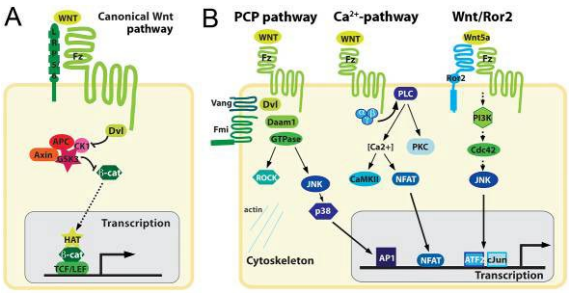

Fig. 1: Simplified cartoons of the canonical Wnt/β-catenin (A) and the non-canonical, Planar-cell-polarity (PCP), Ca^{2+}- and Wnt5a/Ror2 pathways (B).

RESULTS: Chondrogenesis is a multi-step process, which is differentially regulated by the canonical Wnt/β-catenin pathway in the following ways: differentiation of mesenchymal precursors into chondrocytes is negatively regulated [2, 3]. Intracellular activation of the pathway by stabilization of β-catenin in proliferating chondrocytes leads to chondrocyte dedifferentiation associated with down-regulation of the transcription factor *Sox9*. In prehypertrophic chondrocytes activation of the pathway appears to be required to control the expression of *Indian hedgehog* (*Ihh*), which is a central regulator of vertebrate skeletogenesis [4]. Concomitantly, previous experiments in the chicken had hinted already at a positive role of this pathway in chondrocyte maturation [5]. It is currently under debate, whether this positive effect is mediated through β-catenin stimulated degradation of Sox9 protein [6, 7]. Whether the Wnt/β-catenin pathway, which has been reported to be active in hypertrophic chondrocytes [8], plays a role in those cells, is currently unknown. Components of the different non-canonical signaling pathways have also been implicated in chondrogenesis. Increased intracellular Ca^{2+} levels can on the one hand activate calcineurin/NFAT, which influences chondrogenesis [9, 10]. On the other hand, it can activate CaMKII, which affects chondrocyte maturation [11]. JNK and AP1 have also been implicated in regulating chondrogenesis [12, 13, 14]. Wnt5a and Ror2 are also involved in chondrogenesis [15, 16, 17, 18], as are the GTPases, Cdc42, Rac1 and RhoA, and the RhoA-associated kinase Rock [19, 20, 21].

DISCUSSION & CONCLUSIONS: Based on the experimental evidence a complex picture is emerging on the divergent roles of the different Wnt-signaling pathways in chondrocyte differentiation and maturation.

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ACKNOWLEDGEMENTS: Research in the laboratory of Christine Hartmann is supported by Boehringer Ingelheim and grants by the Austrian Science Foundation (FWF Grant No. P19281-B16) and the Arthritis Research Campaign (ARC Grant No. 18075).

Chondroitin sulphate sulfation motifs in intervertebral disc development

Jim Ralphs, Bruce Caterson, Clare Hughes and Anthony Hayes
School of Biosciences, Cardiff University, Wales, UK

INTRODUCTION. The intervertebral disc develops from two key components: the embryonic notochord and sclerotomally derived mesenchyme. Initially, the mesenchymal cells surround the notochord and form regular repeating dense annular condensations of cells interspersed with more widely spaced cartilage precursor cells of the early developing vertebral bodies. As the vertebral body cartilage differentiates, the annular condensations shows evidence of differentiation into inner and outer regions. Shortly afterwards, the notochord rapidly bulges in the region of the developing disc, and thins in the vertebral bodies, from which it eventually disappears. The bulges form the foetal nucleus pulposus, and as they enlarge the annular condensations differentiate into the cartilaginous inner and fibrous outer annulus fibrosus. The ends of the vertebral bodies on either side form the cartilage endplates. The subsequent fate of the nucleus pulposus is species dependent. In human discs, most notochordal cells are lost by about 8 years old and the nucleus becomes populated by a chondrocyte like cell population whose origin has not been conclusively demonstrated, but is likely to be from the surrounding cartilaginous inner annulus fibrosus and endplates.

Chondroitin sulphate (CS), associated with glycosaminoglycan (GAG) chains of extracellular matrix proteoglycans of connective tissues, is composed of repeating disaccharide units of glucuronic acid and N-acetylgalactosamine. The hydroxyl groups on these disaccharide units are differentially sulfated, producing enormous structural heterogeneity in CS chains. Growth and differentiation factors are known to bind to chondroitin sulphate, and the sulfation motifs play critical roles in determining CS affinity for growth factors, enzymes, chemokines and adhesion molecules. Using monoclonal antibodies that bind to different sulphation motif epitopes, we describe their distribution during development and ageing of the rat intervertebral disc.

METHODS. Lumbar spines were removed from embryonic day (E) E15-E20 foetuses and neonates, fixed with 10% neutral buffered formol saline, decalcified, wax embedded and sectioned at 10 microns thickness. After dewaxing they were labeled using indirect immunoperoxidase (Vector ABC universal kit with NovaRed substrate) using monoclonal antibodies 3B3(-), 4C3, 6C3 and 7D4, each of which recognizes a distinct sulphation motif epitope on native CS GAG chains. Controls were incubated with non-immune mouse immunoglobulins or the primary antibody was omitted. These were negative in all cases. Sections were photographed using a Leica DM6000 brightfield microscope.

RESULTS AND DISCUSSION. The CS sulphation variants showed complex distributions that varied temporally and according to tissue type and differentiation. ***At E15***, 4C3 and 7D4 labelled the disc condensations, 6C3 just the notochordal sheath and 3B3(-) was absent. ***At E17***, when overt differentiation into inner and outer annulus and notochordal nucleus pulposus had occurred, all were present. 4C3 and 7D4 were in the inner annulus, nucleus and vertebral body cartilage, 3B3(-) in the inner annulus, nucleus and insertional region of outer annulus into the vertebral body, along with the cambial layer of the perichondrium of the vertebral bodies. 6C3 was also present in the latter, but only on the ventral parts of the ends of the vertebral body cartilages. ***At E19*** 3B3(-) and 6C3 were essentially the same as above, whereas 4C3 and 7D4 were in the nucleus, and in a region that extended from the perichondrium of the vertebral body through into the interface region between the inner and outer annulus. ***In the neonate*** all antibodies labeled the inner annulus and nucleus, with 6C3 and 7D4 also labeling cartilage of the developing vertebral endplate.

The label patterns seem to reveal unexpected relations between tissue types (e.g. continuity between inner annulus and vertebral body perichondrium), and appear to occupy developmentally significant regions. For example, perichondrium mediates growth of developing cartilage and bones, and the expression of similar motifs in inner annulus/outer annulus boundary may have implications for understanding growth and differentiation of the annulus itself. This, and the expression of different epitopes in the different tissue components of the disc at different stages may indicate a role of the different motifs in binding of other molecules, for example growth factors and cytokines, that may in turn mediate growth and differentiation processes.

Acceleration of post-natal articular cartilage maturation

Ilyas M Khan[1], Sam L Evans[2], Nick Avery[3] & Charles W Archer[1]

[1]*Connective Tissue Biology Labs, School of Biosciences,* [2]*School of Engineering, Cardiff University, Wales, UK.*
[3]*Department of Clinical Veterinary Science, University of Bristol, Langford House, North Somerset, UK.*

INTRODUCTION: Articular cartilage form and function is the result of continuous developmental maturation from the initial specification of a synovial joint in the foetal cartilage analagen to an entity that allows practically frictionless articulation of bones and that conveys large forces through these structures. From the perspective of post-natal growth and development, the morphology of articular cartilage can be segregated into two distinct structures based principally on the distribution of chondrocytes within the tissue. An isotropic (random) distribution of chondrocytes defines the immature state, whereas an anisotropic configuration of cells consisting of well demarcated superficial, transitional, deep and calcified zones defines the mature state of articular cartilage[1]. The transition between these states has been shown to occur within 3 months following birth in the New Zealand white rabbit and is hypothesised to represent a balance between neoformation from a surface growth plate and resorption from below[1]. There is indirect evidence to suggest that the latter hypothesis is correct, precluding the possibility that internal reorganisation of the tissue occurs to any significant degree.

We demonstrate for the first time that when grown *in vitro* in a defined medium containing specific combinations of growth factors that aspects of the post-natal maturational program are accelerated in immature bovine articular cartilage explants.

METHODS: Immature articular cartilage was obtained from 7-day-old bovine steers (Ensor & Sons, UK) and surgically removed using 6mm diameter biopsy punches. Explants were grown in defined medium containing DMEM-high glucose, 25µg.ml^{-1} ascorbate, 10mM HEPES, 50µg.ml^{-1} gentamicin and insulin-transferrin-selenium,

RESULTS & DISCUSSION: When immature articular cartilage explants were cultured in defined medium containing (100ng.ml^{-1}) FGF2 and (10ng.ml^{-1}) TGFβ1 for 21 days we noted that there was a 52% reduction in height of growth factor treated explants compared to controls (P<0.01). Biochemical analyses showed that there was no change in the GAG, hydroxyproline or water content of explants normalised to DNA, but, the dry weight of the treated explants increased by approximately 40% (P<0.05). Additionally we noted a 45% increase in the ratio of mature/immature collagen crosslink's in growth factor treated compared to untreated explants (P<0.01). Using DQ Gelatin we noted that the normally smooth border between the resorption front and articular cartilage was more ragged and appeared to be advancing in growth factor treated explants.

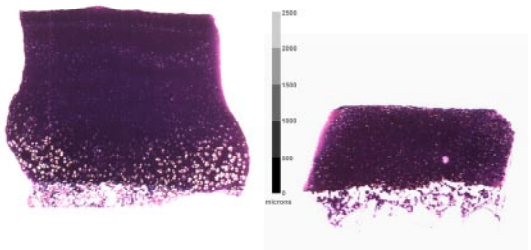

Fig. 1 Effect of growth factor treatment on perinatal immature articular cartilage. The height of the explant decreases by approximately 50% following 21 days in culture (right) compared to untreated explants (left).

From our initial observations we hypothesised that under growth factor treatment explants were undergoing accelerated maturation. As part of the maturational resorptive process, chondrocytes in the lower radial zone adjacent to calcifying spicules undergo apoptosis. Our experiments using caspase-3 antibody labelling and TUNEL clearly show that the zone of apoptotic nuclei has significantly expanded to include cells of the upper radial zone.

Understanding post-natal maturational events in articular cartilage are crucial if we are ever to control spontaneous or directed regenerative processes. The generation of a model system that replicates crucial aspects of the maturation program will aid this effort.

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THE ROLE OF BIOMECHANICAL FACTORS IN OA DEVELOPMENT

Annika Burleigh, Olga Boruc, Jerry Saklatvala and Tonia Vincent
Kennedy Institute of Rheumatology, Imperial College, London.

INTRODUCTION: Excessive or abnormal mechanical loading of the joint is arguably the most important aetiological factor in the development of osteoarthritis (OA). Although such changes could occur by excessive wear and tear leading to attrition of the articular surfaces, it is now generally accepted that OA is not a passive process, but relies upon activation of specific matrix proteases which lead to break down of the two major extracellular matrix proteins, aggrecan and type II collagen. In the mouse a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS)-5 is crucial for this process[1]. We investigated how biomechanical factors influence expression of proteases within the joint following destabilisation of the medial meniscus (DMM), a well characterized model of murine OA.

METHODS: We studied the progression of OA in mice in which joint loading had been altered by cutting the sciatic and/or femoral nerves at the same time as performing DMM surgery. Gait and activity levels were measured in the neurectomised mice. Joints were examined histologically 12 weeks following surgery. Genes expressed by the joint at early points following surgery (DMM or sham surgery with or without neurectomy) were initially assessed by microarray, then validated by RT-PCR. Gene expression was also examined in joints which had undergone DMM, but which had been completely immobilized (by prolonged anaesthesia).

RESULTS: Mice that had undergone sciatic neurectomy were still able to weight bear, but walked on a fully extended knee by flexing at the hip. Mice that had undergone femoral neurectomy walked with a slapping gait – with exaggerated flexion at both the hip and the knee. Mice that had undergone both femoral and sciatic neurectomy dragged the affected limb behind them. When DMM surgery was performed in combination with neurectomy, the cartilage in the sciatic neurectomised mice was completely protected from cartilage degradation. No protection was seen in the femoral neurectomised mice. Gene expression data from the joint at early time points following DMM surgery (6h, 3 and 7 days) revealed strong regulation of many inflammatory response genes including CCL2, TNF-stimulated gene 6 (TSG6), activin A, serum amyloid A (SAA) and IL-6. There was a low fold increase in ADAMTS5 (1.5 fold) which was highly consistent and significantly elevated above sham operated controls. Following sciatic neurectomy there was selective abrogation of a number of genes including CCL2, SAA and ADAMTS5. Genes such as TSG6 and activin A were completely unaffected when DMM surgery was performed in combination with sciatic neurectomy. When gene expression was studied in joints which had been completely immobilised (by sustained anaesthesia), almost all gene responses following DMM surgery were abrogated.

DISCUSSION & CONCLUSIONS: These results show that expression of disease in a surgically-destabilised joint is highly dependent upon the biomechanical environment and suggest that sheer forces associated with flexion at the knee may be essential for development of disease. The results also reveal that gene expression within the joint following destabilisation is highly mechanosensitive, and that protection against OA is associated with selective abrogation of a number of inflammatory genes including ADAMTS5. The observation that partial and complete immobilisation leads to abrogation of different sets of genes suggest that there may be distinct biomechanical thresholds in vivo which when exceeded turn on different sets of genes and determine disease outcome.

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ACKNOWLEDGEMENTS: This work was supported by Arthritis Research UK.

Eicosapenaenoic acid (EPA) and docasahexaenoic acid (DHA) reduce IL-1β mediated cartilage degradation

AK.Wann[1], J.Mistry[1], A.Michael-Titus[2], MM.Knight[1]

[1]*School of Engineering and Materials Science*, [2] *Blizzard Institute of Cell and Molecular Sciences*, *Queen Mary University of London, UK*

INTRODUCTION: Inflammatory joint disease, such as osteoarthritis (OA), is associated with increased levels of pro-inflammatory cytokines, such as IL-1β. These cytokines stimulate the production of matrix metalloproteinases which lead to the degradation of the cartilage extracellular matrix and the loss of key structural components such as sulphated glycosaminoglycan (sGAG) and collagen II. The aim of this study was to examine the therapeutic potential of n-3 polyunsaturated fatty acids (PUFAs) in an *in vitro* explant model of cartilage inflammation.

METHODS: The anti-inflammatory effects of two specific n-3 compounds were examined, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), each at 0.1, 1 and 10μM. Full thickness bovine cartilage explants, 5mm in diameter, were incubated for 5 days in culture media with or without IL-1β and in the presence or absence of each n-3 compound. The media was replaced every 24 hours and assayed for sGAG content using the DMB method. Chondrocyte viability was determined at the end of the culture period using fluorescence microscopy of cells labelled with calcein AM and ethidium homodimer.

RESULTS: Treatment with IL-1β (10ng.mL^{-1}) produced a large increase in sGAG release compared to untreated controls (Fig. 1), but with no effect on cell viability which was maintained above 80% for all treatments. In the absence of IL-1β, both n-3 compounds induced a mild catabolic response with increased loss of sGAG, particularly at 10μM. By contrast in the presence of IL-1β, both EPA and DHA at 0.1 and 1μM significantly reduced IL-1β mediated sGAG loss (Fig 2). The efficacy of the EPA treatment was maintained at approximately 75% throughout the 5 day period. However, at the same concentrations, the efficacy of DHA, although initially greater, reduced to approximately half that of EPA after 5 days. For both EPA and DHA, the higher dose of 1μM were less effective.

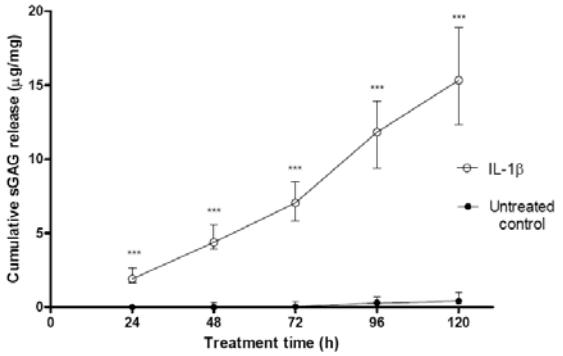

Fig. 1: sGAG loss from cartilage explants cultured for 5 days with and without IL-1β.

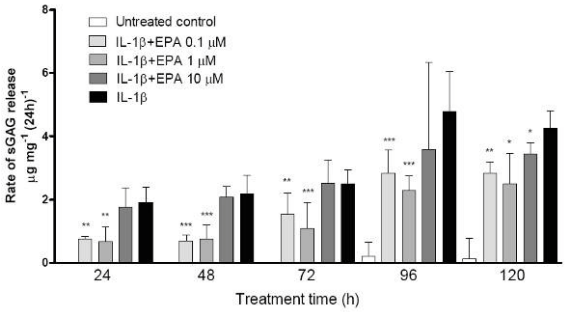

*Fig. 2: Anti-inflammatory effects of EPA at 0.1, 1 and 10μM on sGAG loss in the presence of IL-1β. Statistically significant differences from IL-1β are indicated (*p<0.05)*

DISCUSSION & CONCLUSIONS: The results support the hypothesis that n-3 compounds are anti-inflammatory through competitive inhibition of the arachidonic acid oxidation pathway[1]. This agrees with previous studies reporting that EPA reduces mRNA levels of MMP and other proteolytic enzymes in isolated chondrocytes[2]. Thus we suggest that these n-3 PUFAs, particularly EPA, have exciting therapeutic potential for preventing cartilage degradation associated with chronic inflammatory joint disease.

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Cell interactions of osteoarthritic subchondral bone osteoblasts and articular chondrocytes aggravate MMP-2 and MMP-9 production through the mediation of ERK1/2 and JNK phosphorylation

Indira Prasadam[1], Ross Crawford[1], Yin Xiao[1] (yin.xiao@qut.edu.au)

[1]*Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Australia (*http://www.bee.qut.edu.au/about/schools/engineering/staff/medical/yxiao.jsp)

INTRODUCTION: Matrix Metalloproteinases (MMP) play a key role in osteoarthritis (OA) development. The aim of the present study was to investigate whether, the cross-talk between subchondral bone osteoblasts (SBOs) and articular cartilage chondrocytes (ACCs) in OA alters the expression and regulation of MMPs, and also to test the potential involvement of mitogen activated protein kinase (MAPK) signalling pathway during this process.

METHODS: Normal ACCs (n=3) were obtained from patient's undergoing fracture repair surgery as a result of trauma. OA ACCs (n=4) were sourced from the main defective area of the medial compartment from patients undergoing total knee replacement surgery. OA SBOs (n=4) were sourced from the weight bearing sites from the patients suffering advanced OA, where the cartilage was degraded and showing prominent subchondral bone erosion and density. Normal SBOs (n=3) were collected from the patients mentioned above undergoing fracture repair surgery with no evidence of subchondral bone erosion or cartilage degeneration. Both direct and indirect co-culture was performed to test the effect of soluble and membrane bound factors. Western blotting against the phosphorylated antibodies for p38, ERK1/2 and JNK were used to first see the MAPK signal activation in the indirectly co-cultured vs. non co-cultured SBOs and ACCs. The MAP kinase mediated cellular interactions were further evaluated in the indirect co-cultures by the use of MAPK specific inhibitors (p38: SB203580; ERK1/2: PD98059; JNK: SP600175). The gelatinolytic activity of serum free CM from the co-cultures was separated by electrophoresis in cold room on 10% SDS–PAGE containing 1mg/mL gelatine as a substrate and the gels washed for 30 min with 2.5% Triton X100 and subsequently incubated at 37°C for 12–24hrs in incubation buffer containing 50mM Tris-HCl (pH 7.6), 10mM CaCl2, and 50mM NaCl. The content of MMP-2 and MMP-9 secreted proteins was determined in the co-cultured and non co-cultured cells in the conditioned medium using an ELISA kit. The total cell lysate from co-cultured and non co-cultured ACCs and SBOs was prepared for western blot.

RESULTS: As determined by zymography, ELISA and western blotting methods, our results revealed that the direct and indirect co-culture of OA SBOs with ACCs significantly aggravated the proteolytic activity and increased the expression of MMP-2 and MMP-9 in ACCs. In turn, co-culture of OA ACCs lead to abundant MMP-2 expression in SBOs. Furthermore, addition of ERK1/2 inhibitor PD98059 and JNK inhibitor SP600125 reversed the abnormal MMP-2 and MMP-9 production that was induced during the interactions of OA SBOs and ACCs. In conclusion, our current study is the first to document that signals transmitted between SBOs and ACCs in OA can lead to altered bi-directional interaction which may be related to OA progression.

DISCUSSION & CONCLUSIONS: Our in vitro study is the first to provide direct insight in to the mechanism underlying the cell interaction between subchondral bone and cartilage in OA development. This bi-directional interaction was mediated by the phosphorylation of ERK1/2 and JNK pathways.

Roles of Inflammatory and Anabolic Cytokines in Cartilage Metabolism

MB.Goldring, M.Otero, DA.Plumb, C.Dragomir, K.Hashimoto

The Hospital for Special Surgery , Weill Cornell Medical College, New York, NY

INTRODUCTION: Proteins produced in response to excessive mechanical loading and inflammation in joints not only stimulate the production of enzymes that break down the cartilage but also impair the ability of the chondrocyte to repair the damage. We have used several strategies for identifying and characterizing mediators involved in the pathogenesis of osteoarthritis (OA), including culture models of primary human and mouse chondrocytes and cell lines, mouse models, and human cartilage samples. During OA the normally quiescent chondrocytes with low matrix turnover are activated and undergo phenotypic modulation due to the actions of both inflammatory and anabolic cytokines.

METHODS: Matrix metalloproteinase (MMP)-13, type II collagen gene (COL2A1), and other promoter responses are analyzed by co-transfecting human or mouse cell lines with luciferase reporter constructs and expression vectors encoding signaling molecules and transcription factors. In vivo DNA binding is analyzed by chromatin immunoprecipitation. Selected signaling molecules and transcription factors are analyzed along with MMP-13 and type II collagen cleavage products by immunohistochemical staining in cartilage samples from human OA tibial plateaus, wild type mice subjected to DMM surgery, and cho/+ mice, a genetic OA model. The mRNAs of interest are also analyzed in cartilage tissues by in situ hybridization using available probes. For knockdown (KD) of expression, human primary or immortalized chondrocytes are transfected with specific siRNA oligo-nucleotides against or control nonspecific siRNA and incubated for 72 to 96 h. The KD is confirmed by real time PCR and Western blotting, and target mRNAs are analyzed by real time PCR.

RESULTS: We have identified new genes, not known previously to act in cartilage, including growth arrest and DNA damage (GADD) 45β and the ETS transcription factor, ESE1/ELF3, induced in chondrocytes by bone morphogenetic protein (BMP)-2 and inflammatory cytokines, respectively. Both GADD45β and ESE1/ELF3 are induced by NF-κB and in turn, upregulate MMP13 and suppress COL2A1 gene expression (1,2). A microarray study to compare IL-1β and BMP-2-induced genes resulted in the discovery of a novel role for GADD45β, an anti-apoptotic factor during genotoxic stress and cell cycle arrest, as a mediator of MMP-13 and type X collagen (Col10a1) gene expression during chondrocyte terminal differentiation (2,3). Since GADD45β is present in quiescent chondrocytes in normal cartilage and in early OA cartilage at sites peripheral to the lesion in chondrocyte clusters and in deep zone chondrocytes, it may promote chondrocyte survival, while promoting cartilage calcification during tidemark advancement (4).

DISCUSSION & CONCLUSIONS: Current studies involve both in vitro analysis of signaling and transcriptional mechanisms that regulate the expression and activities of GADD45β and ESE-1 and in vivo analysis of the consequences of knockout and transgenic overexpression of these genes in mouse models, using surgical OA (good matrix with abnormal loading) and genetic models with OA-like pathology (bad matrix with normal loading) during aging. In further studies, we are examining the epigenetic regulation of MMP-13 and using proteomics and genomics approaches to map the signaling networks and microRNA targets that impact on gene expression programs during the onset and progression of OA. These studies may to lead to the identification of critical targets for therapy to block cartilage damage and promote effective cartilage repair.

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ACKNOWLEDGEMENTS: This work was supported in part by National Institutes of Health grant R01-AG022021.

MOLECULAR MECHANISMS OF CARTILAGE REGENERATION

Francesco Dell'Accio

William Harvey Research institute, Barts and the London Queen Mary School of Medicine and Dentistry

Joint surface defects (JSD) involving the articular cartilage and the subchondral bone are a common clinical problem in rheumatology and orthopaedics. The current therapeutic modalities are still inadequate either for limitations related to the size of the defects and repair durability (microfracture) or for the elevated costs (autologous chondrocyte implantation).

In spite of the long held view that the adult articular cartilage can never heal spontaneously, the recent availability of sophisticated imaging in longitudinal studies has revealed that, particularly in young individuals, acute full thickness defects can undergo spontaneous repair. Those defects failing this early repair window reach the attention of the clinician and, indeed, have little residual repair capacity. The evidence that some of these lesions can heal spontaneously has raised important questions as to which lesions should be treated, when, and how. Evidence of repair of some of these lesions has also stimulated research into which factors contribute to successful healing and which ones determine chronic evolution and development of osteoarthritis (OA). The recent availability of large scale gene expression data following cartilage injury and during regeneration processes, new molecular tools, and in vivo models have revealed a complex cellular and molecular response of cartilage to focal defects, which could explain differences in healing responses between individuals, and may provide clues to stimulating intrinsic tissue repair. The combined use of traditional cell biology, novel in vivo models, and mouse genetics is not only providing increasing details on the molecular response to cartilage injury, but also represents a fantastic experimental platform to unravel the molecular basis of cartilage regeneration in vivo. This knowledge will be precious to develop a pharmacological approach to cartilage regeneration without the need of *ex vivo* cell manipulations.

Angiogenic factors, mechanical loads and oxygen supply: possible interactions in the regulation of degeneration associated disc angiogenesis?

C. Neidlinger-Wilke[1], A. Mietsch[1], U.M. Mauer[2], H.J. Wilke[1], A. Ignatius[1]

[1]Institute of Orthopaedic Research and Biomechanics, University of Ulm, [2]Department of Neurosurgery, German Armed Forces Hospital of Ulm, Ulm, Germany

INTRODUCTION: Disc degeneration is often associated with ingrowth of nerves and blood vessels into the degenerated disc tissue[1]. The reduced intradiscal pressure due to aggrecan degradation and the thereby diminished anti-angiogenic influence of aggrecan[2], as well as angiogenic factors such as pleiotrophin (PTN)[3], midkine (MK) and VEGF might play a role in the regulation of disc vascularization. As both mechanical loads[4] and alterations of the oxygen environment can influence gene expression of angiogenic factors, we hypothesize that these factors interact and thus influence disc angiogenesis via alteration of gene expression of pro- and anti-angiogenic factors by IVD cells.

METHODS: Human annulus fibrosus (AF) cells (n=6) were exposed to variations of the oxygen environment (21%, 6%, 1% pO_2) and to mechanical stimulation by cyclic strain (CS: 1%, 1Hz, 1hr). AF cells were supplemented in additional experiments by PTN and MK to investigate a possible autocrine stimulation of pro- and anti-angiogenic factors at unloaded conditions and after exposure to cyclic strain. Gene expression was analyzed by real-time RT-PCR using specific primers for cfos, HIF-1α, aggrecan, VEGF, PTN, and MK. Statistical evaluations were performed by Wilcoxon-signed rank test (level of significance $p<0.05$).

RESULTS: We found a significant up-regulation of VEGF-expression (+1.7-fold, $p=0.03$) at low oxygen supply (1%) compared to the disc normoxic condition of 6% pO_2. All other target genes showed higher variations with a tended increase of aggrecan expression (+1.5-fold increase) at low oxygen supply. CS did not alter the effects at different oxygen conditions. A significant up-regulation of cfos-expression by CS was observed at standard culture conditions (+6.2-fold), as well as in cultures supplemented by MK (+6.7-fold) and PTN (+6-fold). Expression of the angiogenic factor VEGF was increased at unloaded conditions by medium-supplementation with MK (median +1.3-fold, $p=0.03$) with quite big individual variation (up to +1.9-fold). There was a tended increase of VEGF expression after exposure to CS that varied between AF cells from different donors. Responses to mechanical loads were not significantly altered by MK- or PTN-supplementation of the medium due to high patient to patient variability. However, IVD cells from some patients exhibited an increased VEGF expression (up to +1,8-fold) while others were not influenced by the applied mechanical stimulus.

DISCUSSION & CONCLUSIONS: Our findings that oxygen deprivation increased VEGF expression by AF cells, and that both supplementation of the medium with angiogenic factors such as PTN and MK as well as mechanical stimulation by CS tended to increase VEGF expression suggest possible interactions of these factors. Together with our previous findings of an increased PTN-expression by AF cells after exposure to CS[4], an enhancement of these effects via an autocrine mechanism could be assumed. However, the high individual differences in these interactions make it difficult to find a general angiogenic regulation mechanism. Obviously, genetic differences might determine whether disc cells respond to angiogenic stimuli or not. This might explain the big variations in histological findings of discs of the same degenerative situation with some of them showing ingrowth of blood vessels and others not. Ongoing studies are necessary to investigate the role of other genetically determined mechanisms that play a role in the regulation of degeneration-associated disc angiogenesis.

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ACKNOWLEDGEMENTS: This project was funded by EU project GENODISC (HEALTH-F2-2008-201626).

Identification of Human Nucleus Pulposus (NP) and Articular Cartilage (AC) Marker Genes and their Expression in the Degenerate NP.

BM. Minogue, SM. Richardson, JA Hoyland.

School of Biomedicine, Faculty of Medical and Human Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT, UK.

INTRODUCTION: Nucleus pulposus (NP) cells have a phenotype similar to articular cartilage (AC) cells [1]. However, the matrix of the NP is clearly different to that of AC suggesting that specific cell phenotypes exist. To date, studies have reported potential markers for rodent, canine and bovine NP cells and importantly have highlighted species differences in marker profiles [2-4]. Using Affymetrix microarrays and qRT-PCR we have identified human gene markers that distinguish NP cells from their closely related AC cells and have further determined their expression in normal and degenerate human intervertebral disc (IVD) cells.

METHODS: Human IVD tissue was obtained during post-mortem examination and human AC was obtained during total knee arthroplasty, both with informed consent from relatives or patients and local ethical committee approval. Cells were released by enzymatic digestion and RNA extracted. Hybridisations for each cell type were performed in triplicate from three separate individuals using Affymetrix GeneChip® Human Genome U133 Plus 2.0 Arrays. Genes identified were validated in normal human NP and AC samples and further characterised in degenerate NP samples using qRT-PCR.

RESULTS: Microarray comparisons between NP and AC cells identified 189 probesets (152 genes) that were differentially expressed ≥10 fold, of which 42 genes showed high differential expression in NP cells and 110 genes showed high differential expression in AC cells. A subset of these genes was verified by qRT-PCR and demonstrated significant differential expression between NP and AC cells (Figure 1). In particular PAX1 showed significantly higher levels of expression in NP cells when compared to AC cells (>1000 fold, P<0.0001). All of the NP gene markers tested (PAX1, FOXF1, HBB CA12 and OVOS2) showed decreased expression in degenerate human NP cells relative to normal NP cells. Furthermore the NP negative marker FBLN1 was significantly increased in degenerate human NP cells (50 fold, P<0.0001) relative to normal NP cells.

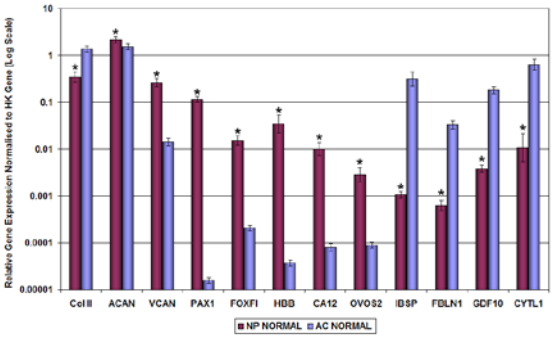

Fig. 1: Quantitative real-time PCR for novel marker genes in human NP and AC cells. Relative gene expression for Col II, ACAN, VCAN, PAX1, FOXF1, HBB CA12, OVOS2, IBSP, FBLN1, GDF10 and CYTL1 was normalised to the house-keeping gene and plotted on a log scale. Represents statistical significance between NP and AC cells (P<0.05).*

DISCUSSION & CONCLUSIONS: This is the first study to have used microarray technology on human NP and AC cells and has identified a number of novel genes that characterise the human NP and allows for the discrimination between AC and NP cells. The use of this gene expression signature will benefit tissue engineering studies where defining the NP phenotype is paramount. Furthermore, changes in the expression profile of these genes were observed in the degenerate NP suggesting that cell phenotype is altered and that they may also be involved in one of the many cellular/tissue events characterising the degenerate IVD.

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ACKNOWLEDGEMENTS: Array analyses were performed by the Microarray and Bioinformatics Core Facilities in the Faculty of Life Sciences, University of Manchester. This research was funded by the Arthritis Research Campaign (Grant ref No:18046).

Burst fracture of the vertebral endplates but not equienergetic impact load promotes in vitro disc degeneration

S. Dudli[1], D. Haschtmann[1,2], S.J. Ferguson[1]

[1] *Institute for Surgical Technology and Biomechanics, University of Bern, CH*
[2] Schulthess Klinik, Zürich, CH

INTRODUCTION: Endplate (EP) burst fractures in disc/EP specimens induce disc degeneration (DD) *in vitro* [1]. The aim of this study was to elucidate whether trauma without EP fracture but with equienergetic impact load induces DD. In order to reproduce more *in vivo* like conditions, the organ model was extended to include partial adjacent vertebral bodies.

METHODS: 76 spinal segments (intervertebral disc (IVD) with one third of adjacent vertebral bodies) were isolated from eight New Zealand White rabbits (6 months old) and cultured in standard media. Trauma induction was performed one week post-harvest in half of the specimens, using a dropped-weight (steel ball, 0.76 J) fracture device. DD mediators [1-2] were measured for the nucleus pulposus (NP) and the annulus fibrosus (AF) 1, 3 or 7, 14 and 28 days post trauma by assessing cell viability, gene transcription using quantitative real-time PCR, GAG/DNA ratio (whole specimen), and Caspase 3/7 activity (only AF). Lactate dehydrogenase (LDH) activity in the media was measured 1, 3, 6, and 8 days post-trauma. All data are relative to control samples of the corresponding day. 6 µm sagittal sections of PMMA-embedded specimens were stained with Movat's Pentachrome.

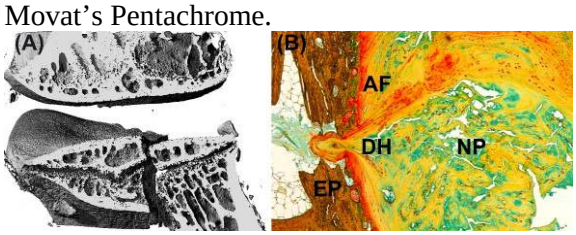

Fig. 1: (A) µCT scan and (B) sagittal section with herniated disc (DH) of group A specimen.

RESULTS: The applied trauma-protocol induced an EP burst fracture in 21 specimens (group A, Fig. 1) and minor vertebral bone fractures or no visible fractures in 17 specimens (group B). Cell viability stayed for all specimen at a high level for 28 days (avg. 92 % ± 4 %, n = 39) except for the AF on day 28 (-8.5 %, n = 3, p = 0.2). LDH activity was significantly increased in group A one day post-trauma (207 %, n = 9, $p < 0.01$) but not in group B (111 %, n = 11, p = 0.76). Caspase 3/7 activity was elevated in trauma group A up to 28 days (avg. 182 %, n = 8, p = 0.05), but not in group B (avg. 102 %, n = 4, p = 1.00). GAG/DNA ratio was significantly reduced in group A after > 7 days (avg. -29 %, n = 4, p = 0.05) but not in group B (avg. +15 %, n = 5, p = 0.35). Gene transcription of DD mediators were strongly enhanced in the NP 1 and 3 days post trauma in group A and less pronounced in group B (Fig. 2). All levels but Collagen I of group A were normalized after 28 days. Gene transcription in the AF remained unchanged (not shown).

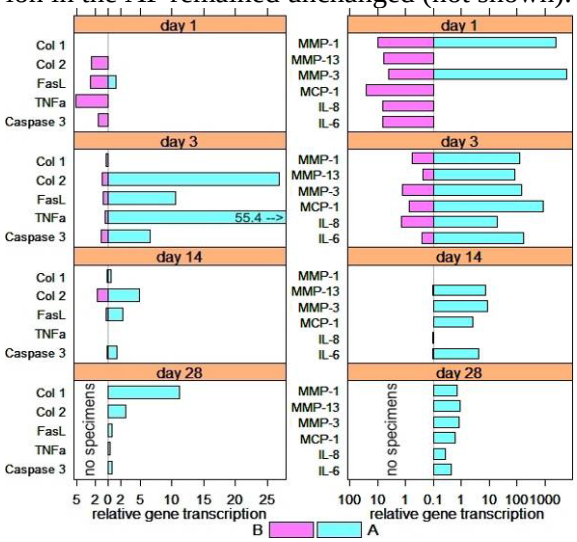

Fig. 2: Relative gene transcription levels in the NP in group B (no EP fracture) and A (EP fracture) of DD mediators, normalized to GAPDH (n(A+B) = 3/day).

DISCUSSION & CONCLUSIONS: A single impact load on spinal segments induces intradiscal expression of DD-associated mediators *in vitro*. Effects are stronger in the NP and in IVD with burst EPs. Therefore we conclude that burst EPs are pivotal for the onset of the vicious cycle of DD which occurs first in the NP and may extend to the whole organ.

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ACKNOWLEDGEMENTS: Funded by Swiss National Science Foundation #310000-122105.

Hierarchical Functionalisation of Scaffold- Macro to Nano

Abhay Pandit
Network of Excellence in Functional Biomaterials
National University of Ireland, Galway
Abhay.pandit@nuigalway.ie

Biomaterials are no longer considered innate structures and using functionalisation strategies to modulate a desired response whether it be a host or implant responsive is currently an important focus in current research paradigms. Using functionalisation strategies such as enzymatic and dendrimeric linkers, we have been able to link biomolecules to different structural moieties.

The programmed assembly of biomolecules into higher-order self-organized systems is central to innumerable biological processes and development of the next generation of functionalized scaffolds. Recent design efforts have utilized a bottom-up approach toward both understanding and engineering supramolecular protein assemblies.

We have synthesized a wide variety of functionalisation systems but have also contributed broadly to the physical characterization and the development of applications of these dendritic macromolecules. Studies involving rheological, thermal, optical, and other methods have revealed that these polymers have unique properties that diverge widely from the established patterns of conventional macro-molecules. These properties, and the unique ability to tailor the polymers at the molecular level, have led to explorations of the use of dendritic polymers in a host of innovative applications.

These include functionalisation of nanoparticles with biomolecules that include designed peptide motifs, growth factors and a multitude of gene vector systems. Structural moieties have taken a variety of different forms such as nanofibers and nanoparticulate. Functionalisation on a microscale and macroscale has also been successfully attempted. Such strategies with examples from in vitro and in vivo studies will be illustrated. Development of complex geometrical structures and quantification of these geometries that have aided these investigations will be exemplified.

Acknowledgement
Science Foundation Ireland, EU FP7, Health Research Board (Ireland)

Modulation of cell behaviour through nanoscale architecture

M. M. Stevens

Department of Materials and Institute for Biomedical Engineering, Imperial College London, London. M.stevens@imperial.ac.uk

OVERVIEW: The highly interdisciplinary field of Tissue Engineering (TE) can benefit from advances in the design of bio-mimetic materials. The molecular and physical information coded within the extracellular milieu is informing the development of a new generation of biomaterials for tissue engineering. Several powerful extracellular influences have already found their way into cell-instructive scaffolds, while others remain largely unexplored[1].

The ability to control topography and chemistry at the nanoscale for example offers exciting possibilities for stimulating growth of new tissue through the development of novel nanostructured scaffolds that mimic the nanostructure of the tissues in the body. Recent developments from our group in this context will be discussed [2,3,4,5].

Since an important aim of regenerative medicine is to restore tissue function with implantable, laboratory-grown constructs that contain tissue-specific cells that replicate the function of their counterparts in the healthy native tissue – a thorough analysis of the regenerated tissue is required. For example it remains unclear, whether cells used in musculoskeletal regeneration applications produce a material that mimics the structural and compositional complexity of native tissue. By applying multivariate analysis techniques to micro-Raman spectra of mineralized nodules formed in vitro, we reveal cell-source-dependent differences in interactions between collagen and multiple bone-like mineral environments[6]. Understanding the biological mechanisms of tissue formation in vitro that contribute to cell-source-specific materials differences may facilitate the development of clinically successful engineered tissue.

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ACKNOWLEDGEMENTS: Funding from the EPSRC, ERC, Wellcome Trust amongst others is gratefully acknowledged.

Injectable thermoreversible hyaluronan-based hydrogels for interveterbral disc repair.

M.Peroglio, D.Mortisen, M.Alini, D.Eglin

[1]*AO Research Institute Davos (ARI), AO Foundation, Clavadelerstrasse 8, 7270 Davos, CH*

INTRODUCTION: Hyaluronan (HA) is an important constituent of the intervertebral disc (IVD) extracellular matrix; hence HA-based hydrogels may offer an appropriate environment for disc cell therapy. The properties of thermoreversible hydrogels (e.g. injectability, stiffness, water retention) are influenced by its composition and architecture. In this study, the synthesis, rheology and water retention of HA grafted poly(N-isopropylacrylamide) (PNP) hydrogels is reported, together with disc cell survival in the gels after 1 week of culture.

METHODS: Synthesis: HA-PNP hydrogels were synthesized by a procedure reported elsewhere [1]. Briefly, N_3-terminated PNP of Mn equal to 10, 20 and $35x10^3$ $g{\cdot}mol^{-1}$ were synthesized by RAFT polymerization and grafted to propargylamide functionalized HA by copper(I) catalyzed alkyne-azide cycloaddition at a degree of substitution (DS) of 25% of the alkyne subunits. In the following, HA grafted with PNP 10, 20 and $35x10^3$ $g{\cdot}mol^{-1}$ and DS=25% will be named A25, B25 and C25 respectively. Characterization: The rheological behaviour of HA-PNP solutions was determined with a CVOR-Rheometer Bohlin instrument with Piezo-Rotary-Vibrator (PRV) option and plate-plate geometry at 1°C/min heating from 25 to 40°C at 2 Hz. The water retention after 1 hr in PBS at 37°C was calculated as the ratio of the wet-dry difference of the sample weight and wet-dry difference of the blank weight. Disc cell encapsulation: Primary nucleus pulposus (NP) cells isolated from 4 months old calves were seeded in the hydrogels ($3x10^6$ cells/ml). Gel drops were formed with an autoinjector (Harvard Apparatus) and plunged in a PBS bath at 37°C and then transferred in 12 well plates containing DMEM-10% FCS (Gibco). After 1 week, cell viability was assessed by live-dead assay. Cell morphology was evaluated on 10 µm thick cryosections, post-fixed in 4% buffered paraformaldehyde and stained with toluidine blue.

RESULTS: The lowest viscosity at 25°C was measured for B25 solution (4.6 Pa·s). At 37°C A25 and B25 showed similar G' (100-200 Pa), while G' of C25 was almost two orders of magnitude higher (~10 kPa). A25 showed an increase in water retention of ~50% after 1 hr in PBS at 37°C, B25 water retention was constant and C25 shrank of almost 50%. Cell viability after 1 week was high (>90%) in B25 hydrogel, while was ~50% in the case of C25. NP cells maintained their round morphology in B25 hydrogels, while their appeared stretched in C25 hydrogels, as observed on toluidine blue stained sections.

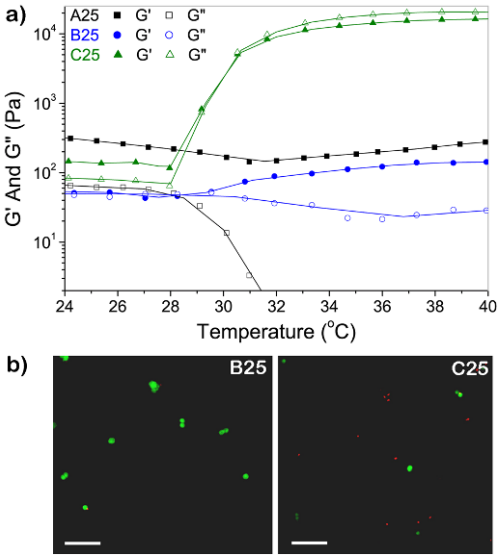

Fig. 1: a) A25, B25 and C25 G' and G'' evolution upon heating from 25 to 40°C (2 Hz); b) live-dead assay on NP cells cultured in B25 and C25 for 1 week (scalebar=100 µm).

DISCUSSION & CONCLUSIONS: RAFT polymerization and "click" chemistry permitted the synthesis of HA-PNP with adjustable properties (e.g. stiff or compliant, presenting swelling or no volume change upon gelling), Hydrogels with varying stiffness and water retention were obtained by slight changes in graft length. A composition adequate for the encapsulation of NP cells was identified and could be used for cell therapy in IVD repair.

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TAILORING SELF-ASSEMBLED OCTAPEPTIDE SCAFFOLDS FOR *IN-VITRO* CARTILAGE REPAIR

A Mujeeb, J Gough & A Saiani

Materials Science Centre, University of Manchester, Grosvenor Street, Manchester, M1 7HS, UK

INTRODUCTION: Nature has evolved a variety of creative approaches to many aspects of materials synthesis and microstructural control. One such approach is self-assembly, which represents a simple and efficient route to the construction of large, complex structures. *De novo* designed peptides are in particular attracting considerable interest due to their structural simplicity, diverse functionality and their ability to self-assemble into a variety of structures and form hydrogels.[1-2] We have recently investigated the self-assembling and gelation properties of a series of ion-complementary peptides based on the alternation of non-polar hydrophobic and polar hydrophilic residues.[3] In this work we focus on two specific octapeptides: FEFEFKFK and FEFKFEFK (F: phenylalanine, E: glutamic acid, K: lysine). These two peptides were shown to self-assemble in solution and form β-sheet rich nanofibres which, above a critical gelation concentration (CGC), entangle to form self-supporting hydrogels for cartilage repair (Figure 1).

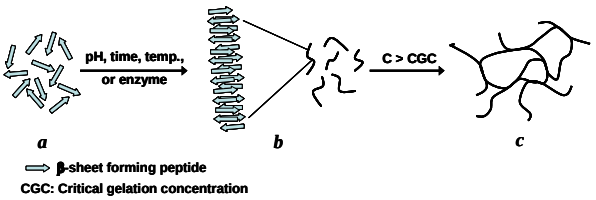

Figure 1: Schematic representation of self-assembly and gelation mechanisms of β-sheet forming peptides

METHODS: The octapeptides were synthesised using a solid phase peptide synthesiser (a ChemTech ACT 90 peptide synthesiser). The fibre morphology of the hydrogels was analysed using TEM and Cryo-SEM. The mechanical properties of the hydrogels were determined using oscillatory rheology. Bovine chondrocytes were used to assess the biocompatibility of the scaffolds under 2D/3D cell culture conditions, particularly looking into cell morphology and proliferation using light microscopy, live-dead and immunochecmical staining, MTS ([3-(4,5-dimethylthiazol-2-yl)-5(3carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), LDH (lactate dehydrogenase), Alamarblue and DNA assays.

RESULTS: These hydrogels consist of a dense fibrillar network of nanometer size fibres which present similarities with the extra cellular matrix (ECM) (Figure 2B). The gelation dynamics and mechanical properties of these gels could be controlled through concentration and processing. Protocols for the preparation of scaffolds for 2D and 3D (homogeneous incorporation of cells) cell culture have been developed using the cell culture medium properties (pH and ionic strength) to trigger the gelation. The hydrogel developed were subsequently used to culture bovine chondrocytes over 21 days. The live-dead staining and the collagen antibody-staining results show the presence of living chondrocytes in the scaffold. The cell proliferation results demonstrated the scaffolds to be cytocompatible with the cells showing varying metabolic activity.

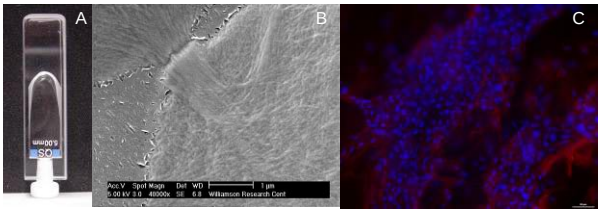

Figure: 2. (A) FEFEFKFK Hydrogel formed at 20 mg ml^{-1}; (B) Cryo-SEM micrographs of hydrogel showing a dense fibrillar network (scale bar = 1 μm); (C) collagen I antibody staining of cell-seeded gel (scale bar = 50 μm).

DISCUSSION & CONCLUSIONS: We have created scaffold exploiting the self-assembly properties of short ionic-peptides. These scaffolds were shown to be biocompatible and sustain the culture of chondrocytes over 21 days in 2D and 3D. The research is now focusing on functionalising these gels using biological signal to trigger specific cell behaviors.

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Substrate elasticity modulates TGF beta stimulated re-differentiation of expanded human articular chondrocytes

D. Vonwil[1], A. Barbero[1], A. Trüssel[1], O. Haupt[1], I. Martin[1]

[1]*Department of Surgery and Biomedicine, University-Hospital Basel*

INTRODUCTION: Culture of mesenchymal progenitor cells on substrates with different elasticity has been shown to modulate cell fate/commitments[1]. We aimed this study at investigating whether substrate elasticity modulates TGFβ stimulated chondrogenic re-differentiation of expanded/de-differentiated human articular chondrocytes (HAC).

METHODS: Expanded HAC from 4 donors (43-77 years) were seeded onto Type I collagen (CI) functionalized poly acrylamide (PA) films (100-150 thickness) with a Young's modulus of 0.26±0.08 kPa (*soft*), 21.32±0.79 kPa (*intermediately stiff*) and 74.88±5.13 kPa (*stiff*) and induced to re-differentiate in a defined serum free medium containing or not TGFβ-3 for 7 days. CI coated tissue culture treated plastic was considered as an *infinitely stiff* substrate and HAC aggregate cultures served as a standard re-differentitation control. HAC were assessed for attachment, proliferation, morphology and mRNA expression (type I and II collagen).

RESULTS: In the presence of TGFβ-3, HAC attached similarly on the different substrates and accomplished less than one total doubling within 7 days. On *intermediately stiff* to *infinitely stiff* substrates HAC assumed a fully spread fibroblastic morphology (shape factor ϕA = 0.23-0.27), whereas on the soft substrate, they remained more spherical (ϕA = 0.35±0.02) and had a reduced spreading area (up to 3.2-fold). F-actin organization on the *soft* substrate was restricted cortically, while on the stiffer substrates, F-actin assembled into stress fibres (Fig. 1). Type II collagen mRNA expression on the *soft* substrate was similar to that in aggregate culture and 18.1-fold higher than on *infinitely stiff* substrates (Fig. 2).
However, in the absence of TGFβ-3, type II collagen mRNA remained at levels expressed by expanded/de-differentiated HAC (Fig. 2).

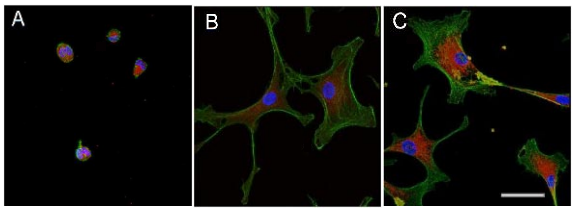

Fig. 1: Representative confocal laser scanning microscopy images of HAC cultured on soft *(A),* intermediately stiff *(B) and* infinitely stiff *(C) substrates. Green: F-actin, red: vinculin, blue: nuclei. Scale bar = 50µm.*

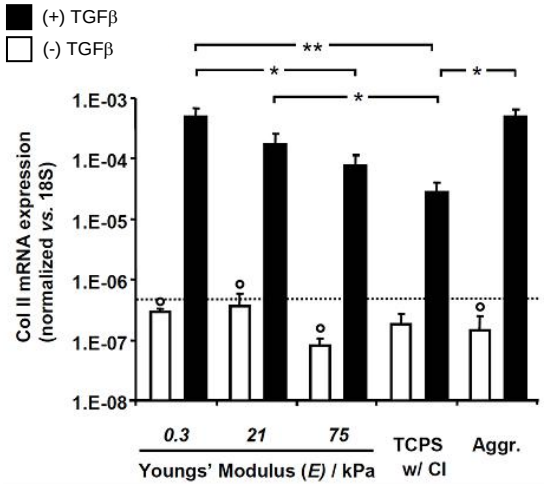

*Fig. 2: Type II collagen mRNA expression levels of HAC cultured on the different substrates or as aggregates. TCPS: tissue culture treated plastic. ····· =expression level in post-expanded HAC, * = p<0.05, ** = p<0.01 from substrate groups, ° = p<0.05 from (+) TGF (same substrate group)*

DISCUSSION & CONCLUSIONS: Substrate elasticity modulated the re-differentiation response of expanded/de-differentiated HAC to the chondrogenic stimulus TGFβ-3, and thus underscores that mechanical compliance in combination with appropriate soluble signals is an important parameter in designing biomaterials for cartilage repair.

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Effects of Shearing on Intervertebral Disc Mechanics and Biology

J.C.Iatridis, A.J. Michalek, A. Barbir

Spine Bioengineering Lab, University of Vermont, Burlington, VT, USA

INTRODUCTION: Torsion is an important loading mode in the intervertebral disc (IVD) and increased torsional range of motion is associated with clinical symptoms from disc disruption. The response of the IVD to torsion should be highly sensitive to anulus fiber tensioning, yet few studies have investigated torsion loading effects on the mechanical and biological response of IVDs. This study reviewed recent papers focusing on torsion loading effects on biological and mechanical behaviors.

METHODS: The first study investigated effects of torsion loading on IVD mechano-biology using a rat tail model subjected to varying levels of cyclic torsion loading with measurements of mRNA expression [1]. The second study examined how torsional shear affects the microscale mechanical response of annulus fibrosus (AF) tissue using in vitro shear testing under confocal microscopy [2]. The third study measured how needle puncture injury affects microscale shearing behaviors under confocal microscopy [3].

RESULTS: Cyclic torsion upregulated elastin expression in the annulus, with little up-regulation of other anabolic genes (Fig. 1). Cyclic torsion at large magnitudes increased proinflammatory cytokine expression for TNF-α and IL-1β, but no changes to in vivo or in vitro biomechanics were observed. Shear loading of isolated AF tissue in vitro induced fiber stretching and rotation. Since elastin was upregulated during in vivo testing, we evaluated effects of elastase digestion on this microscale response. Under shearing, elastase digestion increased fiber rotation and altered fiber load carriage mechanisms. Annular disruption using needle puncture significantly disrupted microscale shear strains with small areas of increased shear strain at fiber bundle boundaries and large areas of strain shielding within the fiber bundles.

DISCUSSION & CONCLUSIONS: Several important findings arise from these works. First, torsional loading does not stimulate generalized protein biosynthesis as does dynamic compression, yet does have the capacity to increase expression of proinflammatory cytokines. Second, elastin expression is upregulated with torsion loading and elastin plays an important role in influencing AF fiber tension under circumferential shear. Third, puncture injuries locally disrupt shear strain through altered fiber tension. That torsion loading upregulates elastin, and that elastin structure has a large impact on AF fiber tension and shear load carriage mechanisms leads us to conclude that shearing influences both IVD mechanics and biology in specific and interactive ways.

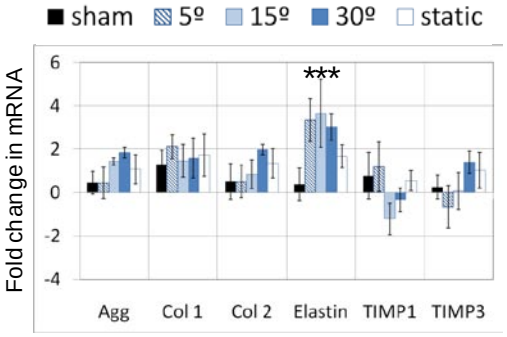

Fig. 1: Fold changes in mRNA levels relative to internal control levels (mean ± SEM) for anabolic genes in the annulus region subjected to cyclic torsion of different amplitudes.

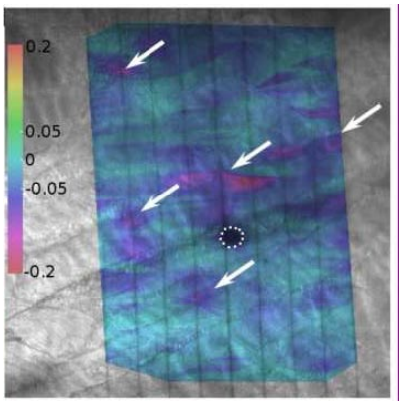

Fig. 2: Localized shear strain map surrounding a needle puncture injury in bovine AF tissue.

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ACKNOWLEDGEMENTS: Funded by the National Institutes of Health & AO Foundation.

Mechanical Stimulation Enhances Functional Mesenchymal Stem Cell Chondrogenesis in 3D Culture

AH Huang, MJ Farrell, IE Erickson, M Kim, BM Baker, JA Burdick, RL Mauck

Departments of Orthopaedic Surgery and Bioengineering, University of Pennsylvania, Philadelphia, PA 19104 USA

INTRODUCTION: Articular cartilage is a load-bearing surface whose mechanical function arises from its inhomogeneous (depth dependent) and anisotropic (direction dependent) material properties[1]. Tissue maturation is directed by mechanical forces; loading induces remodeling of the immature matrix, leading to increases in compressive and tensile properties. Limitations in intrinsic cartilage repair have engendered numerous efforts to engineer functional replacements. As mesenchymal stem cells (MSCs) undergo chondrogenesis in 3D culture, this cell type has been increasingly utilized in such pursuits; however, generating MSC-based constructs with the mechanical complexity and integrity of native cartilage remains a challenge[2]. To enhance this process, we applied mechanical stimulation, through two different bioreactor systems, to enhance the functional development of MSC-seeded hydrogels.

METHODS: Bovine MSCs were isolated and encapsulated in 2% agarose at a density of 20 million cells/mL and pre-cultured in TGF-β3 supplemented chondrogenic induction media (CM+) for 3 weeks[3]. For dynamic compressive stimulation, cylindrical disks (Ø4 x 2.25 mm) were formed. Constructs were loaded in a custom bioreactor system for 5 days per week, 4 hours daily at 1 Hz and to 10% axial strain for 3 weeks (weeks 4-6). Free swelling controls were maintained similarly. Constructs were evaluated at week 6 for biochemical, histological, and mechanical characteristics as in[3]. To better replicate joint loading, and to specifically investigate developing tensile properties, a bioreactor was developed to apply sliding contact (SLC) to MSC-seeded hydrogels. As above, SLC was initiated following 3 weeks of chondrogenic pre-culture; loading was applied through a translating spherical indenter to MSC-seeded strips (41.5 x 7.5 x 1.5 mm) in CM+, 5 days per week, 3 hours daily at 2.5 mm/s and 20% axial strain for 3 weeks (weeks 4-6). Tensile properties and histological features were evaluated[4] in comparison to free swelling controls.

RESULTS: By week 6 of dynamic compressive loading (initiated at week 3), the compressive modulus of loaded samples was greater than non-loaded controls (152±29 kPa vs. 94±17 kPa). GAG and collagen contents were comparable between these groups, though collagen distribution was more uniform in the loaded group. When MSC-seeded strips were subjected to sliding contact, the tensile modulus likewise improved, reaching 783±79 kPa compared to 634±83 kPa for free-swelling controls. Interestingly, while free-swelling constructs exhibited punctate staining of type II collagen, SLC constructs showed more continuous distribution through the depth, with more intense collagen staining observed in the surface region.

DISCUSSION & CONCLUSIONS: Overall, these data demonstrate that dynamic mechanical loading can accelerate the functional maturation of MSC-seeded hydrogels. When dynamic compression was initiated after a 3 week period of pre-culture, construct properties improved dramatically compared to free swelling controls. In a new sliding contact bioreactor, construct tensile and biochemical properties were improved, and evidence of developing matrix inhomogeneity through the depth was observed. Future work will focus on application of SLC over longer culture durations, and evaluate the efficacy of this loading system in directing depth dependent compressive properties and collagen fiber alignment in MSC-based engineered cartilage.

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ACKNOWLEDGEMENTS: This work was supported by the National Institutes of Health and Penn Center for Musculoskeletal Disorders.

Primary cilia are essential for chondrocyte mechanotransduction

AK.Wann[1], SR.McGlashan[2], CJ.Haycraft[3], CA.Poole[4], MM.Knight[1]

[1]*School of Engineering and Materials Science, Queen Mary University of London, UK*
[2]*Department of Anatomy with Radiology, University of Auckland, New Zealand*
[3]*Department of Craniofacial Biology, Medical University of South Carolina, SC, USA.*
[4]*Department of Medicine, University of Otago, New Zealand*

INTRODUCTION: The primary cilium is an organelle found in virtually all eukaryotic cells including chondrocytes[1]. In other cell types primary cilia function as mechanoreceptors. This study tests the hypothesis that chondrocyte primary cilia are required for mechanosensitive up-regulation of sGAG synthesis.

METHODS: The study used chondrocytes extracted from Wild-type (WT) and *Tg737* Oak Ridge Polycystic Kidney (*Tg737*^{orpk}) mice. Hypomorphic allele mutation of the *Tg737* gene (IFT88) disrupts polaris expression, interrupting ciliogenesis and resulting in severely stunted or absent primary cilia[2]. WT and ORPK cells also contain a temperature sensitive mutant of the SV40 large tumor antigen (T-Ag) under the control of the interferon-γ-inducible *H-2Kb* promoter. Cells were expanded in monolayer with 10 ng.mL^{-1} interferon–γ at 33°C and then transformed into chondrocytes by withdrawing interferon–γ and culturing at 37°C. Both WT and ORPK chondrocytes were seeded into 3% agarose constructs at 10x10^6 cells.mL^{-1}. In addition, primary articular chondrocytes were isolated from the bovine metacarpal-phalangeal joint and seeded in identical constructs. Cell-agarose constructs were subjected to 24hrs cyclic compression at 1Hz, 0-15% strain. Controls constructs remained unloaded. Immuno-fluorescence was used to visualize primary cilia in monolayer cells labeled anti-alpha-acteylated tubulin. Matrix synthesis was quantified by measuring the total sGAG content in the agarose constructs and associated media using the DMB assay.

RESULTS: Immunofluoresecence showed primary cilia expression in primary and WT chondrocytes but almost complete absence of cilia in ORPK cells (Fig. 1). All three cell types synthesised sGAG over the 24hrs in agarose. Cyclic compression induced a statistically significantly up-regulation of sGAG synthesis in both primary and WT chondrocytes but not in ORPK cells (Fig. 2).

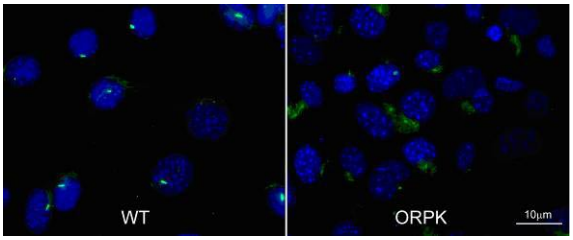

Fig. 1: Immunofluorescence showing primary cilia (green) in WT but not in ORPK cells. Nuclei labelled with Dapi (blue).

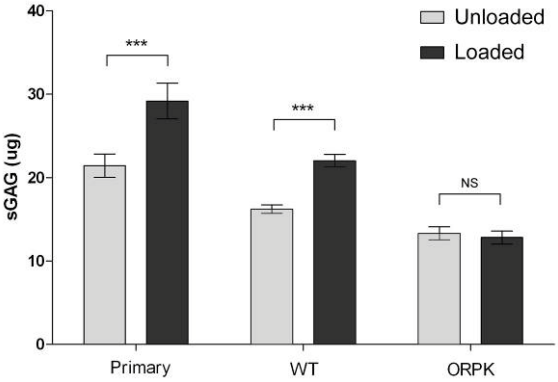

*Fig. 2: Cyclic compression up-regulates sGAG synthesis in primary and WT cells but not in ORPK cells. Values represent mean sGAG synthesised per construct. (***p<0.001).*

DISCUSSION & CONCLUSIONS: Primary and wild type chondrocytes exhibit an anabolic response to cyclic loading similar to that previously reported[3]. However this response was completely absent in ORPK cells with stunted or missing primary cilia. Although the mechanism is still unclear, this is the first study to provide evidence that the primary cilium is essential for chondrocyte mechanotransduction.

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ACKNOWLEDGEMENTS: Angus Wann is supported by the Wellcome Trust, UK.

Development Of An *Ex Vivo* Disc Model System To Test Novel Therapies: Comparison Of Loaded Isolated Discs *Vs* Whole Motion Segments.

E.A.Mitchell[1,] A.M.White[1], S.M.Richardson[1], J.A.Hoyland[1]

[1] School of Biomedicine, University of Manchester, Manchester, England.

INTRODUCTION: Currently there is a substantial amount of interest in developing novel cell-based tissue engineering therapies for intervertebral disc (IVD) degeneration. Such therapies need to be tested *in vitro* or *in vivo* before translation to clinic. However, current animal models do not accurately mimic the human IVD or disease pathophysiology and thus a more appropriate model system for efficacy testing is required. We have previously described a unique bioreactor which can mimic the load and humoral environment of the human IVD and shown that IVD explants can be maintained in this system for 2 weeks with maintenance of cell viability and tissue integrity [(1)]. Here we have expanded this work to develop an optimal whole disc model system which can be maintained for extended periods of time in a loading environment pertaining to that of the human spine.

METHODS: Bovine caudal IVDs (ages 9-18 months) were isolated in the presence of heparin, and either excised as discs and subsequently enclosed within an apparatus to mimic endplates (isolated discs) or as whole motion segments with intact endplates (intact discs) (figure 1).

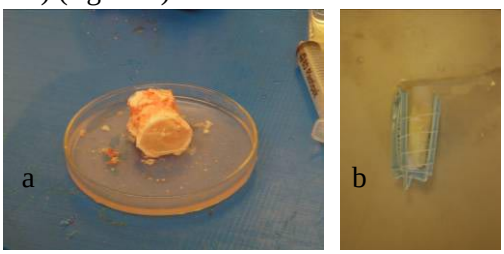

Figure 1: (a) intact discs; (b) isolated discs artificial endplates to prevent swelling of IVD.

Samples were then subjected to a daily hydrostatic loading regime (as detailed previously [(1)]) for set time points up to 5 weeks. Unloaded control discs were cultured for the same period.

Following loading 6mm punch biopsies were taken from the nucleus pulpous (NP) and the outer annulus fibrosis (AF) and assessed to determine cell viability, gylcosaminoglycan (GAG) and collagen content. Atomic force microscopy was used to assess ultrastructural changes

RESULTS: Cell viability: PolyA *in situ* hybridisation demonstrated that in isolated discs cell number and viability was substantially decreased in both the loaded and unloaded samples. Cell viability in intact discs was maintained throughout the culture period and there were no significant differences between the loaded or unloaded samples.

Tissue integrity: Alcian blue staining confirmed that loading helped to maintain GAG content in isolated discs. GAG content was maintained up to 35 days in intact discs and staining was similar in both loaded and unloaded intact discs. Immunohistochemistry demonstrated strong immunopositivity for type I collagen in the AF of loaded isolated discs and intact discs which was corroborated by AFM data demonstrating that the collagen fibril bundles were more orientated in these samples. Likewise there was strong type II collagen immunopositivity in the NP of isolated and intact discs with more intense staining (cellular and pericellular) in loaded intact discs. AFM showed that collagen fibrils were orientated while in unloaded samples collagen fibrils were less orientated and not as well demarcated.

CONCLUSION: Cell viability and tissue integrity was maintained both in loaded isolated and intact discs throughout the 5 week culture, although the data suggests that intact discs are a more optimal model. While loading does not appear to significantly affect cell viability in intact discs loading causes changes in ECM structure.

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A**CKNOWLEDGEMENTS:** This work was funded by the ARC.

Animal Models for Cartilage Regeneration

C. W. McIlwraith, D.D. Frisbie,
Orthopaedic Research Center, Colorado State University, Fort Collins, CO 80523, USA,

The aims of articular cartilage repair techniques are: 1. generation of repair tissue with structural characteristics approximating articular cartilage, 2. integration with adjacent tissue (articular cartilage and subchondral bone). Prior to initiation of human trials, information must be gained in non-clinical models. The use of pre-clinical models ideally should reflect information needed for the product in question and should involve adequate cartilage thickness in an appropriate, critically sized defect. This prompts issues of anatomy, size and, therefore biomechanical forces, articular cartilage thickness, subchondral bone density, clinical monitoring and ability to control exercise. Various models in animals have been reviewed but the author focused on currently used equine models.[1]

The equine models offer advantages as in that the horse gets clinical disease similar to human, the articular cartilage thickness is comparable, large multiple defects can be created, the arthroscope can be used to create lesions and do reexaminations, controlled exercise post-operatively can be done and the horses can be monitored clinically. A study looking at the articular cartilage thickness over five locations on the medial femoral condyle and femoral trochlear ridges showed the following results for cartilage thickness; human 2.2 – 2.5 mm, rabbits 0.3 mm, sheep 0.4 – 0.5 mm, dogs 0.6 – 1.3 mm, goats 0.7 – 1.5 mm and horse 1.5 – 2.0 mm.

In the medial femoral condyle the thickness of noncalcified and calcified cartilage is 2.05 mm.[2] Studies using 1.0 cm^2 defects in the femoral condyle have been used to demonstrate the long term and early effects of subchondral microfracture as well as the differential effects of retaining or removing calcified cartilage,[3] and intraarticular AdIL-1ra/AdIGF-1 gene therapy.[4] These studies have shown significant increase in the amount of repair tissue with microfracture, upregulation of type II collagen expression at 8 weeks with microfractured defects, superior healing with removal of the calcified cartilage and superior aggrecan content in repair tissue with gene therapy supplementation.

Models have been used with 15 mm defects in the medial or lateral trochlear ridges. A recent study testing the ability of a cartilage autogenous implantation system (CAIS) demonstrates the ability to create two 15 mm defects, do sequential arthroscopic examinations at 3, 6, 9 and 12 months with the horses being athletically exercised from 8 to 12 months. Cartilage fragments on PDS foam showed good healing on MTR defects at 4, 8 and 12 months compared to PDS foam alone and empty defects.[4]

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Non-destructive evaluation of human mesenchymal progenitor cell transcriptional activity within osteochondral defects

R.M. Porter[1], F.J. Liu[1], E. Ferreira[1], Z. Shen[1], and C.H. Evans[1]

[1]*Center for Advanced Orthopaedic Studies*
Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, USA

INTRODUCTION: Cartilage loss through traumatic injury remains an important clinical challenge. Because sources of autograft are limited, the generation of cartilage repair tissue *in situ* using endogenous mesenchymal progenitor cells (MPCs) represents an attractive alternative. However, this approach requires MPC differentiation to occur within a potentially refractory environment. For example, pro-inflammatory cytokines produced in response to injury or surgical intervention can inhibit chondrogenesis of human MPCs through elevated nuclear factor (NF)-κB signaling [1]. In order to determine how the traumatized joint environment affects human MPC activity *in vivo*, we have developed a nude rat model to measure transcriptional activity of hMPCs within osteochondral defects using bioluminescence imaging.

METHODS: Cell culture. MPCs were recovered from the intramedullary canal of patients undergoing hip hemiarthroplasty according to an IRB-approved protocol [2]. Cells passaged 2-3 times were transduced with (i) lentiviral reporter constructs encoding firefly luciferase under control of either cytomegalovirus (CMV) or NF-κB-responsive promoters and (ii) adenoviral constructs encoding either no transgene (Ad.null) or human bone morphogenetic protein-7 (Ad.BMP-7). Nude rat model. Osteochondral defects (1.5-mm diameter, ~1.5-mm depth) were generated in both patellar grooves of 12-week old male NIHRNU rats as previously described [3]. hMPCs (500,000) seeded onto Gelfoam scaffolds were implanted into the defects, and animals were allowed free cage activity upon recovery. At one-week intervals post-surgery, reporter levels were measured after D-luciferin injection (150 mg/kg) using a Xenogen IVIS-50 imaging station. Bioluminescent flux was quantified within regions of interest. Animals were sacrificed at 8 weeks for histological/immunohistochemical evaluation of defect repair.

RESULTS: hMPCs were efficiently modified by viral vectors while retaining their chondrogenic potential. When implanted into osteochondral defects, CMV reporter cells produced abundant luminescent signal as early as 2 days post-surgery. Although the signal diminished with time and was typically undetectable from outside the joint by 5 weeks, luminescence could still be detected in freshly explanted grooves at the 8-week endpoint (Fig. 1). Within some knees, NF-κB activity remained elevated or even increased prior to eventual signal attenuation.

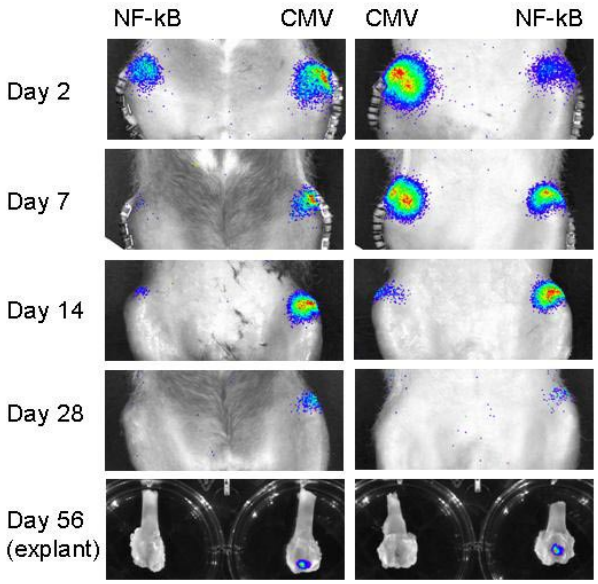

Fig. 1. Longitudinal analysis of reporter expression from hMPCs implanted into osteochondral defects. Images from two representative rats are shown.

DISCUSSION & CONCLUSIONS: These findings confirm that it is possible to monitor gene expression by hMPCs implanted within osteochondral defects during the initial weeks of repair. Preliminary data indicate that NF-κB activity is enhanced within the defect site during this timeframe, potentially inhibiting the chondrogenic differentiation of local progenitor cell populations.

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ACKNOWLEDGEMENTS: RMP was supported by a Pathway to Independence award (K99 AR057105) from NIH/NIAMS.

Cellular synergy for one step cartilage repair

J. Hendriks, R. Schotel, M. van den Doel, J. Riesle

CellCoTec BV, Bilthoven, The Netherlands

INTRODUCTION: One of the current treatments for cartilage defects is based on expanded chondrocytes (Autologous Chondrocyte Implantation, ACI). Expansion of chondrocytes is, however, expensive, requires a complex two step procedure, and hampers cellular quality as a result of cell de-differentiation. This has prevented widespread acceptance of ACI. We have previously established a combination of primary and expanded chondrocytes to substantially enhance cartilage formation compared to expanded chondrocytes alone [1, verified by 2], based on a cell synergy mechanism. To address the drawbacks of ACI, we hypothesized that, within this cell synergy system, de-differentiated expanded chondrocytes can be replaced by un-differentiated cells (bone marrow derived cultured MSCs), and, ultimately, by non cultured bone marrow cells, compatible with a intra-operative one step procedure. We further hypothesized that the current standard 16-22 hour chondrocyte isolation method can be shortened to less then 1 hour. Based thereon, a simple one surgery treatment for cartilage defects may be developed.

METHODS: For the first study, we combined adult bovine or human primary chondrocytes and MSCs or non cultured bone marrow cells, into micromass pellets or into porous PEGT/PBT polymer scaffolds, cultivated in vitro in standard media for 2-4 wks, or implanted subcutaneously into nude mice for 8 wks, and analyzed for glycosaminoglycans (quantitative GAG, safranin O, and, for some samples, aggrecan) and collagen type II. For the second study, we dissected human cartilage, enzymatically digested for various time periods, and analyzed for cell number and viability.

RESULTS: For the first study, in vitro results showed micromass pellets comprising 20% primary chondrocytes and 80% MSCs to contain significantly more GAG compared to the expanded chondrocyte and MSC controls (both controls lacked safranin O staining), and a comparable (not significantly different) amount of GAG compared to the primary chondrocyte control. In vivo results were consistent with in vitro results. Moreover, we detected collagen type II staining, and, a 7 fold increased GAG content per added primary chondrocytes (for a 10/90 ratio), compared to the primary chondrocyte control. Results for non cultured bone marrow cell combinations were essentially consistent with these findings, and, demonstrated significantly enhanced GAG from as little as 2% primary chondrocyte content in the cell mix compared to the bone marrow control, and, a 20 fold increased GAG content per added primary chondrocytes, compared to the primary chondrocyte control.
For the second study, comparable (not significantly different) amounts of viable chondrocytes could be isolated in 25 fold less time (45 min) compared to the current standard method. The outcome of both studies has been robust to donor and age variations.

DISCUSSION & CONCLUSIONS: We have demonstrated a combination of primary chondrocytes and bone marrow cells to synergize in enhancing cartilage formation, compared to expanded chondrocytes, in vitro and in vivo. We further demonstrated an increased GAG content per added primary chondrocytes for the cellular synergy system compared to primary chondrocytes alone. Finally, we have established chondrocyte isolation within less then an hour. The combination of cellular synergy and rapid chondrocyte isolation may therefore enable a one surgery cartilage defect treatment. A clinical trial has been started.

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ACKNOWLEDGEMENTS: The authors greatly acknowledge the support of the TeRM Smart Mix Program of the Dutch Ministry of EconomicAffairs and the Dutch Ministry of Education, Culture and Science.

Runaway Remodelling and Its Implications for Treatment of Osteoarthritis in the Clinic

James B Richardson

Robert Jones & Agnes Hunt Orthopaedic Hospital, Oswestry, Shropshire, SY10 7AG UK
http://www.oscell.co.uk/

INTRODUCTION: Primary osteoarthritis of the knee has no generally accepted cause. Mechanical factors and inherited biology both play a part. Biological abnormalities resulting from inherited abnormalities of collagen or proteoglycan production may be present.

Many of the changes of osteoarthritis are in the subchondral bone where high rates of remodelling lead to poor quality with immature collagens. The overall stiffness of the subchondral bone plate and the subarticular spongiosa are increased through increased density and also increased thickness[1]. (Ding 2007).

OBSERVATIONS: One aspect of modern life is a diet that leads to reduced calcium absorption and increased loss. This in turn leads to raised parathyroid hormone levels and increased remodelling rates. Heaney describes this as fundamental to the causation of osteoporosis.[2] Could an acidic diet also be a factor in osteoarthritis?

Radin and Rose (1986) proposed that stiffening of the subchondral bone would eventually lead to loss of overlying cartilage.[3] No satisfactory explanation has been generally accepted of why the stiffening should arise and progress.

Normal bone remodelling in the skeleton is controlled by Wolf's law, where high local loading leads to increased bone formation. This in turn reduces load, a negative feedback loop.

In an incongruous joint such as the knee or hip (Simon, 1970; Shepherd, 1999), the problem is that the transmission of forces is localised to the region of joint contact.[4,5]

NEW THEORY: In this paper we propose a mechanism where progressive thickening and increased stiffness of the subchondral bone is driven by an abnormal positive feedback loop.

We define a 'tipping point' as the point beyond which increased subchondral bone formation results in increased load, creating a positive feedback loop that steadily increases remodelling in the direction of increased stiffness.

The implications of such a theory will be discussed, both in respect of mechanism and therapeutic options. Proposals of how this theory can be tested will be discussed.

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Disc degeneration: current surgical techniques

C.SCHIZAS

Centre Hospitalier Universitaire Vaudois (CHUV), Site Hôpital Orthopédique, Orthopaedic Department, University of Lausanne, Switzerland

Chronic Low back pain attributed to lumbar disc degeneration poses a serious challenge to physicians. Conservative measures fail often to relieve symptoms and spinal surgeons are increasingly under pressure from patients and colleagues to offer a cure for symptoms that can severely affect daily living. For decades the only surgical option has been spinal fusion. It consists of enhancing bony union across a functional spine unit. This goal can be achieved either by an anterior approach or through a posterior procedure. The use of instrumentation has improved the rates of radiological fusion and is now becoming the most commonly used technique. There is no hard evidence though that surgical technique influences final outcome. Furthermore results of spinal fusion are inconsistent and there is some questioning on its efficacy with some prospective trials showing superiority over usual conservative measures and others failing to demonstrate its advantages. In an effort to improve results of fusion and to decrease the incidence of degeneration of the adjacent segments, disc replacement techniques have been introduced. Nucleus replacement has gained some popularity initially but evidence on its efficacy is scarce. Total disc arthroplasty has been studied extensively. Even though short terms results have shown superiority over some fusion techniques medium term results tend to show that this approach yield results equivalent to those of spinal fusion. Dynamic stabilization involving less rigid implants than in spinal fusion without bone grafting while retaining some form of mobility, represent another surgical option but again evidence is lacking on its superiority over other surgical strategies and conservative measures. Insertion of interspinous devices posteriorly aiming at redistributing loads and relieving pain have been used as an adjunct to disc removal surgery in a disc herniation setting but to date there is no clear data on their efficacy. Finally minimally invasive intradiscal thermocoagulation techniques have been used but evidence of their effectiveness is questioned. Surgery using newer biological solutions might be the future of discogenic pain treatment. Research in biological solutions is now focusing on four broad categories of therapeutic modalities using either protein injections (e.g. Rh BMP) gene transfer, cell therapy (using autologous disc cells, articular chondrocytes, mesenchymal stem cells/MSCs or fetal cells) and finally tissue engineering. Clinical studies have been initiated already in the protein injection and cell therapy fields. All the above biological solutions are nevertheless still a long way from routine clinical use with several questions still in need of answering including possible effectiveness in clinical setting and side effects.

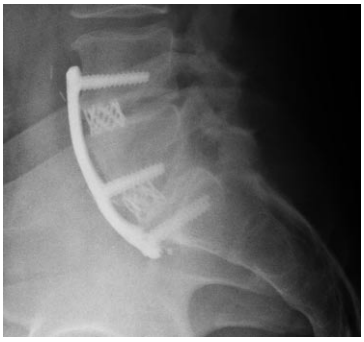

Fig.1: example of anterior lumbar fusion

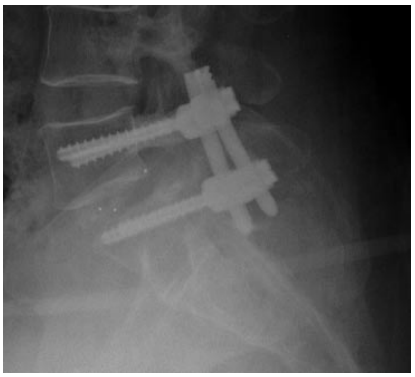

Fig.2: example of posterior instrumented fusion

Stem cells and cartilage repair: turning science into medicine

Professor Anthony P. Hollander

University of Bristol, UK

From a tissue engineering perspective, cartilage is a relatively simple structure as it lacks vasculature and has just a single cell type, the chondrocyte. It is therefore an ideal model for developing therapeutic approaches using stem cells. This presentation will provide an analysis of the use of MSCs in cartilage engineering for osteoarthritis and in the repair of torn meniscal cartilage. The application of cartilage engineering to tracheal reconstruction will also be considered. On June 12th 2008 the first tissue engineered trachea, created using the patient's own cells and a decellularised donor trachea as scaffold, was implanted into Claudia Castillo's airway, thereby avoiding the need to remove her left lung and allowing her to lead a near normal life. Lessons can be learned from this single case that are important for the broader field of cartilage engineering.

Challenges for Cell Based Regeneration of the Degenerate Intervertebral Disc

J.A. Hoyland, S.M Richardson, B.Minogue, A.J Freemont.

School of Biomedicine, Faculty of Medical and Human Sciences, University of Manchester, Oxford Road, Manchester, M13 9PT, UK

INTRODUCTION: Loss of intervertebral disc (IVD) matrix, predominantly in the central nucleus pulposus (NP), as a result of degeneration has been implicated as a major cause of low back pain. Current treatments are purely symptomatic and do not address the aberrant cell biology driving these matrix changes. However, cell based regenerative therapies offer an attractive solution to current interventions as through the implantation of appropriate cells it is possible to replace poorly functioning cells and repair and synthesize a functional tissue with similar features to the non-degenerate IVD. However, in order to develop a successful strategy a number of issues must be considered.

Choice of cells. The obvious choice would be the use of autologous NP cells, as with autologous chondrocyte implantation (ACI) for repair of cartilage defects. However, although clinical studies are underway in Europe (EuroDisc trial) with re-implantation of such cells, evidence from our laboratory and others would suggest that these cells are not ideal. For example, we have shown that cells from a degenerate disc show an altered phenotype (enhanced catabolism) and increased cell senescence which affects their expansion ability in culture (a prerequisite to generate sufficient numbers for re-implantation). Consequently attention has focussed on the use of mesenchymal stem cells (MSCs) because of their easy acquisition, rapid proliferation and differentiation potential.

MSC Differentiation: Data from our laboratory and others has shown that cells can be manipulated *in vitro* to differentiate to NP like cells by the addition of specific growth factors, manipulation of the culture environment and by seeding on suitable biomaterials (e.g. chitosan). Interestingly, recent data from our own co-culture studies would suggest that the interaction between MSCs and NP cells is sufficient to drive MSC differentiation to an appropriate phenotype and enhance matrix gene expression in degenerate NP cells. This, together with our data showing that cells injected into IVD tissue spontaneously differentiate into NP like cells implies that the IVD niche itself may be sufficient to direct cell differentiation and synthesis of appropriate matrix. A caveat to this however, is the environment of the degenerate IVD where oxygen tension and pH are low, nutrition is compromised, cells are exposed to mechanical load and there is a catabolic cytokine milieu, all of which may have detrimental effects on MSC differentiation. The effects of such factors on MSC differentiation to an NP phenotype are currently being investigated.

NP phenotype Although the NP cell shares many similarities with articular chondrocytes (AC) the matrix it produces and the environment in which it resides is distinctly different. Thus, for these therapies to be successful and appropriate matrix produced differentiated cells must have the correct NP phenotype. Using Affymetrix microarrays and qRT-PCR we have identified human gene markers that distinguish human NP cells from AC cells and have used these gene signatures to identify the differentiation of MSCs (derived from bone marrow (BM-MSCS) and Adipose tissue (ASCs)) towards an NP phenotype. Importantly we have demonstrated that our novel human phenotypic NP marker genes (e.g. PAX1 and FOXF1) and AC marker genes (IBSP and FBLN1) can be used to identify the *in vitro* differentiation of BM-MSCs and ASCs to an NP-like rather than an AC-like phenotype in biomaterials suitable for tissue regeneration of the human IVD. Furthermore our results indicate that ASCs may be a more appropriate cell type than BM-MSCs for repairing the human IVD.

ACKNOWLEDGEMENTS: This work was funded through grants from the ARC, BBSRC, MRC, EPSRC and NWDA.

Downregulation of Superfluous Genes on Commitment to Chondrogenic Differentiation of Mesenchymal Stem Cells is associated with DNA Hypermethylation

C.ÓFlatharta[1], E.Mooney[1], M.Berdasco[2], G.Shaw[1], M.Esteller[2], FB.Barry[1], JM.Murphy[1]

[1]*Regenerative Medicine Institute, NUI Galway, Ireland,* [2] *Cancer Epigenetics and Biology Program, Bellvitge Institute for Biomedical Research L'Hospitalet de Llobregat, Barcelona, Spain.*

INTRODUCTION: Bone marrow-derived mesenchymal stem cells (MSCs) differentiate to connective tissue lineages. As they differentiate they undergo epigenetic changes that allow for the variety of intermediate cells that can be formed throughout differentiation. DNA methylation is an epigenetic process whereby methyl groups are added to CpG dinucleotide residues resulting in reduced gene expression. The aim of this project was to investigate global changes in gene promoter methylation patterns as MSCs differentiated to various lineages to obtain a better understanding of the relationship between stem cell differentiation and the epigenetic process.

METHODS: Genomic DNA, extracted from undifferentiated, chondrogenic, osteogenic and adipogenic MSCs, was analysed for promoter methylation patterns using a high throughput epigenomic discovery platform with custom CpG island arrays (Infinium Human Methylation 27BeadChip Assay). Analysis of 14,000 gene promoter regions yielded beta values depending on level methylation (0=unmethylated, 1=methylated) for each CpG analysed. Stringent selection generated a differentiation-specific methylation signature for validation by real-time PCR and bisulfite sequencing.

RESULTS: Although the majority of the 28,000 CpGs analysed were constitutively unmethylated in undifferentiated MSCs, a cohort of CpGs were hypomethylated relative to that in differentiated cells (Figure 1A). Global analysis of the methylation patterns indicated that chondro- and osteo-differentiated MSC clustered closer than to undifferentiated MSC or to adipogenic progeny, with the latter two groups least similar (Figure 1B-D).

Lineage-specific, differentially methylated genes were selected for were selected for array validation and further study. Of the nine chondrogenic genes chosen, eight were hypomethylated following differentiation.

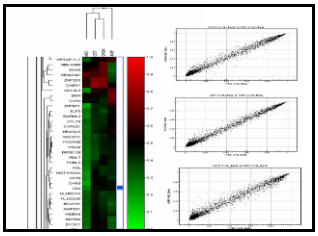

Fig. 1: (A) Heat map of representative genes illustrating relative hypomethylation of undifferentiated MSC promoter region. Scatter plots showing clustering of undifferentiated MSC methylation patterns to (B) chondrocytes, (C) osteocytes and (D) adipocytes.

Five of the genes showed changes in gene expression corresponding to their methylation signature. Of the hypomethylated genes, MIA/CD-RAP and SCRG1 showed increased expression in chondrogenesis over 14 days with a concomitant rapid downregulation of gene expression on differentiation to the adipo- and osteo- lineages. The hypermethylated target, EGFL7 was downregulated in all 3 lineages within 2 days differentiation. In order to further analyze the EGFL7 methylation pattern, the EGFL7 proximal promoter region of the promoter was subjected to bisulphite sequencing. Significantly increased methylation of the promoter was seen in differentiated versus undifferentiated MSCs.

DISCUSSION & CONCLUSIONS: **D**ifferentiation of MSCs may involve a wave of hypermethylation to promote rapidly down regulate expression of genes involved in self-renewal or alternative differentiation pathways. Additionally, epigenetic mechanisms represent an important aspect of control to ensure the sequential and temporal expression of critical genes throughout the differentiation process.

ACKNOWLEDGEMENTS: Funding was from Science Foundation Ireland.

Direct, rAAV-mediated Overexpression of Human IGF-I Enhances Articular Cartilage Repair *in Vivo*

M. Cucchiarini[1,*] MD. Menger[2] H. Madry[1,3]
[1]*Institute for Experimental Orthopaedics, Saarland University Medical Center, University of Saarland, Homburg, Germany,*
[2]*Department of Clinical and Experimental Surgery, Saarland University Medical Center, University of Saarland, Homburg, Germany,*
[3]*Department of Orthopaedic Surgery, Saarland University Medical Center, University of Saarland, Homburg, Germany,*
*Corresponding author: mmcucchiarini@hotmail.com

INTRODUCTION: Therapeutic gene transfer might be a means to enhance the reparative activities in articular cartilage lesions. We previously reported that recombinant adeno-associated viral (rAAV) vectors are capable of delivering transgene sequences to articular cartilage defects in experimental models over extended periods of time [1-3]. In the present study, we tested the hypothesis that efficient and sustained overexpression of IGF-I via direct application of rAAV enhances the healing of osteochondral defects created in the knee joints of rabbits *in vivo*.

METHODS: rAAV were packaged, purified by dialysis, and titrated by real-time PCR [1,4,5]. Titers averaged about 10^{10} functional units/ml. rAAV-*lacZ* carries the *E. coli* beta-galactosidase (β-gal) marker gene (*lacZ*) controlled by the CMV-IE promoter/enhancer [1,4,5]. A human insulin-like growth factor-I (hIGF-I) cDNA (536 bp) [6] was cloned in rAAV-*lacZ* instead of *lacZ* to produce rAAV-hIGF-I. Animal experiments were conducted under an Institution Animal Studies Committee approved protocol. Two osteochondral defects (3.2-mm in diameter) were created in each patellar groove of Chinchilla bastard rabbits (n = 8) [1-3,7]. Each animal received alternatively 10 µl rAAV-hIGF-I per defect on one knee (IGF-I-treated defects) and 10 µl rAAV-*lacZ* per defect on the contralateral knee (control defects). At 3 weeks post operation, cartilage repair was assessed based on safranin O/hematoxylin eosin-stained sections using a histological grading system [8]. A total of 145 paraffin-embedded sections (5 µm) were scored independently by two individuals that were blinded with respect to the treatment. Points for each category and total score were compared between the groups using a mixed general linear model with repeated-measures analysis of variance. Indirect immunohistochemical staining was also performed to detect β-gal (Sigma), hIGF-I (R&D Systems), and type-II collagen (Acris). The DNA, proteoglycan, and type-II collagen contents of the repair tissue within the defects were measured using Hoechst 33258, by binding to DMMB dye, and by ELISA [4], respectively. Data are expressed as mean ± SD. The t-test and the Mann-Whitney Rank Sum Test were employed where appropriate.

RESULTS: Following direct application of the vectors *in vivo*, there were no signs of synovitis, adhesions, or adverse reactions, and no macroscopically descriptive differences between the IGF-I-treated and control knees. β-gal activity was restricted to the control defects, whereas IGF-I expression was present only in the IGF-I-treated defects (Fig. 1).

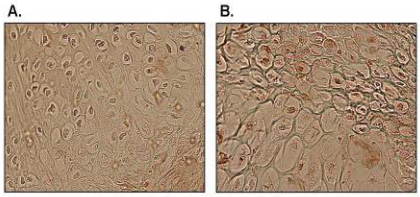

Fig. 1. Detection of IGF-I expression in osteochondral defects 3 weeks after vector application (**A**: rAAV-*lacZ*; **B**: rAAV-hIGF-I). Magnification x20.

After 3 weeks, enhanced tissue healing was observed in the IGF-I-treated defects (Fig. 2). Improved individual parameter scores were observed for defect filling, integration, matrix staining, cellular morphology, defect and surface architecture, new subchondral bone formation (all $P < 0.001$) and tidemark ($P < 0.01$) of the IGF-I-treated defects, with also a significantly improved total score vis a vis control treatment ($P < 0.001$) (Table 1). Immunoreactivity to type-II collagen was more intense and regular in the IGF-I-treated defects (Fig. 2).

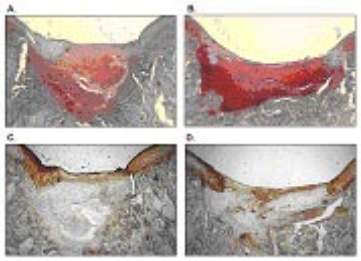

Fig. 2. Safranin O staining (**A**, **B**) and type-II collagen immunoreactivity (**C**, **D**) in osteochondral defects 3 weeks after vector application (**A**, **C**: rAAV-*lacZ*; **B**, **D**: rAAV-hIGF-I). Magnification x2.

Table 1. Effects of IGF-I overexpression 3 weeks after rAAV application *in vivo* on the histological grading of articular cartilage repair tissue

Category	rAAV-*lacZ* Mean (95% CI)	rAAV-hIGF-I Mean (95% CI)
Filling of defect	0.37 (0.21 - 0.54)	0.14 (0.00 - 0.31)*
Integration	1.65 (1.44 - 1.86)	1.07 (0.86 - 1.29)*
Matrix staining	2.03 (1.76 - 2.31)	1.47 (1.19 - 1.74)*
Cell morphology	2.69 (2.39 - 2.98)	1.90 (1.60 - 2.19)*
Architecture of defect	1.41 (1.23 - 1.60)	0.87 (0.69 - 1.06)*
Architecture of surface	2.44 (1.98 - 2.90)	1.03 (0.57 - 1.49)*
Subchondral bone	2.95 (2.64 - 3.25)	1.96 (1.65 - 2.26)*
Tidemark	4.00 (3.95 - 4.06)	3.95 (3.90 - 4.00)*
Average total score	**17.5 (16.8 - 18.2)**	**12.4 (11.7 - 13.1)***

CI = confidence interval. *Statistically significant vis a vis control treatment.

Biochemical analyses performed on the repair tissue from the defects revealed that treatment with rAAV-hIGF-I promoted a significant increase in the DNA (3.2-fold; $P < 0.001$), proteoglycan (1.2-fold; $P = 0.01$), and type-II collagen contents (2.8-fold; $P < 0.001$) (Table 2).

Table 2. Effects of IGF-I overexpression 3 weeks after rAAV application *in vivo* on the biochemical parameters of articular cartilage repair tissue

Parameter	rAAV-*lacZ*	rAAV-hIGF-I
DNA (µg/mg dry weight)	0.02 (0.01)	0.04 (0.01)*
Proteoglycans (µg/mg dry weight)	2.63 (0.41)	2.95 (0.11)*
Type-II collagen (ng/mg dry weight)	1.20 (0.38)	3.12 (0.61)*

Data are given as mean (SD). *Statistically significant vis a vis control treatment.

DISCUSSION & CONCLUSIONS: The data indicate that IGF-I can be overexpressed in osteochondral defects *in vivo* via rAAV transduction, leading to the production of a recombinant IGF-I factor that is capable of significantly improving the articular cartilage defect repair, stimulating both cell proliferation and extracellular matrix synthesis. The results suggest that therapeutic rAAV may have value in enhancing cartilage repair by application to sites of cartilage damage. Further studies are required to evaluate the long-term properties of the repair tissue.

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ACKNOWLEDGMENTS: This research was funded by grants from the German Research Society (DFG CU 55/1-1, 1-2, 1-3 to MC, HM) and the German Osteoarthritis Foundation (DAH to MC, HM). We thank E. Kabiljagic for helping with the animal experiments.

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Modalities of interaction between human primary chondrocytes and mesenchymal progenitor cells responsible for enhanced in vitro cartilage formation

C. Acharya[1], A. Adesida[1,2], P. Zajac[1], Stefan Schaeren[1], M. Jakob[1], J. Riesle[2], A. Barbero[1], I. Martin[1]

[1]*Department of Surgery and Biomedicine, University-Hospital Basel, CH,* [2]*CellCoTec B.V., Bilthoven, The Netherlands*

INTRODUCTION: Co-culture of mesenchymal stromal cells (MSC) with chondrocytes (Ch) has been reported to improve cartilaginous matrix accumulation[1] (phenomenon here named chondro-induction, CI). In this study, we investigate the type(s) of communication between the two cell types responsible for CI.

METHODS: Expanded bone marrow-MSC or fibroblast (as control cells) and freshly isolated Ch were cultured in pellets alone (pure pellets) or after being mixed (co-culture pellets, Ch:MSC ratio 25%:75%). Selected pellets were generated combining: human MSC with bovine Ch, and MSC from HLA-A2+ with Ch from HLA-A2- human donors. MSC and Ch were also cultured in transwells, with the two cell types physically separated. Pellets were assessed biochemically [to quantify CI as a ratio $GAG_{measured}/GAG_{expected}$ ($GAG_{expected}$ = $75\%GAG_{pure\_MSC}$ + $25\%GAG_{pure\_Ch}$)], by RT-PCR using human and bovine specific primers and probes for collagen-II, and cytofluorimetrically. Tissues formed in the inserts of the transwells were assessed histologically and biochemically.

RESULTS: CI was higher when Ch were co-cultured with MSC (1.6±0.1) than with fibroblasts (1.3±0.1). RT-PCR of pellets generated by bovine Ch and human MSC showed an increase in the expression of human collagen-II following co-culture (Fig.1). FACS quantification with antibodies specific for HLA-A2 indicated that: Ch number increased (4.2-fold) in the co-culture pellets while remaining constant in pure pellets, MSC number decreased in the co-culture and pure pellets to a similar extent (5.0-fold) (Fig.2). GAG content of tissues formed by MSC or Ch in the inserts of transwells were not modulated by the presence of either cell types in the bottom layer of the same transwells.

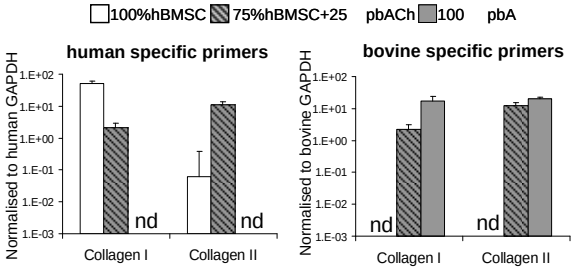

Fig. 1: RT-PCR with specific primers and probes. nd = not detected

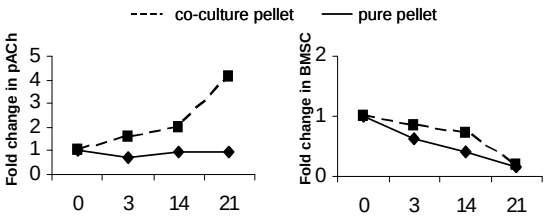

Fig. 2: FACS quantification of HLA-A2+/- cells.

DISCUSSION & CONCLUSIONS: Mutual communication between Ch and MSC occurs in co-culture: Ch stimulate MSC for higher collagen-II expression and, in turn, MSC stimulate Ch to proliferate. These effects are not mediated by soluble factors alone but require cell-cell contacts. In vivo studies are necessary to assess the clinical relevance of our findings in the context of cartilage repair.

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ACKNOWLEDGEMENTS: Authors thank Marie Curie Host Fellowships (TOK 042418, EU) and Fondazione Cariplo.

HUMAN MESENCHYMAL STEM CELLS DIFFERENTIATION INTO INTERVERTEBRAL DISC-LIKE TISSUE - COMPARISON OF DIFFERENT BIODEGRADABLE MATRIXES

A. Bertolo[1], N. Aebli[2], M. Baur[3], S. Ferguson[4], J. Stoyanov[1]

[1]*Swiss Paraplegic Research, Nottwil, CH,* [2]*Swiss Paraplegic Center, Nottwil, CH,* [3]*Cantonal Hospital of Luzern, Luzern, CH,* [4]*Institute for Surgical Technology and Biomechanics, University of Bern, CH*

INTRODUCTION: Intervertebral discs (IVD) can be altered by different factors, such as trauma, which can lead to degeneration of the tissue, causing pain and possibly disabilities[1]. To date, there is not a cure against IVD degeneration, but tissue engineering applications carry the potential to offer a solution to the problem. Bone marrow derived mesenchymal stem cells (MSCs) are multipotential cells widely used in tissue engineering applications, as they have the capacity to give rise to IVD-like cells when stimulated by growth factors in a three-dimensional culture.
Here we compare how four biodegradable matrixes influenced the differentiation *in vitro* of MSCs derived from iliac crest and vertebral body over a 30-day culture period. We also compare MSCs performances to that of human IVD cells isolated both from annulus fibrosus (AF) or nucleus pulposus (NP).

METHODS: Four sponge-like matrixes made of derived equine and porcine collagen type I, gelatine (hydrolyzed collagen) and chitosan (de-acetylated chitin) were used as support for MSCs. Matrix materials are all approved as medical devices either for wound management or as haemostats. Differentiation of the tissue engineered constructs was assessed by qRT-PCR, Western Blot and histological and immunohistochemical stainings.

RESULTS: Gene expression analysis showed that the highest levels of IVD specific markers (aggrecan, collagen type I and II) were expressed by MSCs seeded in gelatine scaffolds, followed by equine collagen, porcine collagen and chitosan. MSCs express much lower levels of collagen type I compared to AF or NP cells, while collagen type II level is higher. Aggrecan expression levels were comparable between MSCs and IVD cells. IVD cells did not express ostepontin, a marker for bone tissue. Histological and immunohistochemical analysis of the cell constructs with staining typical of extracellular disc matrix molecules showed aggrecan, collagen type I and II accumulation (*Figure 1*).

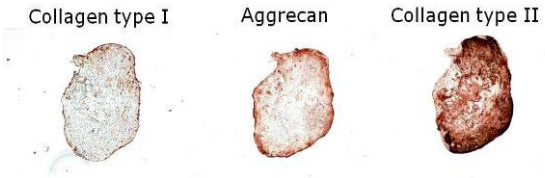

Fig. 1: Immunohistochemical staining of equine collagen/MSCs constructs with collagen type I, aggrecan and collagen type II monoclonal antibodies.

DISCUSSION & CONCLUSIONS: We demonstrate that there is a difference between different biodegradable scaffolds in their capacity to support MSCs differentiation in IVD-like cells *in vitro* and that gelatine matrix can be a practical and effective carrier for advanced cell therapies. Under the tested conditions, MSCs derived from the vertebral body had higher gene expression levels compared to MSCs derived from iliac crest.

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ACKNOWLEDGEMENTS: This work was supported by the Swiss Paraplegic Foundation.

Pullulan Enhances Progenitor Cell Attachment on OA Cartilage

Bulman SE[1], Coleman CM[1], Murphy M[1] & Barry F[1]
[1]Regenerative Medicine Institute, National University of Ireland, County Galway, Ireland

INTRODUCTION: Delivery of mesenchymal stem cells (MSCs) to the articular joint in a goat model of osteoarthritis (OA) results in retardation of cartilage destruction, however in vivo MSCs do not show engraftment to either intact or fibrillated cartilage (Murphy 2003). Interestingly, MSCs have previously shown the potential to engraft to an OA cartilage explant model (Curtin 2006). It is therefore hypothesized that enhanced adhesion of MSCs at the surface of the joint will increase cellular engraftment. Pullulan a non-toxic biocompatible exopolysaccharide, (Rekha 2007) was evaluated for enhancing MSC adhesion to OA cartilage.

METHODS: MSCs were isolated from the bone marrow of consenting human donors using traditional methods (Murphy 2003). MSCs in monolayer were exposed to 0, 2 or 5% pullulan in α-DMEM with 10% serum for 1, 3 and 7 days, after which viability and proliferation were assessed by MTS and picogreen analysis, respectively.

OA cartilage biopsies from femoral head and tibial plateau of consenting human donors undergoing arthroplasty were coated with 0, 2 or 5% pullulan prior to the addition of $0.5x10^6$ MSCs labeled with PKH26 cell surface dye. Cells were allowed to adhere for 20 minutes and then excess cell suspension removed and explants washed. Explants were analysed for progenitor cell adherence using histology and fluorescence microscopy to identify labeled cells (Curtin 2006).

RESULTS: MSC treated explants were cryosectioned and stained with H&E, revealing MSC adhesion on all explants with a greater number of MSCs adhering to pullulan treated tissues (Fig 1).

Assessment of MSCs in monolayer culture for alterations in metabolic activity normalized to DNA content indicated increased cellular metabolism at day 1 in MSCs exposed to 5% pullulan and at day 7 when exposed to 2% pullulan. This trend was not statistically significant (Fig 2).

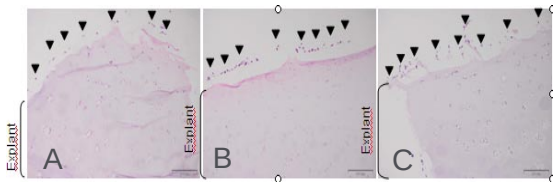

Fig 1: Histological staining of OA cartilage explants after MSC adhesion*. Cartilage explants were exposed to (A) 0%, (B) 2% or (C) 5% pullulan and incubated with MSCs. Cellular adhesion was observed on all explants (arrowheads), with an increase in cellular adhesion with the addition of pullulan.*

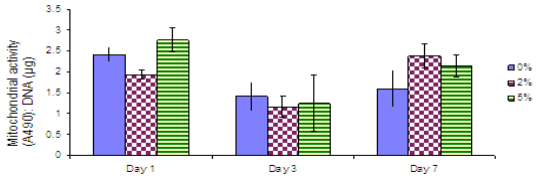

Fig 2: Ratio of mitochondrial activity to DNA concentration of MSCs cultured with pullulan. *Although there is a reduction in mitochondrial activity per cell over time, as demonstrated by control cells, there is an increase in mitochondrial activity per cell when the cells are exposed to pullulan at days 1 and 7.*

DISCUSSION: When pullulan was applied to OA cartilage prior to MSC addition, cellular adhesion was enhanced. MSC exposure to pullulan in vitro resulted in an increase in mitochondrial activity and an initial mitogenic response after 3 days (data not shown). An initial pullulan-stimulated increase in MSC proliferation at the cartilage surface after adherance may also prove favourable to increase cell numbers for engraftment and matrix repair. Together, these data suggest pullulan as a novel adhesive material for enhancing MSC adhesion and retention to OA cartilage.

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Effect Of Short Term Torsion To The Intervertebral Disc: An Organ Culture Study

S.C.W. Chan[1], S. J. Ferguson[1], K. Wuertz[2], B. Gantenbein-Ritter[1]

[1]ARTORG, Institute for Surgical Technology and Biomechanics, University of Bern, Bern, CH
[2] Competence Centre for Applied Biotechnology and Molecular Medicine, University of Zurich, CH

INTRODUCTION

It has been suggested that a low degree of axial rotation of the spine at low frequency might increase the spinal length, and therefore, enhance the nutrition and waste exchange,[1] whereas a high torsion angle would cause damage to the disc.[2] In this study, the biological response of the intervertebral disc (IVD) to three different degrees of continuous torsion were tested in a bovine organ culture model during 5 days of culture.

METHODS

Organ culture. Bovine caudal IVDs (diameter 16-22 mm) with endplates were isolated as previously described.[3] Orientation of the disc was marked with a surgical pen to distinguish the anterior and posterior side of the disc. Disc explants were cultured in custom-made polycarbonate chambers supplied with 45 mL Dulbecco's Modified Eagle's Medium (DMEM) with 10% Foetal Bovine Serum (Gibco, Switzerland) at 37 °C, 5% CO_2 for 5 days. The discs were secured in the chamber by two custom-made serrated titanium plates. Static compression (20 N) was applied to the chamber during the entire culture period.

Mechanical stimulation. To apply mechanical stimulation, a combined loading of 20 N static compression and either 0° (control) or ± 2° (low), ± 5° (medium) or ± 10° (high) torsion at 0.1 Hz was applied (Mini-Bionix System 858, MTS Systems Corp, Eden Prairie, Minn). Loading was applied to the disc for 1 h per day and for four consecutive days starting from the second day of culture.

Evaluation. Disc tissue was divided into 3 parts: nucleus pulposus (NP) and inner and outer annulus fibrosis (IA and OA). Each part of the disc was further divided into 4 pieces for analysis: 1) cell viability analysis by Live/Dead stain and confocal microscopy[3,4] (Fluka, Sigma, Switzerland), 2) metabolic activity by Alamar Blue assay (Invitrogen, Switzerland), 3) measurement of glycosaminoglycan (GAG) and DNA content, and 4) gene expression analysis by real-time RT-PCR (n =6).

RESULTS

Results showed a significantly higher cell viability in the IA regions of the disc with 2° of torsion applied, compared to static loading controls (LSD *post-hoc* test, IA: Control vs 2° $p = 0.033$). A trend of decreased metabolic activity in the NP with increasing torsion angle has been observed, while no significant differences were observed in AF cell metabolic activity between torsion groups or the control group (figure 1). No significant difference in GAG/DNA content was found among the groups. Gene expression of the disc tissue, including anabolic genes (Collagen I, Collagen II, Aggrecan, Versican, Elastin), catabolic genes (Adamts 4, MMP3, MMP13) and small leucine rich proteoglycans (Biglycans, Decorin, Lumican) were tested by real-time PCR relative to fresh controls; however, no significant differences were found between the control and the torsion group.

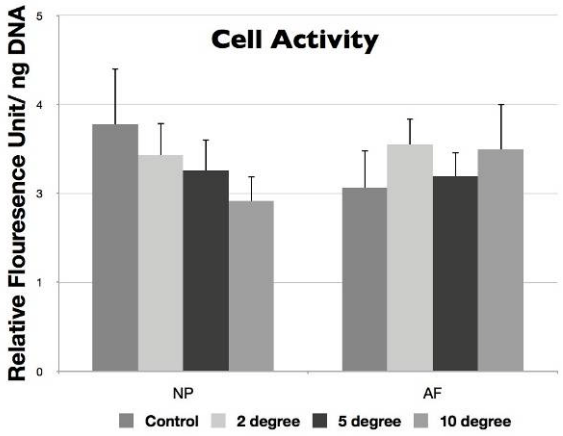

Figure 1: Cell metabolic activity of the IVD after 4 consecutive days of torsion for 1h. Plot of mean ± SEM.

DISCUSSION & CONCLUSION

Results showed that short-term physiological low angle torsion improved cell viability in the inner annulus of the disc in organ culture as compared to static loading alone, which could be due to increased nutrition and waste exchange to the inner region of the disc. This is a similar finding to uni-axial compression with diurnal loading (mimicking day-night rhythm from previous organ culture studies[5,6]). However, larger torsion angles, which were "hyperphysiological" have decreased the general metabolic activity of the NP cell. This repetitive loading may also cause damage to the laminate structure in the annulus. These findings will be further studied by histological evaluation and investigation of disc cell apoptosis.

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ACKNOWLEDGEMENTS

This project was supported by the Swiss National Science Foundation (SNF # 310030-127586/1).

Analysis of TGF-β3 and BFGF Induced Chondrogenic Differentiation In Human Bone Marrow-Derived Mesenchymal Stem Cells

Pan-Pan Chong, Azlina A. Abbas, T. Kamarul

Tissue Engineering Group, National Orthopaedic Centre of Excellence for Research and Learning (NOCERAL), University of Malaya, 50603 Kuala Lumpur

INTRODUCTION: Due to its ability to differentiate into different mesenchymal cell lineages, it has been suggested that for the repair of focal cartilage damage, the use of mesenchymal stem cells (MSCs) as a therapeutic agent may be beneficial. However, the complex mechanisms that regulate the process of chondrogenesis (cartilage cell forming) from MSCs remain largely unexplained. A study was thus conducted to better understand the extracellular matrix and the regulation of genes expression during chondrogenic differentiation of MSCs.

METHODS: Two ml of bone marrow (BM) aspirates from femur or tibia were harvested from patients (n=6) undergoing orthopaedic procedures. Mononuclear cells were isolated from human BM aspirates using Ficoll–Paque PREMIUM method. Cells were characterized and identified using flowcytometry. MSCs were cultured in alginate scaffolds using chondrogenic medium containing transforming growth factor-β3 (TGF-β3) and fibroblast growth factor-basic (BFGF) to promote chondorgenic transformation. Chondrogenic-MSCs (C-MSCs) were examined and histologically compared using Safranin O staining to that of human chondrocytes as a means to determine chondrogenic transformation. Microarray gene chips (Affymetrix Gene 1.0 ST Array: 28,869 Well–Annotated Genes) were used to compare the genes expressed in MSCs and C-MSCs cultures. The data attained were analyzed using Agilent GeneSpring analysis platform and R language in combination with Bioconductor packages.

RESULTS: MSC characteristics of the isolated cells were confirmed and described elsewhere[1,2]. The formation of proteoglycan extracellular matrix for the chondrogenic-MSCs were observed in Haematoxylin-Fast Green-Safranin O staining, which had similar positive expression of proteoglycan extracellular matrix staining as that of human articular chondrocytes. However, these depositions were not observed in non-treated MSCs that were cultured as monolayers using basal growth medium.

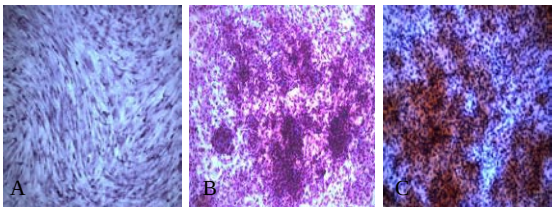

Fig. 1. The proteoglycan was stained orange to red. The cell nuclei were stained black, while the background was stained blue green. A: MSCs. B: CMSCs. C: Clonetics® Normal Human Articular Chondrocytes

Table 1. Summary of the differences in gene regulation between MSC and C-MSC. Log ratio=+1 denotes 1 fold increase. (Significance: P<0.05)

Correlation: *p*-val < 0.05		**Correlation: *p*-val < 0.001**	
Log-ratio > 1	*Log-ratio* < -1	*Log-ratio* > 1	*Log-ratio* < -1
260	240	223	187

Microarray analysis revealed a wide range of differences in genes expressed in MSCs and C-MSCs, denoting a major change in gene regulation.

DISCUSSION & CONCLUSIONS: Significant changes in the expression of more than 400 genes occur dynamically during the course of chondrogenic transformation of MSCs. This dynamic response should be considered when interpreting the overall changes in the genetic expression during chondrogenesis.

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Injectable Type II Collagen-Hyluronan Hydrogel As Reservoir System For Nucleus Pulposus Regeneration

E. Collin[1], S. Grad[2], D. Zeugolis[1], J. Guicheux[3], P. Weiss[3], M. Alini[2], A. Pandit[1]

[1]Network of Excellence for Functional Biomaterial, National University of Ireland, Galway, IE, [2]AO Research Institute Davos, CH, INSERM U791, LIOAD, Nantes, F

INTRODUCTION: Degeneration of the intervertebral disc (IVD) is the main cause of neck and low back pain [1]. During this irreversible process, the nucleus pulposus (NP) loses its gel-like consistency and becomes more fibrotic [2]. The hypothesis of this work is that a supplementation of the degenerated IVDs with cell seeded composite hydrogels which mimic the native environment may provide the ideal environment to conduct the regeneration of the NP tissue. The objective of this study was to develop an optimally stabilised type II collagen-hyaluronan hydrogel acting as a reservoir system of cells for NP regeneration.

METHODS: Different molar ratios of type II collagen (Symatese, France) and hyaluronan (HA) (CPN, Czech Republic) were tested in this study (9:0, 9:1, 9:4.5, 9:9). After neutralisation, NP cells, extracted from 5-month old bovine caudal discs, were added ($2x10^6$for 270µl of hydrogel). Hydrogels stabilisation was performed using poly(ethylene glycol) ether tetrasuccinimidyl glutarate (4S-StarPEG) (JenKem Technology, USA). Hydrogels were incubated for one hour at 37°C before the surface addition of DMEM 10% FBS, 1% P/S. Hydrogel cellular compatibility and cell distribution were observed by live/dead assay. Type I collagen, type II collagen and aggrecan mRNA expressions were quantified using real-time PCR. Rabbit adipose tissue derived stem cells (ADSCs) were cultured for 14 days within the hydrogel. Cell viability and proliferation were quantified to evaluate the cyto-compatibility of the hydrogels.

RESULTS: A significant decrease of free amine groups was observed following the reaction between 4S-StarPEG and collagen (4S-StarPEG 0mM-16.94±0.25 nmol, 0.5mM-12.96±3.15 nmol, 1mM-5.19±2.7 nmol, 2mM-3.62±1.04 nmol). A gel point at 8.9±1.81 was observed for the optimal hydrogel (1mM 4S-StarPEG cross-linked hydrogel). Stability in culture was noted and confirmed by the absence of mass loss after 14 days in culture (Fig. 1). Good viability of bovine NP cells (Fig. 2) and rabbit adipose derived stem cells (ADSCs) could be shown after 14 days in culture. No impact of HA after 7 days on the NP cell phenotype was observed. Maintenance of type I collagen mRNA expression was noted after 7 days in culture independently of the concentrations of HA used.

Figure 1. Hydrogels composed of different ratios of coll II:HA after 14 days in culture

However, NP cell proliferation was seen after 14 days in culture in absence of HA. ADSCs growth and proliferation were observed within the hydrogel after 14 days.

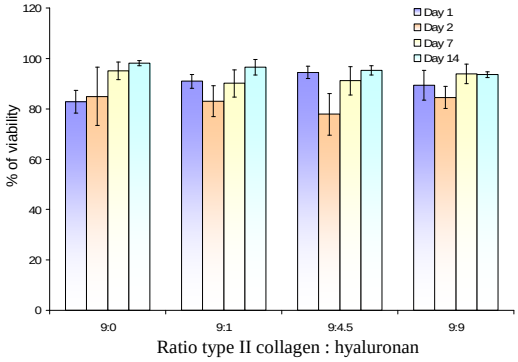

Figure 2. NP cell viability after 14 days in culture in the type II collagen/Hyaluronan hydrogel. (n=3; +SD; p-value<0.05).

CONCLUSIONS: Type II collagen/hyaluronan hydrogel showed rapid gel formation and no toxicity for cells. While no impact of the HA concentration was observed on NP cells, its presence seems to prevent a loss of phenotype. Stability in culture and capacity to support cell growth in addition to its injectable properties makes it a promising candidate as a reservoir of cells for intervertebral disc regeneration.

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ACKNOWLEDGEMENTS: Science Foundation Ireland, Research Frontiers Programme (07/RFP/ENMF482)

Selection of Chondroprogenitors from Bone Marrow by Adhesion to Chondroitin Sulfate

[1]O' Sullivan J; [1]Coleman CM; [1]Murphy M; [1]Barry F
[1]Regenerative Medicine Institute, National University of Ireland Galway, Galway, Ireland
Senior author: Frank.Barry@Nuigalway.ie

INTRODUCTION: Osteoarthritis is a disease of the joints characterized by progressive destruction of articular cartilage. As cartilage is composed primarily of extracellular matrix (ECM) with limited capacity for self-repair, the therapeutic application of mesenchymal stromal cells (MSCs) offers potential to regenerate cartilage. It was therefore hypothesized that a chondrogenic population of MSCs within the bone marrow (BM) can be isolated by preferential adhesion to cartilaginous ECM proteins.

METHODS: Proceeding MSC isolation, culture flasks were coated with 1mg/ml hyaluronan (HA) or chondrotin sulphate (CS) at 4°C overnight (Fig.1). BM from the iliac crest of healthy consenting human donors was exposed to uncoated, HA or CS coated plates for 24hrs or 5 days before non-adherent cell removal as per traditional methods. Confluent MSCs were passaged and expanded on uncoated plastic. At P2, MSCs were induced towards chondrogenesis, adipogenesis and osteogenesis.[1]

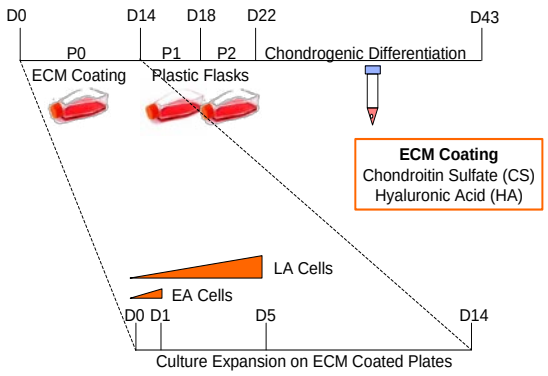

Fig.1. MSC isolation from bone marrow using CS or HS coated plates. *Bone marrow was exposed to ECM or control tissue culture flasks. Non-adherent cells were removed from the flask 24 hours or 5 days after exposure to isolate early or late adherent cells respectively. MSCs were then expanded using traditional, uncoated tissue culture flasks.*

RESULTS: All isolated cells retained a fibroblastic morphology with comparable expansion characteristics. Adipogenic differentiation was significantly increased in all ECM isolated MSCs, especially in the early adherent HA and CS isolated cells. Osteogenic potential was enhanced in all ECM selected MSCs especially in early adherent CS cells. Chondrogenic differentiation was enhanced in all ECM isolated groups, particularly in CS isolated cells (Fig.2).

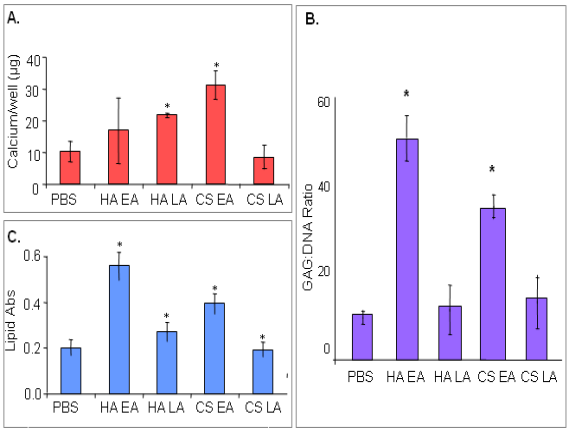

Fig.2*. Tri-lineage differentiation potential of ECM isolated MSCs. A) Osteogenic differentiation indicated increased potential in HA and CS isolated MSCs. B) Chondrogenic differentiation of ECM isolated cells was consistently significantly enhanced as compared to control populations C) Adipogenic differentiation was also significantly enhanced in all ECM isolated MSCs.*

DISCUSSION & CONCLUSIONS: Cartilaginous ECM molecules (HA and CS) were utilized to isolate MSC subpopulations from BM. Cells adhering to CS had the greatest capacity for chondrogenic differentiation, suggesting cells expressing receptors for CS in the bone marrow may be more potent chondroprogenitors. Specific isolation of these subpopulations for clinical application will enable a reduction in the number of MSCs required for clinical efficacy and tissue regeneration.

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Fetal Epiphyseal Chondrocyte Master Cell Banking for Osteochondral Repair and Regeneration

SE. Darwiche[1,2], C. Scaletta[1,2], MC. Osterheld[3], W. Raffoul[2], DP. Pioletti[1], LA. Applegate[1,2]

[1]*Laboratory of Biomechanical Orthopedics, EPFL, Lausanne, CH,* [2]*Cellular Therapy Unit, Service of Plastic and Reconstructive Surgery, Department of Musculoskeletal Medicine,* [3]*University Institute of Pathology, CHUV, Lausanne, CH*

INTRODUCTION: The avascular, aneural and alymphatic nature of articular cartilage has challenged surgeons and tissue engineers alike for many decades. Indeed, we are still far from determining a gold standard for osteochondral therapeutic strategies with the choice of cell source remaining a central and controversial issue. Adult mesenchymal stromal cells (MSCs) have quickly become the most popular contenders as of late, despite concerns of phenotypic homogeneity, reliability and stability. To address these challenges, we present here a feasibility study for the isolation, expansion and development of a fetal epiphyseal chondrocyte (FEC) master cell bank from a single tissue donation. Fetal cartilage has a remarkable ability for repair[1] and, much like MSCs, fetal cells exhibit immune-modulatory activity[2] and significant wound healing capabilities[3]. These aspects, in combination with their natural osteochondrogenic ability following epiphyseal ossification and the findings of this study makes FECs a very interesting cellular choice for osteochondral repair and regeneration.

METHODS: The proximal ulnar epiphysis was processed following strict cGMP guidelines for organ donation and screening to create an FEC parental cell bank for tissue engineering applications (CHUV Ethics Committee protocol # 62/07). The tissue biopsy (~2mm^3) was micro-dissected and dispersed by mechanical attachment to scalpel scored surfaces. FEC outgrowth was observed at one week and expansion was accomplished at two weeks. The parental cell bank was established with 100 vials of 5-10 x10^6 cells and stored in the vapor-phase of liquid nitrogen following cGMP stocking conditions. A cGLP pre-clinical FEC master cell bank was then created for in vitro and in vivo characterization and validation.

RESULTS: Isolated cells exhibit a remarkable homogeneity in monolayer culture as well as a notable proliferative potential with a propensity for overlapping, all the while maintaining the same susceptibility for trypsin-mediated re-suspension. FECs do not seem to exhibit notable phenotypic variations within the first six passages and are able to spontaneously coalesce when placed in pellet culture form, deposit matrix and express fundamental chondrogenic markers. Flow cytometric analyses revealed unimodal distributions indicative of a homogenous population. A comparison with adult bone marrow derived MSCs yielded FEC surface marker profiles consistent with a chondrogenic rather than an undifferentiated progenitor phenotype.

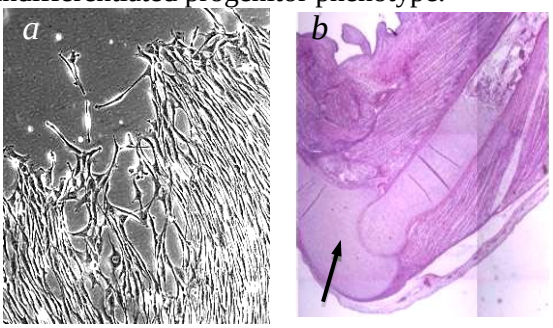

Fig. 1: (a) FEC expansion on tissue culture plastic. (b) Fetal ulnar epiphysis (black arrow).

DISCUSSION & CONCLUSIONS: The thorough cGMP isolation and processing combined with the observed homogeneity and stability of this FEC population in culture allows for the reliable expansion of FECs for in vitro and in vivo animal testing. It also makes it possible to use the same master cell bank for clinical applications, much like that developed with skin cells[4], opening the door to novel osteochondral therapies.

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ACKNOWLEDGEMENTS: These studies were funded in part by the S.A.N.T.E. foundation, the Sandoz foundation and the Inter-institutional Center for Translational Biomechanics EPFL-CHUV-DAL.

Disc Regeneration - Automation for a New Cell-based Approach

N. Franscini[1], E. Bono[1], R. Durner [2], K Wuertz[3], N. Boos[3] and U. Graf-Hausner[1]

[1]Institute of Chemistry and Biological Chemistry, Zurich University of Applied Sciences, Wädenswil, CH, [2]Tecan Schweiz AG, Männedorf, CH, [3]Spine Research Group, Center for Applied Biotechnology and Molecular Medicine, University of Zurich, Zurich, CH

INTRODUCTION: Intervertebral disc degeneration (IVD) is one of the leading musculo-skeletal disorders confronting the health system. Despite current therapeutic approaches (i.e. surgery), the IVD disease is still problematic.
Tissue Engineering represents a promising alternative. A recent study performed in our group assessed the potential of a new cell-based approach for the IVD augmentation. IVD cells seeded and cultivated in a combination of polyurethane (PU) and fibrin gel were able to proliferate and re-differentiate (1).
Nevertheless, for a wide implementation of regenerative medicine into clinical routine a standardization and reduction of costs and risks is fundamental. This can be achieved only by automation. Therefore an automated system was developed in order to isolate, propagate and characterize in large scale human IVD cells for the PU-fibrin constructs production.

METHODS: Disc biopsies were obtained from surgeries on low back pain patients. Disc cell isolation and expansion was either performed manually using standard procedures or automatically. For the automatic process, a liquid handling robot, based on the Tecan® Freedom EVO®, combined with an automated tissue dissociation tool and a cell detection platform (Cellavista®, Roche) was applied.

RESULTS: Cell isolation and expansion. IVD cell isolation: the cell yield (automated: $620*10^3$ +/- $37.5*10^3$ cells per gram of biopsy; manual: $468*10^3$ +/- $134*10^3$) and viability (>90% after 7 days culture by both procedures) were very similar (n=5).
Automated quality controls:
1. Growth kinetic of cells processed with both procedures, determined by automated cell confluence measurement, was very similar.
2. Expression of typical markers of IVD cells, as Collagen I (fig.1), Collagen II and Versican was analysed by immuno-fluorescence with the automated system. No difference between the methods was observed for fluorescence intensity.

Fig. 1: Collagen I expression of manual (A) and automated processed cells (B)

In situ decision on cell splitting:

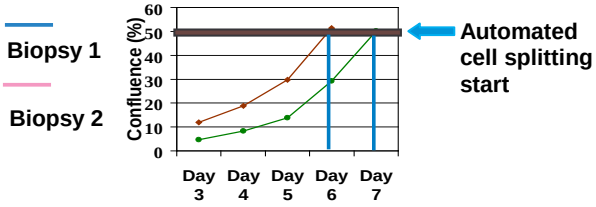

Fig.2: Real time decision on cell splitting is performed by regular cell confluence measurement. The splitting process can be initialized at a set confluence (here >50%).

Cell-based structure: Extracellular matrix synthesis of manually cultivated IVD-like cells in PU-fibrin composites:

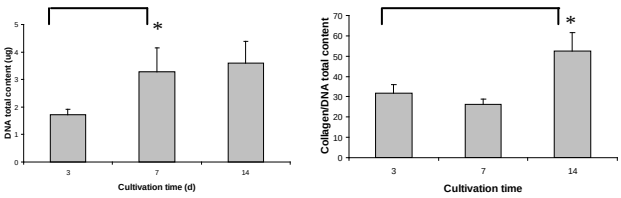

*Fig.3: Biochemical analyses of DNA content (A) and collagen/DNA ratio (B) within PU-fibrin composites up to 14 days (n=3). *p<0.05*

DISCUSSION & CONCLUSIONS: The implementation of the presented automated procedure will allow the application in the clinical routine of a promising cell-based approach using scaffolds like the PU-fibrin structure and autologous cells isolated out of patient's biopsy.

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Quantifying Cell Shape Changes of human Mesenchymal Stem Cells undergoing Differentiation

B. Gantenbein-Ritter[1,2], C. Seiler[2], SCW Chan[1,2], M. Reyes[2], J. Kühn[3], C. Depeursinge[3]

[1]*ARTORG Center, University of Bern, Bern, CH,* [2]*Institute of Surgical Navigation and Biomechanics, University of Bern, Bern, CH,* [3]*Microvision and Microdiagnostics Group, Advanced Photonics Laboratory, École Polytechnique Fédérale de Lausanne , Lausanne, CH*

INTRODUCTION: Cell therapy using autologous mesenchymal stem cells (MSCs) seems promising for a wide variety of tissues of the muscle-skeletal system.

MSC expansion is generally done using selection of density-gradient centrifugation and selection by plastic adherence. [1] Since stemness, i.e. the potential to differentiate into different tissue types of the mesenchyme, is highly dependent on the heterogeneity of the cells and other factors, e.g. donor age, outcome is highly unpredictable. [2] Here, we addressed whether cell morphology could be directly correlated with cell commitment and function. For obtaining non-invasive high resolution images, we imaged live MSCs undergoing differentiation using digital holographic microscopy (DHM).

METHODS: Bone-marrow-derived hMSCs of one human male donor were isolated and expanded as previously described. [3] Passage 3 cells were expanded for 14 days in differentiation media. [1, 2, 3] The day before live imaging the cells were trypsinized and cultured for 24h in glass-bottom-well plates (P35G-1.5-14, MatTek Corp.,Ashland, MA, USA). Chondrocytes were cultured at 4M/ml in 1.2% alginate beads and dissolved in sodium citrate and seeded in 2D monolayer 24h prior cell imaging. Live cells were kept in each of the differentiation media during imaging. At least 10 cells were imaged per group using 40x magnification on a digital holographic microscope (DHM T1000, LyncéeTec SA, Lausanne). The optical resolution of this device in z-axis is ~1 nm. [4] Noise reduction was performed on the phase data using a median filter (Fig. 1). The cell phenotype was monitored using real-time RT-PCR at marker genes.

RESULTS: We found no significant differences in cell volume between groups (Fig. 2) (K-W test, P = 0.11). However, there was a trend that undifferentiated stem cells in expansion with bFGF-2 were larger in "volume" as inferred from phase data.

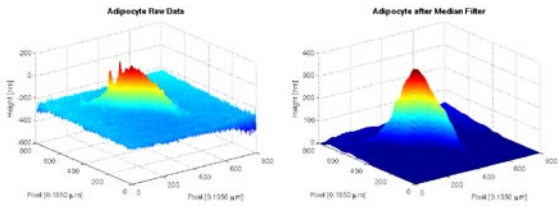

Fig. 1: Noise reduction transformation for volume estimation of human MSCs undergoing differentiation of digital holographic phase-contrast image data (DHM T1000).

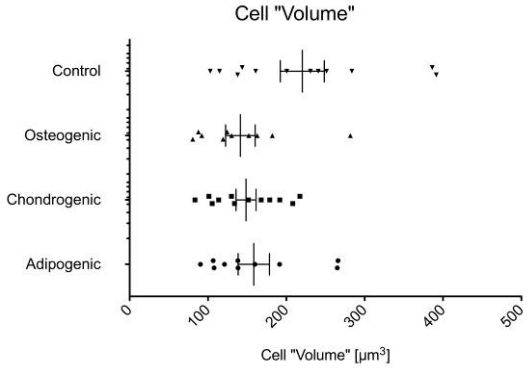

Fig. 2: Cell volume estimates of live human MSCs undergoing differentiation inferred from digital holographic phase-contrast image data.

DISCUSSION & CONCLUSIONS: It seems obvious that, as cells become committed to a specific function, they undergo major structural changes. Here, we found a trend that cell volume as a relatively obvious character could possibly vary among cells undergoing mesenchymal differentiation. However, here we assumed equal refractive indices for the phase data among cells, which might not be true and have to be determined independently in future trials. Also tracking of individual cells through time might be necessary to classify cell types.

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ACKNOWLEDGEMENTS: We thank Ladina Ettinger for histological stains. Lorin Benneker provided the human bone marrow sample.

Whole IVD organ culture systems to study the potential of biological repair

R Gawri[1,4], J Moir[1], Ouellet[2], P Roughley[3], F Mwale[4], J Antoniou[4], T Steffen[1], L Haglund[1*]

[1]Orthopaedic Research Laboratory, [2]McGill Scoliosis and Spine Center, [3]Shriners Hospital for Children, [4]LDI, McGill University, Montreal, Canada

*Corresponding: lisbet.haglund@mcgill.ca

INTRODUCTION: Intervertebral disc (IVD) degeneration is a common cause of back pain, which has a negative impact on the quality of life of the patient and is costly to the health care system. The adult human IVD seems incapable of intrinsic repair once injured, although experiments in small animals suggest that the early stages of disc degeneration can potentially be retarded or even reversed by the administration of growth factors to promote new extracellular matrix (ECM) synthesis. The literature describes a number of organ culture systems ranging from tissue explants to discs with preserved bony endplates that can potentially be used to study IVD repair. Many systems use discs from young healthy animals with a high proteoglycan concentration, as well as discs from smaller animals which retain notochordal cells throughout life. However, the early stage of human disc degeneration is characterized by loss of proteoglycans, and adult human discs do not contain notochordal cells. The purpose of this study was to develop a long term organ culture system suitable for studying repair in the human IVD.

METHODS: Adult human or bovine IVDs were prepared with intact cartilaginous endplates. The bovine discs are of similar structure to the human discs and do not possess notochordal cells. Human discs had mild grade (Thompson grade 2-3) degeneration, while bovine discs came from young animals with no sign of degeneration. Degeneration was therefore induced by a single trypsin injection into the center of the bovine discs. Disc swelling, cell viability, proteoglycan content and extracellular matrix degradation were compared to control discs under a variety of culture conditions.

RESULTS: Human IVDs isolated with intact cartilage endplates but no adjacent vertebral bone retained more than 96% viable cells and did not show altered matrix degradation after 4 weeks of culture with either high and low nutrient levels. In contrast, both human and bovine discs cultured with the cartilage endplates removed or with the vertebral bone retained displayed an excessive cell death after 3-7days of culture. Bovine discs treated with trypsin to induce degeneration showed extensive degradation and loss of aggrecan, but the abundance and degradation of chondroadherin, a collagen-associated protein, was not as greatly affected. Cell survival and morphology was maintained following trypsin treatment throughout 4 weeks of culture. To date, human discs have been cultured for up to 4 months with maintained viability. No loading protocol was needed to maintain disc integrity or viability of either human or bovine discs.

DISCUSSION & CONCLUSIONS:
We have developed a novel human and bovine disc organ culture system with long term maintenance of cell viability. Retention of the cartilage endplates limits tissue swelling and permits efficient nutrient supply, and allows for long term survival. The availability of such systems will permit the repair potential of therapeutic candidates to be studied in bovine discs with induced degeneration followed by verification in adult human discs with naturally occurring degeneration. The bovine system is an excellent tool for initial screening as discs are readily available, and the human system using discs with naturally occurring degeneration would then be the ideal second step for validating candidates that showed potential to induce biological repair. Long culture periods will be needed to measure a significant change in disc ECM composition during repair, and the present system provides an easy and economical means to make this possible. The system is simple to use, as no apparatus is needed to limit the detrimental effects of excessive tissue swelling.

ACKNOWLEDGEMENTS: Funding for this study was obtained through AOSpine, Shriners of North America and CIHR.

A dual chondrogenic and anti-angiogenic approach for cartilage repair

K. Gelse[1,2], J. Gusinde[1], M. Blanke[1], A. Mauerer[1], B. Swoboda[2], F. Hennig[1]

[1]*University Hospital Erlangen, Dept. of Orthopaedic Trauma Surgery, Erlangen, Germany,*
[2] *Dept. of Orthopaedic Rheumatology, University Erlangen-Nuernberg, Erlangen, Germany*

INTRODUCTION:
Microfracture (Mfx) is a simple, minimally-invasive technique that is frequently applied for the induction of cartilage repair tissue, however, this approach mostly generates inferior fibrocartilage. Furthermore, an unphysiological hypertrophy of the subchondral bone plate with vascular invasion and subsequent endochondral ossification within the repair tissue is a frequently observed phenomenon that may interfere with the long-term outcome[1-3]. Therefore, this study followed a strategy to combine Mfx with the application of recombinant Bone Morphogenetic Protein-7 (BMP-7) to promote chondrogenesis, and with the application of recombinant Thrombospondin-1 (TSP-1), a protein with anti-angiogenic properties[4], to prevent excessive endochondral ossification within the repair matrix.

METHODS: In miniature pigs, cartilage defects with 5-mm in diameter in the femoral trochlea in a total of 12 knee joints were treated by the microfracture technique (Mfx). The Mfx-treated defects either received no further treatment (Mfx), or were filled with 35µg collagen-bound BMP-7 (Mfx+BMP7), or 5µg of collagen-bound TSP-1 (Mfx+TSP-1), or with a combination of the two factors (Mfx+TSP-1+BMP7), respectively. The defects were sealed with a layer of fibrin glue. The animals were allowed to move freely in their cages. Six or 26 weeks later, the animals were harvested and the outcome of the repair tissue was assessed by the ICRS histological assessment scale.

RESULTS:

Defects treated by Mfx were typically characterized by fibrocartilaginous repair tissue and hypertrophic reactions of subchondral bone. The average volume-ratio of calcified- versus non-calcified tissue within the repair matrix was 23,8%. The application of BMP-7 (Mfx+BMP7) efficiently induced chondrogenesis of the ingrowing BMSCs and significantly improved the quality of the repair tissue with a hyaline-like, proteoglycan-rich matrix, but was also associated with inadvertant excessive endochondral ossification. In contrast, the application of TSP-1 (Mfx+TSP) completely prevented subchondral bone hypertrophy and matrix calcification, but did not support chondrogenesis within the repair tissue. Furthermore, Mfx+TSP interfered with reconstitution of the subchondral bone plate, that recovered not until after 26 weeks.

Only the combined treatment of microfractured lesions with TSP-1+BMP7 generated a hyaline-like repair matrix but prevented excessive endochondral ossification. Within the period between 6 and 26 weeks, a tendency for maturation of the repair matrices and reconstitution of the subchondral bone plate could could be observed.

DISCUSSION & CONCLUSIONS:

These data indicate that both the induction of chondrogenesis and the prevention of terminal chondrocyte differentiation have to be considered for cartilage repair strategies and may have to be addressed by different factors. Future studies may further optimize this “cocktail” of factors and their release kinetics in order to yield the unique articular chondrocyte phenotype within the cartilage repair tissue.

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ACKNOWLEDGEMENTS: This work was supported by the Interdisciplinary Center of Clinical Research Erlangen (Grant A36) and the German Research Foundation (DFG, Grant GE1975/2-1).

Multiple Neocartilage Cushions supported a long-term subsided Smith-Peterson Hip Arthroplasty

F. Gottsauner-Wolf[1], H. Plenk Jr.[2]

[1] *Orthopaedic Department, Landesklinikum Krems, A,* [2] *Bone Biomater. Res., Histol.Embryol. Institute, Medical University of Vienna, A*

INTRODUCTION: When Smith-Petersen introduced his new method of hip arthroplasty [1], the interposed glass, and later metallic cup mold should allow regeneration of articular surfaces damaged by coxarthritis, leading to "a smooth, glistening fibrocartilage upon densified, more firm underlying bone". Many of these non-cemented hemiarthroplasties, which are predecessors of all later resurfacing hip joint arthroplasties, had a history of long-term success. In this presentation, the skeletal tissue reactions under such a metallic cup will be analyzed after forty years of functional loading.

METHODS: A male patient had suffered for 2 years from chronic deep infection of the right hip after shrapnel wounding in the Second World War. In 1947, at the age of 29 years, the partially coxitis-destructed femoral head had been covered with a metallic (Vitallium®) Smith-Petersen cup. The patient kept all radiographic follow-ups until the hemi-arthroplasty had to be replaced by cementless total hip arthroplasty after 40 years. The resected femoral head and neck was polymethylmethacrylate-embedded, and consecutive undecalcified microtome and ground sections in frontal plane were evaluated by light microscopy.

RESULTS: After normal and sports activities for 13 years, function was impaired by increasing pain and limping. Radiology showed a gradual subsidence and tilting into varus position of the cup (Fig.1). At macroscopic inspection, the surface of the retrieved femoral head appeared smooth and spherical, but with distinctive spots of a whitish tissue protruding from the cartilage-denuded (eburnated) bone.
Light microscopy showed a heavily densified, but viable bone in the cranial load-bearing region, totally masking the original structure of trabecula in the remaining femoral head and neck (Fig.1). While part of the caudal surface was still covered with fibrocartilage (FiCa), in the cranial region only spotty cartilaginous cushions could be observed in larger vascular spaces opened in the sclerotic bone region. These cartilage cushions were protruding over the bony surface, apparently providing additional support for the freely rotating cup.

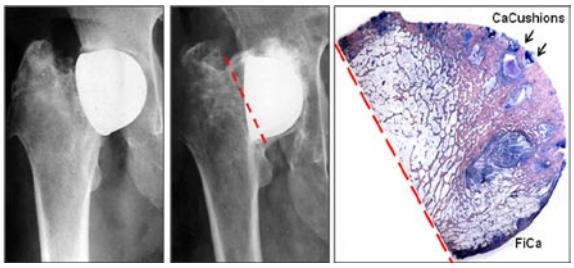

Fig.1: Post-operative (left) and follow-up radiograph after 40 years (middle), showing subsidence and tilting of the Smith-Petersen cup. The red dotted line indicates the resection plane of the retrieved femoral head and neck remnant under the cup (right; undecalcified microtome section, Giemsa-stain).

DISCUSSION & CONCLUSIONS:

Apparently, the femoral head and neck bone survived and remodelled underneath the cup, but the surface was not covered with fibrocartilage in the load-bearing area. Cartilaginous tissue differentiation in this situation seems possible according to the theory of causal histogenesis, put forward in the German literature by Krompecher [2] and Pauwels [3]. Pauwels had observed full resto-ration and endochondral growth of an infant coxitis-destructed femoral head underneath a plexiglass-cup. However, in this adult patient and under full load-bearing conditions bone gradually adapted to biomechanical stresses, but cartilage could only differentiate from granulation tissue in the mini environments of opened vascular spaces in the eburnated sclerotic bone.

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The use of chondrogenic differentiated mesenchymal stem cells in treating focal cartilage damage: preliminary study in animal model

T. Kamarul, Terence Tay, H. Dashtar, Tay L.X., Pan-Pan Chong, R. Ali, Azlina A. Abbas,
Tissue Engineering Group, National Orthopaedic Centre of Excellence for Research and Learning (NOCERAL), University of Malaya, 50603 Kuala Lumpur

INTRODUCTION: The use of mesenchymal stem cells (MSCs) in treating cartilage defects have been previously described and shown to provide good results[1]. However, concerns over the use of non-lineage committed cells which can result in unwanted cellular transformation have limited current proposed methods to be used only in experimental models. It is believed that the use of chondrogenic differentiated MSCs (C-MSCs) may resolve this issue however studies using these lineage committed cells have not been previously described. The current study describes the results of using C-MSCs to repair full thickness focal cartilage defects created in rabbit models and was compared to those using autologous chondrocyte and undifferentiated MSCs.

METHODS: New Zealand white rabbits (n=18) were used in this study an in accordance to the approval obtained from the ethics committee on animal studies in University of Malaya. Full-thickness articular cartilage defects were created on the weight-bearing surfaces on the medial femoral condyles in both knees. Repair of the cartilage defect were then performed 6 weeks after the injury using a modified method described previously by Brittberg et al[2]. Cells sources used in this procedure varied but the numbers were standardized at $1x10^6$ cells per defect site. Rabbits were randomly divided into 3 groups (n=6/group) based on the types of cells used for the repair of the damaged sites: (Group 1) undifferentiated MSCs, (Group 2) chondrogenic MSCs and (Group 3) autologous chondrocytes. Cell characterization and the method of cell preparation for this study were described elsewhere[3]. The untreated contralateral knees were used as controls. Rabbits were sacrificed at 3 and 6 months and biopsy of the repaired and damaged sites were performed to determine the quality of cartilage at these time points. Three assessment methods were used: Brittberg morphological score, quantitative analysis of glycosaminoglycan (GAG) using spectrophotometry analysis and O'Driscoll histological grading were performed for comparative analysis. Statistical analyses were performed accordingly with significance set at $p<0.05$.

RESULTS: The mean Brittberg score, GAG quantification and histological scores were higher in the surgically treated right knee as compared to the untreated left knee at both 3 and 6 months. However, statistically significant differences were only noted for those at 6 months ($p=0.024$). Quantification analyses revealed higher scores in all the 3 assessment methods in Group 1 as compared to the other groups. Histological examination revealed remarkable hyaline cartilage regeneration in the transplanted sites but were almost absent in the unrepaired sites.

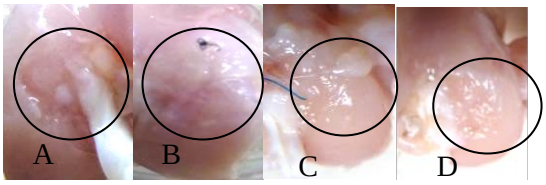

Fig. 1: Gross appearance of the focal defect sites at 6 months: (A) left untreated knee-control (B) undifferentiated MSC treated (C) C-MSC treated (D) autologous chondrocytes

DISCUSSION & CONCLUSIONS: This study shows that the use of undifferentiated MSCs provided better tissue repair outcomes than that of C-MSCs or autologous chondrocytes. Furthermore, cell maltransformation did not appear to be a problem in the MSC treated sites. Thus, the use of undifferentiated MSCs may be the better cell source for future therapies. However; larger number of samples and longer duration of observations may be required in order to make a definite conclusion.

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THE DIFFERENT PREPARATIONS OF HUMAN AMNIOTIC MEMBRANE (HAM) AS A POTENTIAL CELL CARRIER FOR CONDROCYTES

S.R. Kanthan, K. Ganesan, Azura. M, T. Kamarul

Tissue Engineering Group, National Orthopaedic Centre of Excellence for Research and Learning (NOCERAL), University of Malaya, 50603 Kuala Lumpur

INTRODUCTION: Human Amniotic membrane (HAM) is a biological product which has been used in a variety of clinical applications. Despite being recognized as bio-compatibile, bio-degradable, safe and durable, the use of this tissue in cartilage tissue engineering has not been well established although preliminary studies have indicated its usefulness [1,2]. Furthermore, the choice of using different HAM preparations to maximize phenotypic chondrocyte expression has not been previously described. To determine this, a study was conducted to compare the effects of different HAM preparations on the phenotypic expression of chondroctyes seeded on these substrates.

METHODS: Permission to conduct this study was obtained from the ethical committee for use of the human tissue in University of Malaya. Amniotic membranes were derived from placenta of patients who had undergone Cesarean Section after obtaining written consent. Patients were screened for Hepatitis B, C and HIV. Only those with negative results were selected for this study. Harvested amniotic membranes were peeled off from the chorion layer and soaked in 0.05% Sodium Hypochloride and 0.9% normal saline. HAMs were cut in two with each half processed using freeze-dried (FD) or air-dried (AD) methods as described elsewhere. [3]

Chondrocytes were obtained by processing cartilage harvested from 2-3 months old New Zealand white rabbits. The methods used to obtain cultured chondrocytes were described elsewhere [3]. Chondrocytes ($4x10^5$ cells) were seeded on the basement membrane side of FD-HAM and AD-HAM which had been cut into a standard size of 3cm diameter. Non seeded HAMs were also used as controls. GAG concentrations were measured in the cell culture medium at different time points. Histological examination and scanning electron microscopy (SEM) were also performed to determine cell morphology and attachments on substrates. Statistical analyses were performed for the GAG contents with p value<0.05 accepted as significant.

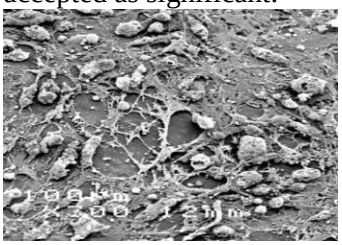

Fig. 1: SEM of chondrocytes attached to the basement membrane of air dried HAM

RESULTS: There were no contaminations observed in cultures using AD or FD HAMs. An increase in GAG content was observed over time in cultures with chondroctye seeded HAMs. Significant differences between the GAG contents in the control and seeded HAM were observed (*$p<0.05$)*. However, No significant differences were noted between the GAG expressed in FD-HAM and AD-HAM groups (*$p>0.05$)*. SEM of cell seeded HAMs showed that chondrocytes were adherent to the basement membranes and formed colonies (fig 1).

DISCUSSION AND CONCLUSION: HAM appears to support cell attachment whilst promoting GAG expression from seeded chondrocytes. The different HAM preparations did not appear to influence GAG expression. Considering that the processes involved in producing AD-HAM is faster, cheaper and less labour intensive than that of FD-HAMs whilst still producing similar GAG expressions, the use of the AD-HAMs may be the more prudent choice for future research or applications.

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A MULTI-LAYER COLLAGEN-BASED SCAFFOLD FOR OSTEOCHONDRAL DEFECT REPAIR

T.J. Levingstone[1], G. Dickson[2], F.J. O'Brien[1], J.P. Gleeson[1]

[1] Tissue Engineering Group, Anatomy Department, Royal College of Surgeons in Ireland, Dublin 2, Ireland; [2] School of Medicine, Dentistry and Biomedical Science, Queen's University, Belfast, Northern Ireland, UK

INTRODUCTION: Damage to articular cartilage and the underlying subchondral bone frequently occurs as a result of disease or trauma. Cartilage is avascular and has poor regenerative capacity and thus osteochondral defects present a significant challenge to the orthopaedic community. Current clinical treatments have limited regenerative capacity [1]. Recently, scaffolds have shown promise for the repair of osteochondral defects. However, long term clinical success has yet to be achieved. The aims of this study were to develop a multilayer scaffold (cartilage, calcified cartilage and bone layers) with optimised structure and composition for osteochondral repair and to investigate the chondrogenic potential of the cartilage layer.

METHODS: Multilayer scaffolds, composed of a base layer optimised for bone repair [2], an intermediate layer for calcified cartilage repair and a top layer for cartilage repair (optimised Type I/II collagen composition), were fabricated using a novel freeze drying 'iterative layering technique' (EPO 9151226.9). Pore structure and layer interface properties of the scaffold were examined using scanning electron microscopy (SEM). Compressive strength and layer adhesion strength were measured using unconfined compression testing and a custom layer adhesion strength test, respectively. *In vitro* performance was assessed using MC3T3 pre-osteoblast cells and rat MSCs. Histological analysis was carried out using H&E and Safranin-O staining techniques.

RESULTS: Scaffolds exhibited a homogenous pore structure (Fig.1), high porosity (>98%), high degree of pore interconnectivity, and seamless layer integration. Mean compressive modulus was found to be 0.51 ± 0.03 kPa. Fibre pullout was observed on the fracture surface during interfacial adhesion strength testing. *In vitro* analysis demonstrated excellent infiltration and proliferation of MC3T3 cells within the scaffold. Safranin-O staining following chondrogenic culture of rMSCs in the scaffold for 28 days revealed evidence of cartilage-specific proteoglycan production.

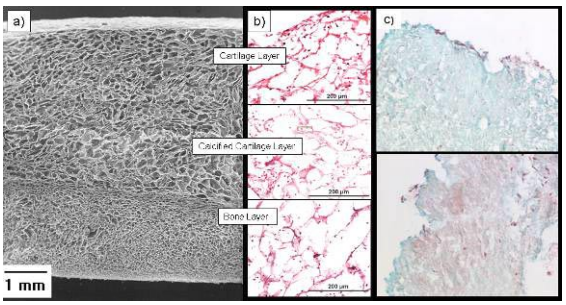

Fig 1: 3-layer scaffold a) SEM of scaffold microstructure b) Infiltration of MC3T3 cells at 14 days c) S-GAG production in optimised top layer compared to collagen only control

DISCUSSION & CONCLUSIONS: In this study, a multi-layered scaffold, which is structurally and compositional similar to osteochondral tissue has been successfully developed. The scaffold is highly porous, has a seamlessly integrated layer structure, a homogenous pore structure and a high degree of pore interconnectivity. *In vitro* analysis has shown this scaffold to be highly biocompatible, enabling a high degree of cellular infiltration and proliferation. The optimised top layer shows significantly increased cartilage-specific proteoglycan production over the collagen control. Further optimisation of this osteochondral defect repair scaffold is ongoing including pre-clinical assessment using a rabbit osteochondral defect model.

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ACKNOWLEDGEMENTS: Enterprise Ireland Commercialisation Fund, POC (PC/2007/331) and TD (CFTD/2009/0104).

Generation of human nasal chondrocytes engineered to express soluble VEGF receptor-2 for cartilage tissue engineering

A.Marsano[1], P.Bernegger[1], U.Helmrich[1], S.Schaeren[1], S.Miot[1], A.Barbero[1], I.Martin[1], A.Banfi[1]

[1]Departments of Surgery and of Biomedicine, University Hospital Basel, Switzerland

INTRODUCTION: Vascular Endothelial Growth Factor (VEGF) is involved in regulating cartilage growth and endochondral ossification during growth plate development [1,2] or in cartilage degenerative processes, e.g. in osteoarthritis [2,3] or following mechanical overloading [4]. Instead, VEGF expression is suppressed during hyaline cartilage formation and maintenance [3]. Therefore, in the present study we tested the hypothesis that blockade of the endogenous VEGF pathway could improve *in vitro* chondrogenesis. Nasal chondrocytes (NC) are a promising cell source for cartilage regeneration since even after culture expansion, they retain a high and reproducible capacity to redifferentiate and generate hyaline-like tissue [5]. For this purpose, nasal chondrocytes were transduced to express soluble VEGF receptor-2 (sFlk-1), which can efficiently block VEGF activity in vivo.

METHODS: NC isolated from two donors were transduced with a retroviral vector carrying the cDNAs for sFlk-1 and a truncated form of CD8a as a cell-surface marker, or with a control vector carrying only CD8a [6]. For each group a CD8a-positive population was FACS-sorted. The functionality of the secreted sFlk-1 was tested by using human umbilical vein endothelial cells (HUVEC). The chondrogenic differentiation of NC was tested in 3D pellet culture at either 20% or 2% of O_2 for 2 weeks [7].

RESULTS: NC were successfully transduced and sorted. The secreted sFlk-1 was active and could specifically inhibit the mitogenic activity of VEGF concentrations up to 10 ng/mL on HUVEC (Figure. 1). The GAG/DNA ratio was also similar in the pellets generated by naïve NC and cells transduced with CD8a alone or linked to sFlk-1 (Figure. 2). Culture at 2% of oxygen tension seemed not to affect the chondrogenic potential of the NC pellets as compared to 20%.

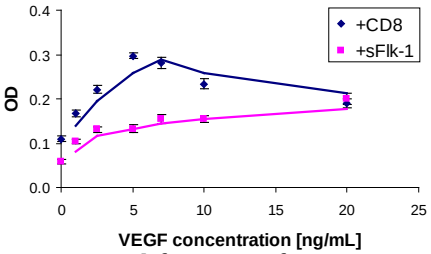

Figure. 1. Proliferation of HUVEC cultured in presence of medium conditioned by transduced cells producing either sFlk-1 or only CD8a.

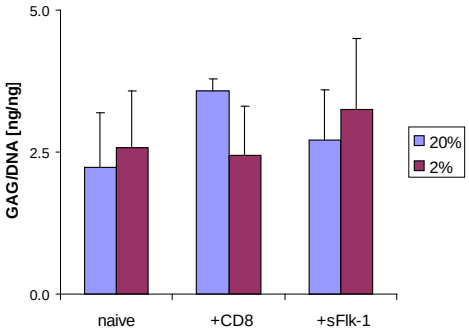

Figure. 2 Biochemical quantification of GAG/DNA ratio in pellets generated by NC from two different donors.

DISCUSSION & CONCLUSIONS: Our preliminary data show that blocking the endogenous VEGF pathway does not affect significantly the in vitro chondrogenic differentiation of NC. Ongoing studies aim to assess the effects of sFlk-1-transduced cells in 3D constructs in in vivo models, where autocrine effects would be coupled to paracrine ones to control angiogenesis [8].

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ACKNOWLEDGEMENTS: Funding from EU FP7 project NMP3-LA-2008-213904 (DISC REGENERATION).

Addition of Hyaluronic Acid Improves Cellular Infiltration and Cartilage-specific Extracellular Matrix Synthesis in a Porous Collagen Scaffold

A. Matsiko[1], T.J. Levingstone[1], F.J. O'Brien[1] J.P. Gleeson[1].

[1]*Department of Anatomy, Royal College of Surgeons in Ireland, Dublin 2, Ireland.*

INTRODUCTION: Tissue engineering provides the prospect of developing scaffolds that present a suitable three-dimensional microenvironment optimised for tissue repair applications. Composition is an essential factor in scaffold development since it regulates cellular adhesion and proliferation[1]. Chondroitin sulphate (CS) and Hyaluronic acid (HyA), forms of glycosaminoglycan (GAG), are natively found in cartilaginous tissue. GAG's are elementary in regulating cell signalling and synthesis of extracellular matrix. Collagen-CS scaffolds, previously developed in our laboratory have been shown to support chondrogenesis[2]. However, we hypothesise that over a long term culture period, HyA may improve stability of newly synthesised cartilage-specific matrix since it acts as a back-bone for the attachment of proteoglycans[3].
The aim of this study was to investigate the effect of HyA and CS, incorporated in our collagen scaffolds, on enhancing early cartilage-specific matrix production, cellular proliferation and infiltration within these highly porous tissue engineering matrices.

METHODS: Porous collagen-GAG scaffolds were fabricated using a freeze-drying technique previously described[4] and subsequently dehydrothermally (DHT) cross-linked at 105ºC for 24 hours. GAG concentration in Collagen-CS and Collagen-HyA was 2.67mg/ml of 0.5M acetic acid[4]. Scaffolds were seeded with rat mesenchymal stem cells and cultured in chondrogenic medium for 14 days. Assessment on cell-seeded scaffolds was carried out using a Hoescht dye DNA and Dimethylmethyline Blue dye sulphated GAG quantification assays. Histological analysis was also carried out using H&E and Safranin-O staining techniques (cell nuclei and sulphated GAG respectively).

RESULTS: At 14 days, cell numbers (Fig. 1) and quantified sulphated GAG production were highest in scaffolds containing HyA. Histological images also show a higher sulphated GAG synthesis in these scaffolds (Fig. 2). The addition of HyA dramatically improved *in vitro* cell infiltration within the scaffolds. In contrast, sulphated GAG production in collagen-CS scaffolds was seen predominantly around the periphery of the constructs (Fig. 2A), with consequently low levels of cell infiltration observed after 14 days.

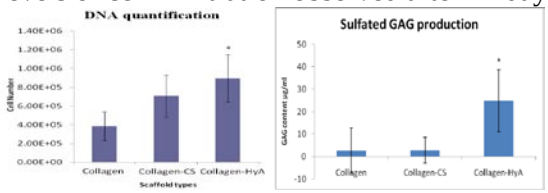

Figure 1. Hoescht dye DNA quantification and Sulfated GAG synthesis after 14 days.

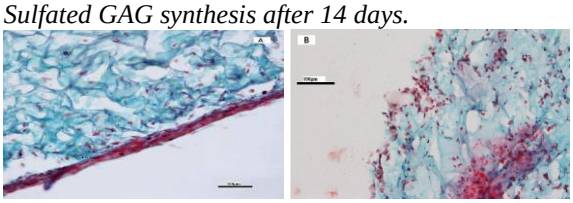

Figure 2. Histological images of A: Collagen-CS and B: Collagen-HyA, after 14 days incubation.

DISCUSSION: The aim of this study was to investigate the effect of GAG addition on the early-stage chondrogenesis within highly porous collagen scaffolds. Our results show that HyA addition into highly porous collagen-based scaffolds significantly improved the quantity and homogeneity of cartilage-specific matrix production *in vitro* and may improve the chondrogenic potential of these scaffolds.
HyA in these scaffolds may play a vital role in supporting synthesised cartilage proteoglycans by mediating migration, proliferation and ultimately chondrogenesis via CD44 cell surface receptors. Work is currently ongoing to investigate the effect of HyA addition on cartilage-specific gene expression, optimisation of the scaffold pore size and pre-clinical assessment using a rabbit osteochondral defect model.

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ACKNOWLEDGEMENTS: Funding was provided by Enterprise Ireland, CFTD/2009/0104.

THE LOSS OF DIFFERENTIATION POTENTIAL OF HUMAN MESENCHYMAL STEM CELLS CAN BE PREDICTED BY USE OF A SET OF SENESCENCE MARKERS

M. Mehr[1], A. Bertolo[1], N. Aebli[2], S. Ferguson[3], J. Stoyanov[1]

[1]*Swiss Paraplegic Research, Nottwil, CH,* [2] *Swiss Paraplegic Center, Nottwil, CH,* [3]*Institute for Surgical Technology and Biomechanics, University of Bern, CH*

INTRODUCTION: Human mesenchymal stem cells (MSCs) are multipotent cells and have emerged as a promising tool for clinical applications. More than 40 years ago, Hayflick discovered that *in vitro* cultured cells, after a given number of divisions, become senescent [1]. Senescence also concerns MSCs, which, due to limited sample size, may have to be expanded *in vitro* to large extent for clinical use. From tissue engineering perspective, the senescence of expanded MSCs has certain implications, such as quality control and setting correct point for differentiation start. Mainly because of patient variability, it is unclear to what extend cell expansion can proceed for each case before the MSCs start to lose their ability to differentiate and whether senescing MSCs lose this ability gradually or as a discrete event [2]. Our aim was to develop a tool based on a set of parameters which can predict a future loss of differentiation capacity in order to provide optimal therapy andprevent waste.

METHODS: We assessed the senescence of hMSCs by monitoring cell division rate, colony forming units, senescence-associated beta-galactosidase activity and expression levels of three senescence-associated marker genes (CDC2, TOP2A and p53) across passages. This data was correlated to differentiation to chondrogenic phenotype of hMSCs cultured in 3-dimensional biodegradable scaffolds. Chondrogenesis of the constructs was assessed by RT-PCR (Aggrecan, Collagen Type II), immunoblotting as well as histological and immunohistochemical stainings.

RESULTS: Analysis of the data showed that hMSCs undergo *in vitro* senescence (*Figure 1*) which reduces their ability to differentiate (*Figure 2*). Senescence can be assessed by several markers but not a single one of them is 'fool proof'. Therefore, it makes sense to combine two or more markers in a battery of tests in order to predict excessive loss of potential to undergo chondrogenesis.

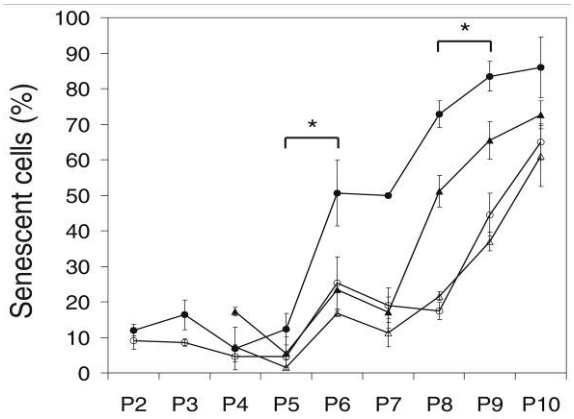

*Fig. 1: Percentage of senescent cells among passages. *, P<0.05 (paired Student's test).*

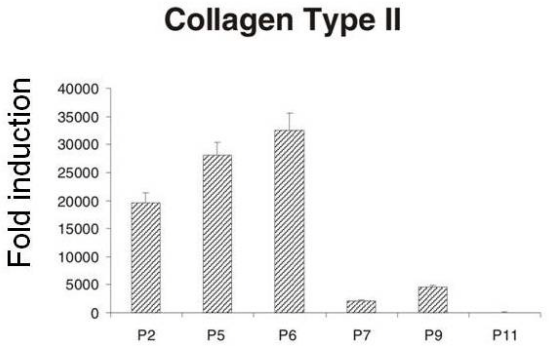

Fig. 2: Normalized expression of collagen type II after 30 days in chondrogenic medium, compared to control medium.

DISCUSSION & CONCLUSIONS: We demonstrated that it is possible to correlate senescence to differentiation at an earlier point when cells still can be used to their full potential. On practical level, it is worth cryopreserving consecutive passages and - once senescence evaluation accomplished - to use the appropriate ones for further differentiation.

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ACKNOWLEDGEMENTS: This work was supported by the Swiss Paraplegic Foundation.

Influence of Oxygen Concentration on Nucleus Pulposus Cells in 3D Pellet Culture During Long-Term Cultivation

A. Mietsch[1], A. Ignatius[1], U. M. Mauer[2], H.-J. Wilke[1], C. Neidlinger-Wilke[1]

[1]*Institute of Orthopaedic Research and Biomechanics, University of Ulm,* [2]*Department of Neurosurgery, German Armed Forces Hospital of Ulm, Ulm, Germany*

INTRODUCTION: Disc degeneration is often associated with an ingrowth of blood vessels and nerves into the intervertebral disc potentially leading to back pain. This ingrowth is fostered by a degraded matrix[1]. In the healthy avascular disc the physiological oxygen tension is about 6% pO_2. Thus, cells are adapted to reduced oxygen supply. With ongoing degeneration and calcification of the endplates oxygen tension can decrease to insufficient 1% pO_2. By contrast, using un-physiological 21% oxygen during cell culture might also harm nucleus pulposus (NP) cells. The aim of our study was to investigate the influence of the oxygen environment on bovine and human NP cells in 3D pellet culture. We investigated the hypothesis that long-term adaptation of NP cells to either disc-normoxic (6%) or atmospheric oxygen (21%) conditions followed by a step-wise reduction to 1% oxygen influences the gene expression pattern of matrix proteins and angiogenic factors. These experiments might contribute to understand the role of the oxygen environment in the pathogenesis of disc degeneration.

METHODS: NP cells isolated from bovine caudal discs (n=6) or from exemplary human disc samples (n=4) were transferred to pellet cultures for 4 weeks in chondrogenic medium[2] for *in vitro* maintenance of their differentiated phenotype at either atmospheric oxygen (21%, group A) or disc-normoxic oxygen (6%, group B) conditions. Then, oxygen was reduced stepwise from 21% to 6% to 1% (group A) or from 6% to 1% (group B) for 24h each to investigate the influence of oxygen deprivation on gene expression of aggrecan, collagen type II and I, as well as HIF-1α and VEGF, analysed by real-time RT-PCR normalised to ribosomal RNA. Chondrogenic differentiation was verified histologically by Alcian blue staining. Statistical significance was tested by Wilcoxon signed-rank test ($p<0.05$).

RESULTS: After 4 weeks of adaptation to different oxygen conditions in 3D pellet culture, bovine NP cells showed a significantly lower expression level of HIF-1α at 6% pO_2 whereas VEGF was not affected. Reduction of oxygen to 1% led in group A to a significant reduction of HIF-1α (-2.8-fold, p=0.0016) whereas VEGF was significantly up-regulated (+2.3-fold, p=0.0032) in both groups. Exemplary human donors showed the same trends with a 2.4-fold down-regulation of HIF-1α and a 5-fold up-regulation of VEGF in group A. Gene expression level of aggrecan and collagen type I was lower at 6% pO_2, whereas collagen type II was not affected after 4 weeks adaption. Influences of oxygen reduction were more pronounced in group A with a significant down-regulation of collagen type II (-2.8-fold, p=0.0016) and type I (-3.4-fold, p=0.0016). Aggrecan expression tended to decrease (-1.8-fold, p=0.078). Exemplary human donors showed same trends. However, Alcian blue staining intensity was similar in both groups.

DISCUSSION & CONCLUSIONS: An increase of VEGF after oxygen reduction points out its angiogenic role in disc degeneration. Our finding of a decreased HIF-1α expression after oxygen reduction is in disagreement to literature findings. Further studies on protein level are needed to verify this effect. Adaption of the cells to 6% oxygen over 4 weeks led to a more moderate decrease of matrix protein gene expression levels after oxygen reduction than in cells adapted to 21% oxygen. Thus, NP cells that are already adapted to low oxygen supply within their native environment might be less sensitive to further oxygen reduction. However, a diminished matrix protein gene expression might influence the balance in matrix turnover towards a faster degradation, that facilitates ingrowth of blood vessels and nerves via VEGF regulation.

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ACKNOWLEDGEMENTS: This project was funded by EU project GENODISC (HEALTH-F2-2008-201626).

Platelet-Rich Plasma (PRP) and Hydrostatic Pressure Influence Cell Differentiation in Nucleus Pulposus Tissue Engineering

A.Mietsch[1], C. Neidlinger-Wilke[1], H. Schrezenmeier[2], U. M. Mauer[3], B. Friemert[4], H.-J. Wilke[1], A. Ignatius[1]

[1]Institute of Orthopaedic Research and Biomechanics, [2]Institute of Clinical Transfusion Medicine and Immuno Genetics, University of Ulm, [3]Department of Neurosurgery, German Armed Forces Hospital of Ulm, [4]Department of Trauma Surgery and Orthopedics, German Armed Forces Hospital of Ulm, Ulm, Germany

INTRODUCTION: Differentiation of autologous mesenchymal stem cells (MSC) to a nucleus pulosus (NP)-like phenotype is precondition for their usage in NP tissue engineering[1]. Adequate differentiation might be further supported by a stimulation of the cells with hydrostatic pressure, thereby simulating *in vivo* loads and motion of the intervertebral disc. Due to its high concentration of growth factors human platelet-rich plasma (PRP) appears to be a suitable autologous substitute during *in vitro* differentiation avoiding animal supplements and recombinant growth factors[2]. The aim of this study was to investigate the influence of different 3D culture systems (pellet culture versus alginate beads) and media compositions (addition of human PRP or TGF-β_1) in interaction with hydrostatic pressure on chondrogenic differentiation of human MSC compared to NP cells.

METHODS: MSC isolated from human bone marrow (n=13) were transferred either to pellet cultures or alginate beads for 4 weeks in chondrogenic medium[3]. This medium was either supplemented with 10 ng/ml TGF-β_1 or 10% human PRP. A part of the samples was subsequently stimulated mechanically by hydrostatic pressure (30 min, 2.5 MPa, 0.1 Hz). Chondrogenic differentiation was verified histologically by Alcian blue staining. Further gene expression of aggrecan, collagen type II and I, and Sox9 was analysed using real-time RT-PCR. All experiments were carried out simultaneously for human NP cells from herniated disc tissue to compare the differentiation pattern. Statistical significance was tested by Wilcoxon test ($p<0.05$).

RESULTS: Both MSC and NP cells showed a considerably higher expression (up to 23-fold) of aggrecan and collagen type II in pellet culture compared to alginate beads. Using TGF-β_1 caused 8000-times higher effects in gene expression levels of the matrix molecules compared to PRP for both cell types with significant results for aggrecan ($p=0.02$) and collagen type II ($p=0.007$). These results were confirmed by Alcian blue staining. Independent of the cell type or culture system used, the application of hydrostatic pressure tended to increase aggrecan, collagen type II, and Sox9 expression (up to 2.5-fold) whereas collagen type I was not affected. However, MSC differentiation as well as NP cell differentiation showed high variability between individual donors.

DISCUSSION & CONCLUSIONS: Human PRP was not able to replace TGF-β_1 with regard to chondrogenic differentiation independent of culture system or cell type used. The mixture of growth factors in PRP appeared to promote proliferation rather than chondrogenic differentiation. In contrast, mechanical stimulation appeared to enhance chondrogenic differentiation. Thus mechanical loading of the 3D constructs might be a promising approach for the development of more functional biological implants. However, variability of the results among individual donors indicates limitations. Not all patients might be suitable for NP tissue engineering and further points such as the genetic background of the patients might influence the application of this potential form of therapy.

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ACKNOWLEDGEMENTS: This project was funded by EU project GENODISC (HEALTH-F2-2008-201626).

Using AFM To Define The Structure Of Collagen Within a Loaded Intervertebral Disc Model System

E.A.Mitchell[1,] A.M. White[1], S.Richardson[1], N. W. Hodson[1], M.J. Sherratt[1], J.A.Hoyland[1]

[1] School of Biomedicine, University of Manchester, Manchester, England.

INTRODUCTION: The intervertebral disc (IVD) consists of an outer annulus fibrosus (AF) composed predominantly of type I collagen organised in lamella like structures, and a central gelantinous nucleus pulposus (NP) composed of proteoglycans and type II collagen. Mechanical loads are thought to be important for maintaining this extracellular matrix (ECM), with physiological and non-physiological loads leading to matrix anabolism and catabolism respectively. As such, mechanical loads may aid tissue remodelling and repair of the IVD ECM. Such changes in ECM structure have usually been qualitatively assessed by conventional histology, immunohistochemistry or standard ultrastructural techniques (i.e. electron microscopy). Here we have used cryosectioning and atomic force microscopy (AFM) to image nanoscale features of the IVD ECM and thus assess changes in ECM (specifically collagen fibril assemblies) that may occur with the application of mechanical load.

METHODS: Bovine caudal discs were isolated with intact endplates. Discs were hydrostatically loaded with a regime designed to mimic normal human IVD loading (encompassing both static and dynamic loads) [(1)] for a period of 6 weeks. Unloaded discs were maintained in culture media for the same time period. Samples of AF and NP were frozen and cyrosectioned at 5µm, and imaged by intermittent contact mode in air using a Veeco Multimode AFM with a Nanoscope IIIa controller.

RESULTS (Figure 1): AFM of these cryosections clearly identified well preserved structural features within the IVD tissue, including thick and thin collagen fibrils embedded in the ECM. The banding of the collagen along with telopeptide ridges was evident on many of the component collagen fibrils.

Freshly isolated AF contained defined individual fibres, which were woven into different layers giving a "basket–weave" appearance. Loaded AF samples had a similar structure to directly isolated disc, except that the bundles of fibrils appeared more tightly bound, with more of a directional orientation. Radial analysis of Fourier transforms of these images confirmed the fibres were orientated and that there was repetition of fibrils through the image. Unloaded cultured discs had a less well defined fibril structure with a small amount of banding, and appeared shorter. Fourier transforms of these sections showed little repetition and orientation of the fibrils.

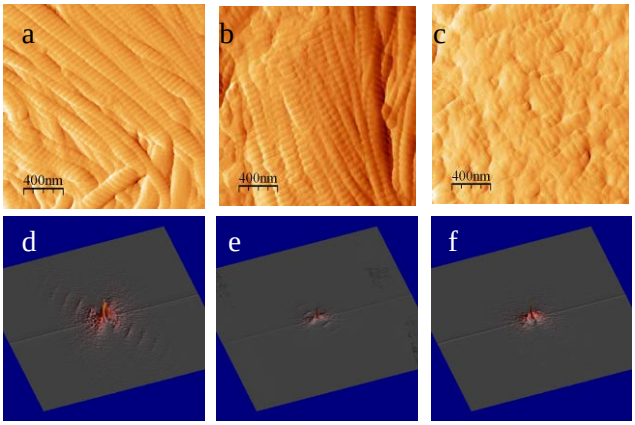

Figure 1: AFM images and their respective Fourier transform analysis. (a) The T=0 sample (b) 6 week loaded IVD AF section (c) unloaded IVD AF sample. (d-f) Fourier transform analyses of AFM images.

Conclusion The use of AFM has enabled ultra-structural imaging of collagen fibrils within the AF and has effectively demonstrated that loading of whole discs induces changes in ECM structure/assemblies. Imaging at the nanoscale level thus provides additional information on changes in ECM structure induced by physiological loading.

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ACKNOWLEDGEMENTS: This work was funded by the ARC.

eCM XI: Multi-axial bioreactor for Nucleus Pulposus Tissue Engineering

M. W. Naing, Y. Liu, E. A. Rayment and D. J. Williams

Healthcare Engineering Research Group, Centre for Biological Engineering, Loughborough University, Loughborough, LE11 3TU, UK

INTRODUCTION: In the body, the intervertebral disc is a tissue that is regularly exposed to mechanical stresses. While back pain due to intervertebral disc degeneration is a common problem, little is understood about the cells that make up the nucleus pulposus, the gel-like component of the disc. Previous research have shown that fluid flow[1], hydrostatic pressure[2], and compressive stresses[3] contribute to the mechanical changes in the disc. In this poster, we present a multi-axial system that is designed to deliver all these stresses simultaneously.

METHODS: In collaboration with Bose Electroforce Test Instruments a multi-axial bioreactor was designed and developed to deliver the following dynamic mechanical stimulation conditions: hydrostatic pressure, pulsatile perfusion flow and uniaxial compression. This mechanical arrangment allows triaxial stimulation of samples. The system can accommodate up to four samples simultaneously in separate sample mounts which allow perfusion of media through the scaffolds. The samples are housed in a hydrostatic chamber which can apply up to 0.3 MPa of pressure. Different combinations of compressive stresses, and pulsatile flow can be dynamically and concurrently administered to the samples.

Real-time data capture of the changes in mechanical properties of the samples can be achieved through a specially-designed software package. The software allows the user to apply a sequence of differing conditions dynamically during the experiment including adapting to the changing properties of the scaffolds. Real-time images can be recorded via a viewing window to the hydrostatic chamber or novel optics. This complex system affords the opportunity to investigate the effect of different combinations of mechanical stimulus on various scaffold and cell types.

RESULTS: Initial results from experiments using Polyurethane scaffolds and human mesenchymal stem cells show that the system is viable for cell-scaffold culture. However, continual detailed improvements in design and operational protocols are necessary to achieve robust experimental procedures and realize the original intent. Mechanical stimulation parameters being optimised include: closed loop control of hydrostatic pressure, media flow rate into the samples, balancing external hydrostatic and the internal sample pressure, and pulsatile flow rate. With multiple interconnected separate components, maintaining the sterility of the system for the extended duration of experiments is a challenge.

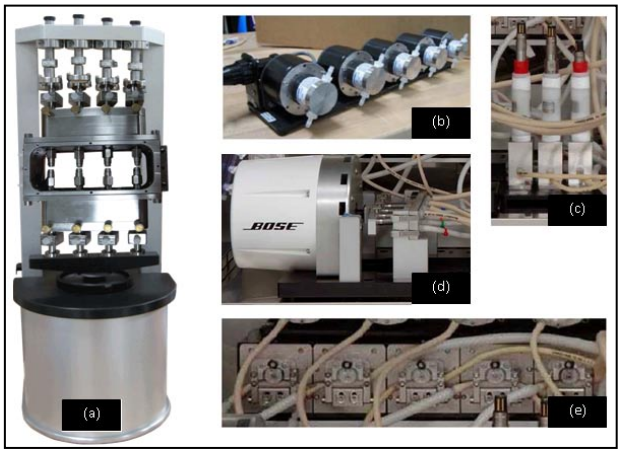

Fig. 1: Components of the system (a) The axial compression unit (b) The mean flow pumps (c) the in-line chemical sensors (d) The pulsatile unit and (e) The restriction valves

DISCUSSION & CONCLUSIONS: As bioreactors attempt to more closely replicate physiological conditions, trade-offs are required between our bio-mimetic aspirations and the practical limitations of engineering, both design and machine building. The construction of demonstrator systems allows the definition of both common ground and key problem areas.

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ACKNOWLEDGEMENTS: Acknowledgements go to Bose Corporation and Dr Gwendolen Reilly from University of Sheffield.

Selection of Chondroprogenitors from Bone Marrow by Adhesion to Chondroitin Sulfate

[1]O' Sullivan J; [1]Coleman CM; [1]Murphy M; [1]Barry F
[1]Regenerative Medicine Institute, National University of Ireland Galway, Galway, Ireland
Senior author: Frank.Barry@Nuigalway.ie

INTRODUCTION: Osteoarthritis is a disease of the joints characterized by progressive destruction of articular cartilage. As cartilage is composed primarily of extracellular matrix (ECM) with limited capacity for self-repair, the therapeutic application of mesenchymal stromal cells (MSCs) offers potential to regenerate cartilage. It was therefore hypothesized that a chondrogenic population of MSCs within the bone marrow (BM) can be isolated by preferential adhesion to cartilaginous ECM proteins.

METHODS: Proceeding MSC isolation, culture flasks were coated with 1mg/ml hyaluronan (HA) or chondrotin sulphate (CS) at 4°C overnight (Fig.1). BM from the iliac crest of healthy consenting human donors was exposed to uncoated, HA or CS coated plates for 24hrs or 5 days before non-adherent cell removal as per traditional methods. Confluent MSCs were passaged and expanded on uncoated plastic. At P2, MSCs were induced towards chondrogenesis, adipogenesis and osteogenesis.[1]

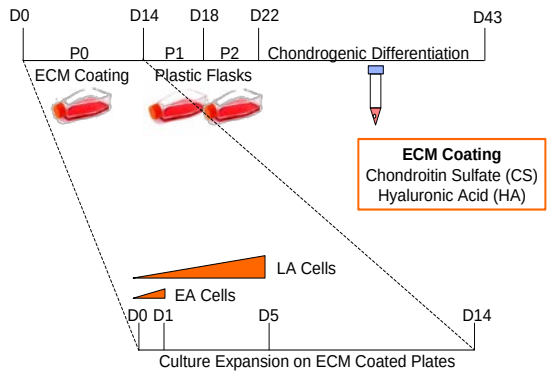

Fig.1. MSC isolation from bone marrow using CS or HS coated plates. *Bone marrow was exposed to ECM or control tissue culture flasks. Non-adherent cells were removed from the flask 24 hours or 5 days after exposure to isolate early or late adherent cells respectively. MSCs were then expanded using traditional, uncoated tissue culture flasks.*

RESULTS: All isolated cells retained a fibroblastic morphology with comparable expansion characteristics. Adipogenic differentiation was significantly increased in all ECM isolated MSCs, especially in the early adherent HA and CS isolated cells. Osteogenic potential was enhanced in all ECM selected MSCs especially in early adherent CS cells. Chondrogenic differentiation was enhanced in all ECM isolated groups, particularly in CS isolated cells (Fig.2).

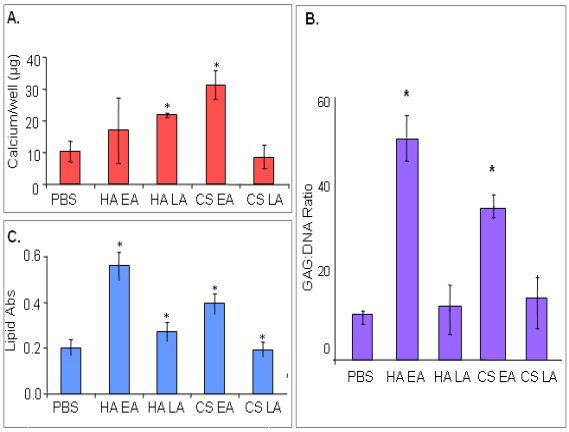

Fig.2. *Tri-lineage differentiation potential of ECM isolated MSCs. A) Osteogenic differentiation indicated increased potential in HA and CS isolated MSCs. B) Chondrogenic differentiation of ECM isolated cells was consistently significantly enhanced as compared to control populations C) Adipogenic differentiation was also significantly enhanced in all ECM isolated MSCs.*

DISCUSSION & CONCLUSIONS: Cartilaginous ECM molecules (HA and CS) were utilized to isolate MSC subpopulations from BM. Cells adhering to CS had the greatest capacity for chondrogenic differentiation, suggesting cells expressing receptors for CS in the bone marrow may be more potent chondroprogenitors. Specific isolation of these subpopulations for clinical application will enable a reduction in the number of MSCs required for clinical efficacy and tissue regeneration.

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Effects of Short Term Loading on Intervertebral Disc Cell viability in a Loaded Disc Culture System

CPL. Paul[1], HA. Zuiderbaan, B. Zandieh Doulabi[1,2],AJ. vd Veen[1], TH. Smit[1], MN. Helder[1], BJ. van Royen[1], MG. Mullender[1]

[1]*Department of Orthopedic Surgery*, [2]*Department of Oral Cell Biology, Vrije University Medical Center Amsterdam, NH, The Netherlands*

INTRODUCTION: The main function of the intervertebral disc (IVD) is to withstand and transfer high magnitude forces, while maintaining flexibility of the spine. Yet, mechanical overloading has been identified as an important extrinsic factor contributing to degenerative disc disease. We have developed a novel Loaded Disc Culture System (LDCS). In this study we aim to culture and load goat IVD's with maintenance of their native cellular and extracellular properties and to investigate the influence of different mechanical loading patterns over a 7- and 14-day culture period.

METHODS: IVD's with endplates attached (L1-6; N=175), were harvested from goats (N=35) under sterile conditions. IVD's were assigned to a day 0 or unloaded control group or one of 4 loading groups (fig. 1). Discs were cultured for 7 or 14 days in the LDCS. Cell viability was assessed in the nucleus (NP) and annulus (AF) regions. Also, water, glycosaminoglycan (GAG) and total collagen content of the extracellular matrix (ECM) were measured.

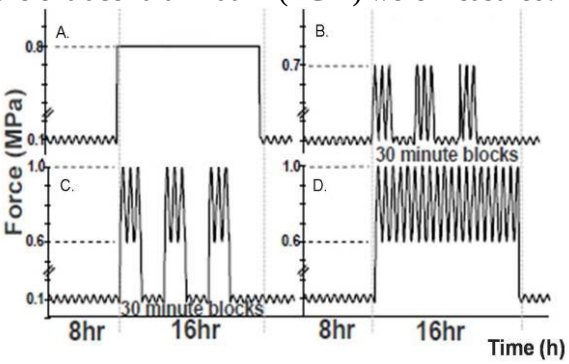

Figure 1: Schematic of loading groups: static (A); low dynamic (B); high dynamic (C); high dynamic prolonged (D).

RESULTS: Fresh discs (day 0) displayed a mean cell viability of 79.4% (NP) and 77.7% (AF). At day seven, cell viability of the unloaded group dropped to half in both NP and AF. The static group showed a drop only in the AF region. Low dynamic load maintained cell viability, whereas high dynamic and prolonged high dynamic loading showed increasing cell death (fig. 2).

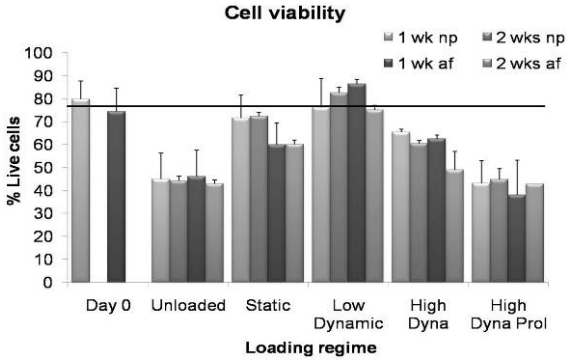

Fig. 2: Mean (+SD) cell viability in NP and AF region of the two control groups and loading regimes during 7 and 14 days.

In the ECM, water-, GAG- and collagen content (fig. 3) remained unchanged after 7 and 14 days in all experimental groups.

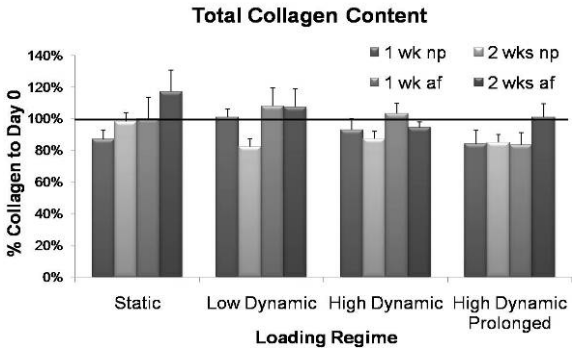

Fig. 2: Collagen content of the NP and AF region normalized to day 0 after 7 and 14 days.

DISCUSSION & CONCLUSIONS: Caprine IVD cells show different responses in cell viability depending on the applied loading condition. We were able to maintain the native properties of the IVD's with low dynamic loading. However, higher loads already induce pathological changes within 14 days of culture. Loss of water, GAG and collagen may happen over time and delay might be due to slow turn-over of ECM proteins.

Acknowledgements: This study was supported by ZonMw grant number 11400090

Unstable Callus-Distraction caused also Growthplate-like Cartilage Islands in Sheep Mandibles

H.Plenk Jr[1] F.Kanz[1] W.Mayr[2] O.Ploder[3]

[1]Bone Biomater. Res., Inst.Histol.Embryol., Med.Univ.Vienna, A, [2]Center Med.Physics Biomed.Engin., Med.Univ.Vienna, A, [3] Dept.Oro-Maxillo-Facial Surg., LKH Feldkirch, A

INTRODUCTION: In many surgical disciplines distraction osteogenesis or callus distraction has become a widely accepted method for long bone lengthening and axis correction [1], as well as for bone regeneration in defects without using bone substitute materials. The aim of a foregoing study [2] was to lengthen the sheep mandible with a fully buried device and to quantitatively analyze the tissue regenerate in the distraction gap. Employing two- and three-dimensional histomorphometry the presence and distribution of pseudarthroses could be demonstrated in the bony callus. For this presentation the cartilage islands were re-evaluated for growthplate-like structures and their 3D-orientation.

METHODS: A custom-made internal device for continuous distraction with a cable-connected external power and control unit was developed (W.M.). In five adult sheep the external device was fixed buccally with three bicortical screws on either side of an osteotomy anterior to the premolar region of the mandible. After a 5-day latency period, distraction was activated every 2 hours and advanced at a rate of 1.01 mm per day for 2 to 17 days. Bone healing was followed by radiographic imaging and computed tomography. After a 6-weeks consolidation period, the harvested mandibles were plastic-embedded without decalcification, and serially ground sectioned for histo-morphometric analysis and 3D-reconstruction.

RESULTS: The resulting distraction gap distances ranged from 1.7 to 17.1 mm (mean, 0.95 mm/day), see Fig.1. Histologic examination demonstrated secondary bone healing with predominantely membranous bone formation in the periosteal callus, bridging the distraction gap mainly on the lingual side (Fig.2). Where the bridging appeared incomplete some of the cartilage remnants not only showed chondral bone formation, but also structures resembling growth plates (Fig.3).

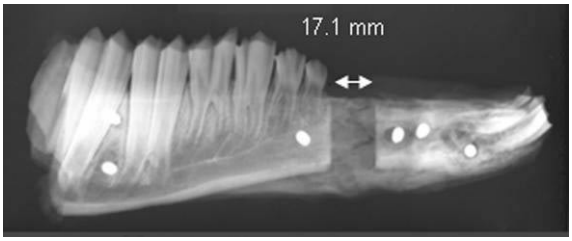

Fig.1: Specimen radiograph. Pseudarthroses in the callus bridging the 17.1 mm distraction gap

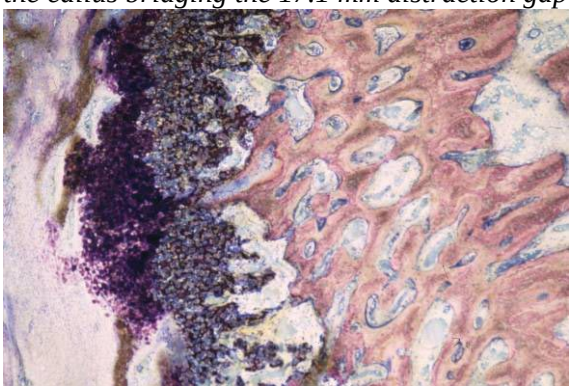

Fig.2: Giemsa surface-stained ground section. Growthplate-structure in the cartilage island.

DISCUSSION & CONCLUSIONS: Callus distraction is as susceptible to unstable osteo-synthetic devices as fracture healing, both resulting in pseudarthroses. However, under distractive forces cartilage remnants in the bony callus seem to exhibit growthplate-like structure with active chondral ossification in direction of distraction. Similar observations were made previously in the pseudarthroses of a dorsal spondylodesis-model with rapidly growing pigs [3]. Apparently, biomechanical influences play an important role for tissue differentiation during bone regeneration and skeletal tissue engineering.

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Repair of Osteochondral Defects with recombinant Human Type II Collagen Gel and Autologous Chondrocytes in a Rabbit Model

H. Pulkkinen[1], V. Tiitu[1], P. Valonen[1], T.S. Silvast[2], J.S. Jurvelin[2], J. Töyräs[2], I. Kiviranta[3], M.J. Lammi[4]

[1]Department of Biomedicine, University of Eastern Finland, Kuopio, FI, [2]Department of Physics and Mathematics, University of Eastern Finland, Kuopio, F, [3]Department of Orthopaedics and Traumatology, University of Helsinki, Helsinki, FI, [4]Department of Biosciences, University of Eastern Finland, Kuopio, FI

INTRODUCTION: Cartilage is a tissue, which has a limited capacity to regenerate spontaneously[1]. Autologous chondrocyte implantations have shown a promise as a procedure to repair cartilage, but it suffers from long recovery times, due to slow filling of the repaired lesion site. Biomaterials may enable the faster filling and recovery of the injured cartilage. Our previous studies showed that recombinant human type II collagen gel was a suitable scaffold for long-term chondrocyte cultivation.[2]

METHODS: In this study, osteochondral lesions in the rabbit knee were treated with recombinant human type II collagen (rhCII) gel seeded with autologous chondrocytes to test the usability of this novel biomaterial in cartilage repair. Cartilage pieces were surgically collected (nonweight-bearing femoral trochlea from contralateral joints), autologous chondrocytes were then enzymatically digested and expanded. rhCII-gels containing $4x10^6$ chondrocytes were cultivated 4 weeks in vitro in prior the next surgical operations, where osteochondral lesions (4 mm in diameter, 3 mm in depth) were created and three groups of samples were collected: 1) the defects filled with pre-cultivated rhCII-gels with autologous chondrocytes, 2) defects left untreated, and 3) Intact tissue from the contralateral femurs (n=13) was used as a reference.

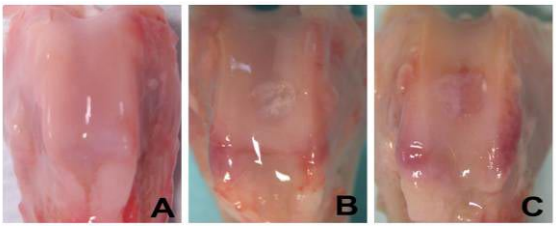

Figure 1. Photographs of rabbit femur: A) intact tissue B) rhCII-gel repair, and C) spontaneous repair, 6 months after operations.

RESULTS: The rhCII gel improved significantly the mechanical stiffness of the neotissue (dynamic modulus 3.9±2.2, 3.7±1.6 and 2.2±0.8 MPa for intact, rhCII repaired and spontaneous repair, respectively). The histological analysis showed no significant differences between the repairs. Both of the repair groups lacked smooth, normal looking cartilage surface and some superficial fibrillation was noticed. The rhCII-gel and spontaneous repair tissue exhibited high proteoglycan content and type II collagen production, but incomplete integration to the adjacent tissue. The microCT-analysis from the underlying bone revealed no major changes in the bone tissue under the lesions.

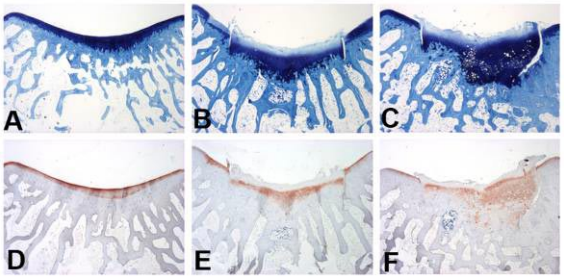

Figure 2. Histological evaluation of the samples: upper row: Toluidine blue, lower row: type II collagen immunohistochemical staining. A and D: intact; B and E: rhCII repair; C and F: spontaneous repair. The poor integration of the repair tissue is revealed in both of the repair groups (rhCII-gel and spontaneous repair).

DISCUSSION & CONCLUSIONS: The present results suggest that the use of recombinant human type II collagen (rhCII) gel seeded with autologous chondrocytes can accelerate the mechanical maturation of the repair tissue, as compared to spontaneous repair.

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Five percent oxygen tension is not beneficial for the neocartilage formation in scaffold-free primary chondrocyte cultures

Chengjuan Qu, Heli Lindeberg, Mikko J. Lammi
Department of Biosciences, University of Eastern Finland, Kuopio, Finland

INTRODUCTION: Articular cartilage is an avascular tissue that lives at low oxygen (O_2) environment *in vivo*. The low level of O_2 in cartilage makes chondrocyte metabolism highly dependent on carbohydrate utilization. Chondrocytes synthesize a large number of proteoglycans (PGs). Our previous study indicated that 5% O_2 increased PGs and type II collagen expression in monolayer cultures of primary chondrocytes (1). In this study, we investigated whether 5% O_2 could be beneficial for the neocartilage formation when primary chondrocytes were cultured in collagen II-coated transwell insert.

METHODS: Primary chondrocytes isolated from articular cartilage of bovine femoral condyles were seeded into insert at the cell density of $6x10^6$, and cultured in DMEM supplemented with 10% FBS and antibiotics (control), or glucosamine sulphate (GS) or hyaluronan (HA) at 5% or 20% O_2 atmosphere for 2, 4, and 6 weeks. The samples were then collected for histological staining of PGs and type II collagen, qRT-PCR of aggrecan and procollagen α_1(II) mRNA expressions, and DMMB assay of PG measurement.

RESULTS: Neocartilage produced at 20% O_2 appeared larger than at 5% O_2 (Fig. 1a), and it was bigger and more homogenous in GS-treated culture than in control or HA-treated culture at 20% O_2 (Fig. 1b). Histological staining showed that more PGs, type II collagen and better native cartilage structure was produced at 20% O_2 (Fig. 2b) compared to 5% O_2. The thickness of the neocartilage increased following the culture period (Fig. 2a). Quantitative RT-PCR showed that aggrecan and procollagen α_1(II) mRNA expressions were significantly higher at 20% O_2 than at 5% O_2. Proteoglycan content increased at 20% O_2. However, no significant difference in gene expression and PG content found between control and GS- or HA-treated culture either at 20% or 5% O_2.

DISCUSSION & CONCLUSIONS: In contrast to 5% O_2 in monolayer cultures (1),

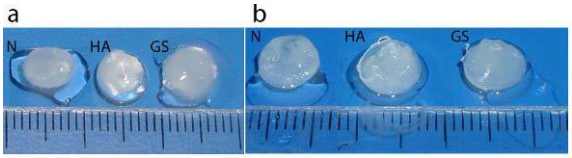

Fig. 1: Neocartilage formed from bovine primary chondrocytes cultured in insert for 6 weeks at 5% (a) or 20% O_2 (b) tension. N: control; HA: hyaluronan-treated; GS: glucosamine sulphate-treated.

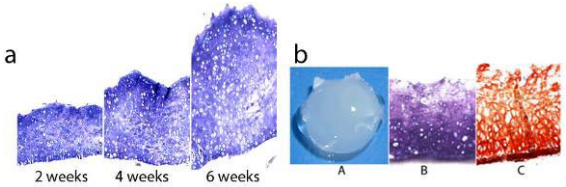

Fig .2: Bovine primary chondrocytes cultured in insert for 2, 4 or 6 weeks (a) at 20% O_2. (b) A: macroscopic appearance of neocartilage; B: Toluidine blue staining; C: Type II collagen staining.

low oxygen did not favour the PG synthesis in 3D scaffold-free culture. Glucosamine/sulphate and HA, a glycosaminoglycan present in cartilage and synovial fluid, have been used to treat osteoarthritic patients as nutraceutical or drug. It has been reported that GS stimulated aggrecan mRNA and protein levels in human OA chondrocytes (2), although reports showing no effect on chondrocytes have been published, too (3). Intra-articular HA increased cartilage breakdown biomarker in OA patients (4). Chondrocytes cultured in insert could form a hyaline cartilage with well-defined cartilaginous zones at 20% O_2 (5). We conclude that, in contrast to monolayer cultures, 5% O_2 was not beneficial for the extracellular matrix production in scaffold-free cell cultures, neither did GS or HA.

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The Effect of Different Culture Environments on Chondrocyte Proliferation, Differentiation and Extracellular Matrix Production

C.M.Rogers[1], T.S.Woolley[2], S.C.Cruwys[2], L.D.K.Buttery[1], F.R.A.J.Rose[1], K.M.Shakesheff[1]

[1]*University of Nottingham, Nottingham, NG7 2RD, UK,* [2] *AstraZeneca, Loughborough, LE11 5RH, UK*

INTRODUCTION: Cartilage defects result in pain and disability for millions of people worldwide. Osteoarthritis (OA) can result from such a defect. Currently there is no cure for this debilitating joint disease [1]. Tissue engineering (TE) has been recognised as a promising technique that may lead to the development of successful *in vitro* models to simulate defective tissues. The ultimate aim of this study is to grow cartilage *in vitro* to mimic the histology and anatomy of healthy rat tissues. Initial studies investigated the potential of different cell sources and culture environments to promote maintenance of phenotypic stability and enhance subsequent extracellular matrix (ECM) deposition within TE scaffolds.

METHODS: Primary rodent foetal and neonatal chondrocytes were isolated and expanded for 10 passages under normoxia or hypoxia (5% O_2). Phenotypic stability of the cells was assessed by determining the maintenance of differentiation markers by reverse-transcriptase polymer chain reaction (RT-PCR). Foetal chondrocytes (passage 4) were seeded ($4x10^5$ cells/ml) on fibrous non-woven poly (lactic-co-glycolyic acid) scaffolds and cultured statically or dynamically in a rotary cell culture system (RCCS™, Cellon) under normoxia and hypoxia for 8 weeks. Cell differentiation and ECM production was assessed using PCR and confocal microscopy.

RESULTS: Cells from rodent foetal limbs expressed chondrogenic markers for at least 10 passages under both normoxia and hypoxia. Expression of these genes in neonatal chondrocytes was lost after 8 passages under normoxia (fig 1). Chondrocytes seeded into scaffolds cultured under normoxia and hypoxia had similar cell proliferation rates over the 8 weeks for both dynamic and static conditions. Expression of collagen type II varied depending on culture environment. Production was lower under hypoxia and was limited to the periphery of scaffolds cultured statically (fig 2 A B and C). High expression was seen throughout scaffolds cultured under normoxia in the RCCS (fig 2 D).

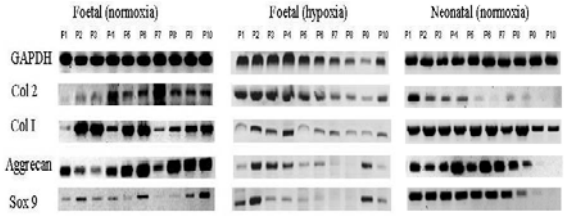

Fig. 1 Gene expression profiles of rodent chondrocytes.

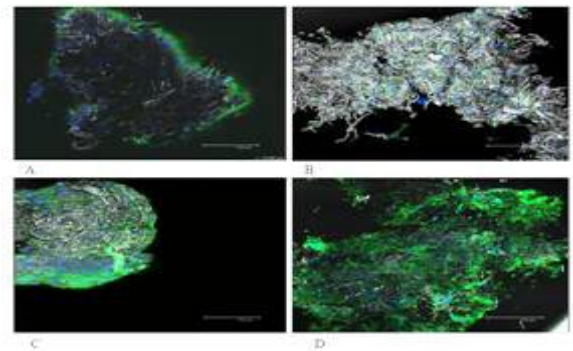

Fig 2 Confocal images of scaffolds at week 8. Cell distribution (DAPI) and collagen type II (FITC) production under (A) Hypoxia Static (B) Hypoxia RCCS (C) Normoxia Static and (D) Normoxia RCCS culture.

DISCUSSION & CONCLUSIONS: Cells from rodent foetal limbs remained in a differentiation state for a higher number of passages than neonatal chondrocytes. Culture of scaffolds under normoxia within a dynamic environment enhanced ECM production compared to static and hypoxic conditions. Future work is now directed at long-term culture within the RCCS to generate a tissue construct suitable to progress into further studies to simulate OA.

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ACKNOWLEDGEMENTS: Many thanks to the EPSRC and AstraZeneca for funding.

Modulating the Materials Properties of Poly(HEMA-co-MMA) Materials and their Potential Use as Meniscal Substitutes.

J. Sague[1] S. Honold[1] Y. Loosli[1] J. Vogt[2] R. Luginbuehl[1]

[1]RMS Foundation, Bettlach, Switzerland [2] PolyPhysConsult, Flüh, Switzerland

INTRODUCTION: Hydrogel materials found a prominent place in modern orthopaedic applications based on their biocompatibility and simple way of modulating their chemical-physical properties [1]. Most hydrogels used as meniscus substitutes are composed of a uniform polymeric body [2-4]. In this work we show that by creating a heterogeneous polymeric body, suitable mechanical compliant hydrogels can be used as synthetic materials in meniscus substitution.

METHODS: Polymeric samples were prepared after degasing of the formulation by reacting 2-hydroxyethyl methacrylate (HEMA), methyl methacrylate (MMA), deionized/distilled water (10% w/w) and 2,2′-Azobis(2-methylpropionitrile) (AIBN, 0.01 mol%) as reaction initiator overnight at 70°C. Polymerized samples were cut in cylinders (10mm high, 12mm diameter) for equilibrium water content (EWC) and mechanical characterization. Tribological testing was performed with an in-house-built pin-on-plate instrument. Hemispherical polymer samples were used as pin and a highly polished CoCrMo plate served as sliding plate. All tests were performed in PBS solution. The unconfined creep modulus (E_c) was determined by mechanical indentation of the hydrogel samples using a Zwicki Z5.0 (Zwick-Roell, Ulm, Germany) [3]. Contact angles were measured in a SURFTENS-Universal contact angle meter.

RESULTS: The EWC decreased linearly with increasing MMA content (R^2=0.99) while the E_c value showed a bi-modal relationship. Samples with less than 35 mol% MMA had a very low E_c whereas at higher MMA content the E_c increased dramatically (Fig 1a. slope of ln E_c vs MMA=0.02 for 10 to 35 mol% MMA and ln E_c vs MMA=25 for 35 to 50 mol% MMA, respectively). The apparent dynamic friction (μ) was very low (μ=0.02) for samples containing less than 20 mol% MMA. The friction increased to a maximum of μ=0.25 for 45 mol% MMA. In the polymer prepared with higher MMA concentration (50 mol%), μ decreased to 0.17 which is close to the values obtained for PMMA (Fig 1b).

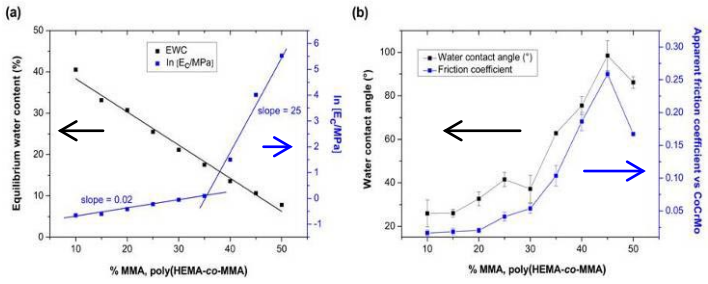

Fig. 1: Influence of MMA concentration in poly(HEMA-co-MMA) materials: a) unconfined creep modulus E_c, and b) the friction force coefficient, μ, and contact angle.

DISCUSSION & CONCLUSIONS: It was found, as expected, that the EWC and E_c can be modulated with the MMA content. The lower surface polarity at higher MMA concentration interfered with aqueous lubrication which resulted in higher friction. Samples with 50 mol% MMA content possess tribological properties similar to PMMA. On a structural point of view, HEMA–*co*–MMA polymers cover a broad range of E_c allowing to reach mechanical and tribological properties similar to those of human menisci.

The tribological and mechanical performance of these biomaterials can be further optimized by creating Interpenetrated (IPN) or Semi-Interpenetrated Polymeric Networks (SIPN) with other hydrogels.

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A Physiologic Robot Reactor System to Simulate *In Vivo* Conditions

V. Schmid[1], R. J. Egli[2], R. Luginbühl[2*], J. Justiz[1]

[1] *Bern University of Applied Sciences, Biel, CH,* [2] *RMS Foundation, Bettlach, CH*

INTRODUCTION: Success in cartilage tissue engineering and cartilage repair strategies depends on the formation of a hyaline like cartilage tissue. The interaction of seeded cells with a surrounding scaffold and biochemical triggers is of utmost importance to induce or maintain cells in a chondrocytic phenotype. Any significant *in vitro* evaluation of cell/scaffold constructs has to be performed under the harsh conditions encountered *in vivo* within synovial joints. Therefore, many different bioreactor systems have been developed with the aim to simulate these conditions. However, two main shortcomings have been identified in these bioreactor systems: (i) the mechanical stimulation units do not operate within a physiological stress range and they are limited in the applicable motion pattern; (ii) most systems lack an ambient control and therefore no hypoxic environment is generated as encountered in synovial joints. We have addressed these shortcomings by designing a fully autonomic modular Physiologic Robot Reactor System (PPRS).

METHODS: We have engineered an autonomous reactor system that comprises a mechanical stimulation unit (MSU), an automatic sample changer (ASC), and a glove box for tight environmental control (ECB). The MSU is designed with three linear (orthogonal axes) and two rotational degrees of freedom (around x- and z-axis) capable of simulating physiological motions under physiological loads (Fig. 1). Highly complex motion patterns, e.g. of the knee-joint, can be closely simulated by individual control of each of the axis. The load generated by the MSU is transferred via an exchangeable anatomically formed plunger on the sample tissue. Highly accurate force-feedback and motion systems are controlled by ultra-fast FPGA and real-time components which continuously monitor all system-parameters. Sample containers will be placed on a carrousel, which allows for individual piloting of the containers with their own stimulation pattern. The ASC and the MSU will be integrated in a sterile ECB in which humidity, temperature, gas-mixture (O_2, CO_2), and pressure are actively controlled.

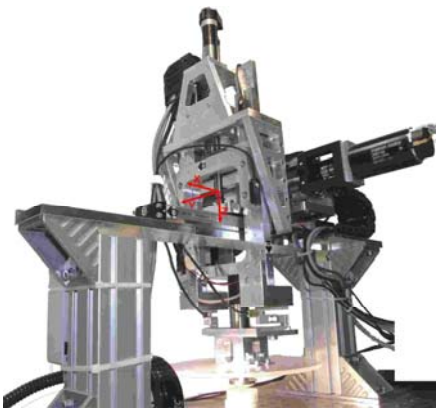

Figure 2: MSU test setup

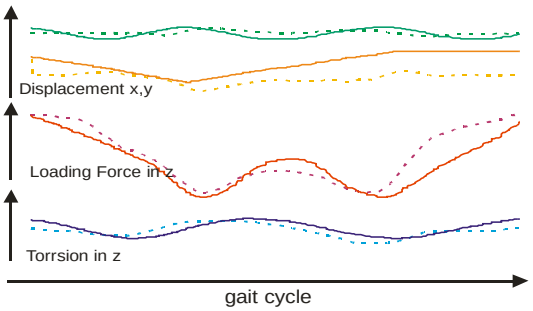

Figure 2: Simulated with MSU (solid) and measured (dashed)[1] motion pattern of a knee

RESULTS and DISCUSSION: First stimulation tests utilizing the three linear and one rotational axes were carried out. Different benchmark tests and realistic motion patterns were executed. The physiological motion and load pattern of a knee joint were simulated by simple sinusoidal and linear motions (Fig. 2). Loading forces of up to 500 N in longitudinal and 100 N in lateral direction may be achieved reflecting the physiological forces encountered in the knee.

The MSU and ASC are currently integrated in the ECB. In addition, an automated media exchange is also implemented which will enable the prolonged uninterrupted cultivation of cell/scaffold constructs. The PRRS provides a convenient and flexible tool for screening and evaluation of the most promising materials and scaffold designs for tissue engineering of articular cartilage.

Reference: [1] www.orthoload.com

The role of mechanical load on the chondrogenesis of human bone derived mesenchymal stem cells in fibrin-polyurethane scaffolds

Stoddart MJ., Li Z., Eglin D., Alini M.

AO Research Institute, Davos, Switzerland.

INTRODUCTION: The differentiation of stem cells into chondrocytes is highly dependant on the signals the cells receive. TGFβ is used to induce chondrogenesis and yet this would not be supplemented within an articular defect. We have developed a biodegradable polyurethane-fibrin scaffold system which has been shown to be highly favorable for chondrogenesis under classical chondrogenic stimuli (TGFβ containing medium). The aim of this study was to determine the effectiveness of this scaffold composite in supporting chondrogenesis in the absence of an exogenous TGFβ signal, but under the influence of a loading regime similar to that which might be experienced during a patient rehabilitation protocol.

METHODS: The scaffolds were prepared by a salt leaching-phase inverse technique consisting of the mixing in equal weight of a porogen (sodium phosphate heptahydrate dibasic salt, particles size range from 90 to 300 μm) with a solution containing a mixture of solvents and the polyurethane synthesized from hexamethylene diisocyanate, poly(epsilon-caprolactone) diol and 1,4:3,6-dianhydro-D-sorbitol in a one step solution polycondensation reaction. P3 hMSCs were suspended in fibrin and seeded at a cell density of $5x10^6$ per polyurethane scaffold. All groups were cultured in medium consisting of DMEM, ITS, Pen/Strep, ascorbate-2-phosphate, 5 μM ε-amino-caproic acid, and 10^{-7} M dexamethasone. 0 ng/ml, 1 ng/ml, or 10 ng/ml recombinant human TGF-β1 was added into the medium of 3 groups respectively. Load was applied using our previously described bioreactor system[1] with ball oscillation of ±25° at 1 Hz and dynamic compression 1 Hz with 10% sinusoidal strain, superimposed on a 10% static offset strain. Mechanical loading was performed 1h a day over 7 consecutive days. DNA content was measured spectrofluorometrically using Hoechst 33258. The amount of GAG in the scaffolds and medium was determined by the dimethylmethylene blue dye method. mRNA expression of collagens type-I (COL1), type-II (COL2), type-X (COL10), aggrecan (AGG), proteoglycan4 (PRG4), osterix (Sp7), transforming growth factors -β1 (TGFB1), and β3 (TGFB3) were investigated and compared to 18S ribosomal RNA as the endogenous control.

RESULTS: Total GAG synthesized was normalized to DNA content. The control samples cultured in medium with 1 ng/ml ($P<0.01$) or 10 ng/ml TGF-β1 ($P<0.001$) had significantly higher GAG/DNA value compared to samples cultured in medium without TGF-β1. In all the 3 groups where samples were cultured in medium with different concentrations of TGF-β1, the total GAG/DNA value showed a trend of up-regulation by mechanical load, this difference was significant in the groups with 0 ng/ml or 10 ng/ml TGF-β1.

As expected, the addition of TGF-β1 led to an increase in chondrogenesis in a dose dependant manner. By day 14, in the absence of load, addition of 1 ng/ml TGF-β1 increased the COL2 ($P=0.004$), AGG ($P=0.002$), COL10 ($P=0.002$) and Sp7 ($P=0.004$) gene expression compared to cells cultured in the absence of TGF-β1. This increase was greater when 10 ng/ml TGF-β1 was added to the medium

When investigating the effect of load on chondrogenesis increasing concentrations of TGF-β1 lead to a diminished response. The greatest response to load was seen in the groups without TGF-β1. When hMSCs were cultured in medium without TGF-β1, mechanical load significantly stimulated gene expression of COL2 ($P=0.018$), AGG ($P=0.004$), COL10 ($P=0.004$) and Sp7 ($P=0.006$). This suggests that under natural in vivo conditions mechanical load would be required to fully realise the chondrogenic potential of stem cells within this scaffold system.

DISCUSSION & CONCLUSIONS: This study demonstrates that the scaffold composite described is able to support chondrogenesis. The requirement for TGF-β1 in the medium can be removed when sufficient mechanical stimulation is applied. This study also shows that to more accurately determine the in-vivo response of a cell-biomaterial implant all stimuli, including mechanical load, must be considered.

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ACKNOWLEDGEMENTS: Supported by Swiss National Fund (SNF 320000-116846/1).

Rapid Prototyping in Cartilage Tissue Engineering

J. Walser[1], M.D. Caversaccio[2], S.J. Ferguson[1]

[1]*Universität Bern, Institute for Surgical Technology and Biomechanics, Bern, CH,*[2]*Universität Bern, Universitätsklinik für HNO, Kopf- und Halschirurgie, Inselspital, Bern, CH*

INTRODUCTION: The successful outcome of reconstructive procedures in Oto-Rhino-Laryngology (ORL) is defined not only by the functional properties of the reconstructed structure, but also by aesthetic aspects that have to be considered when planning and executing the surgery.[1] The potential for tissue-engineered (TE) constructs for ORL applications has already been demonstrated in a number of studies.[2] Autologous cartilage grafts are a preferable tissue for reconstructive surgery, but donor site morbidity remains a concern. Furthermore, insufficient volume of tissue often limits a successful reconstruction.[3] Available scaffolds are not patient specific and a lack of stability and rejection of engineered tissue grafts after implantation remains a problem. In order to assess the feasibility of using a plaster/PMMA-based rapid prototyping (RP) machine within the chain of patient-specific cartilage scaffold production, a cell culture pilot study was performed.

METHODS: The 3D geometry of a miniature ear (length 12mm) was generated, printed with a ZCorporation Spectrum Z510 RP machine and superficially polished to remove printing debris. Silicone was cast into the printed form to obtain the negative ear shape. Calf articular cartilage was harvested and digested. The released chondrocytes were then cast with 1.5% agarose into the prepared silicone form, at a cell-seeding concentration of $4x10^6$ cells/ml. 48 specimens with an average volume of 90µl were produced and cultured for 3 weeks in DMEM/F12, 10% FCS, Vit. C. 3 specimens were taken for each sampling time at days 1, 7, 14 and 21 for the analysis of total DNA content and GAG accumulation.

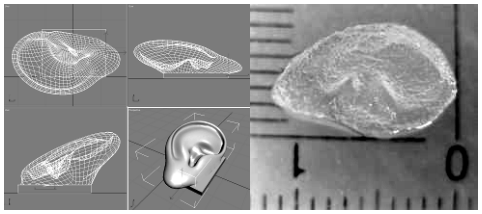

Fig. 1: generated miniature ear (left), cartilage ear after 21 days in culture, scale [1cm] (right)

RESULTS: The metabolic response of the tissue engineered constructs shows a strong increase in cell proliferation (DNA content), ECM (GAG) production and relative cell activity. The TE constructs maintained their initial shape given by the casting process during the whole culture period.

Table 1: DNA and GAG content of produced constructs.

	DNA [ng/mg]	GAG [ng/mg]
day 1	41.9	340.9
day 7	51.2	1362.9
day 14	119.3	3680.1
day 21	195.3	6625.9

Fig. 3: Relative cell activity (GAG production normalized to total DNA)

DISCUSSION & CONCLUSIONS:
Cell viability over an extended period was demonstrated. The feasibility of using the described RP machine for producing complex scaffold shapes is highlighted by rapid cell proliferation and ECM production in the RP TE constructs.

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ACKNOWLEDGEMENTS: This study is part of the NCCR Co-Me project "Cartilage Tissue Engineering for ORL Applications" and funded by the Swiss National Science Foundation. Technical assistance of L. Ettinger is gratefully acknowledged.

Impact of exposing Nucleus Pulposus Cells to Direct Current

PM. Welz[1], K. Ito[2], SJ. Ferguson[1]

[1] *Institute for Surgical Technology and Biomechanics, University of Bern, Bern, Switzerland,*

[2] *Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands*

INTRODUCTION: Major disturbances in intradiscal streaming potentials are believed to be involved in degenerative disc disease (DDD). It is known that electrical fields (*E*) have the potential to support chondrocyte proliferation and ECM gene expression by intervention in cellular signal transduction pathways [1]. Though clinically applied to the spine [2], knowledge regarding the impact of currents and *E* on the cellular level is only rudimentary. The aim of this study was to explore whether morphology and viability of bovine nucleus pulposus (NP) cells in culture can be influenced by the exposure to direct currents.

METHODS: Bovine NP cells were seeded at 5,000 cells/cm^2 into 6-well plates and cultured in DMEM/F12 supplemented with 1% penicillin/streptomycin, 25 µg/mL ascorbic acid, and 10% fetal calf serum for 5 days at 5% CO_2/37°C. At days 2, 3, and 4, currents were applied via carbon electrodes (C-Dish™, IonOptix) to the culture for 30 min. Cells were either exposed to constant current (20 µA, DC), monopolar pulses (400 µA, 49 ms, 20 Hz), or bipolar pulses (200 µA, 1 ms, 20 Hz). The field potential in each well was continuously monitored. While cell morphology was assessed daily by inverted light microscopy, cell viability was qualitatively determined by a fluorescence-based LIVE/DEAD assay on day 5.

RESULTS: During the 30 min of electrical stimulation (ES), all signals generated continuously increasing *E*. The greatest slopes were observed for DC (178%). Monopolar and bipolar pulses generated only minor increases in *E* (12 and 20%, respectively). Peak *E* of 240, 710, and 18 mV/cm were achieved for DC, monopolar, and bipolar signals, respectively. Monopolar pulses provoked successive cellular contraction and detachment starting from day 3 (Fig. 1B). While at this time point, cells were still viable as proven by Trypan Blue (not shown), these cells were dead or severely damaged on day 5 (Fig. 1F). Cells stimulated with bipolar pulses or DC maintained their initial morphology and viability for the entire culture time. No obvious differences were found versus the control (Figs. 1A, C-E, G-H).

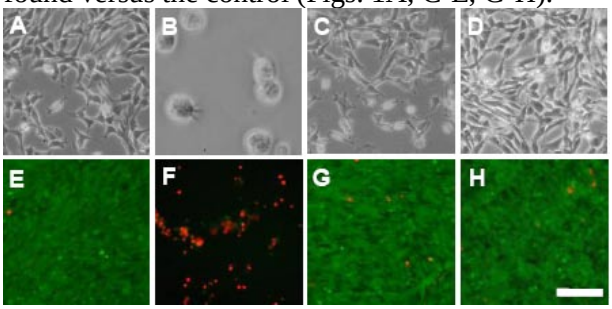

Fig. 1: Comparison of morphology (A-D: day 3) and viability (E-H: day 5) of NP cells after ES. A+E: DC, B+F: monopolar pulses, C+G: bipolar pulses, D+H: Control. Scale bar = 50 µm in B; 100 µm in A, C-H.

DISCUSSION & CONCLUSIONS: Contrary to DC signals, monopolar and bipolar pulses caused only minor polarization effects, limiting the accumulation of chemical compounds on the electrodes. For bovine NP cells, viability windows of 20-200 µA and 18-240 mV/cm were identified. *E* generated by bipolar pulses were in the same range as those promoting the upregulation of ECM genes in bovine articular chondrocytes [1]. As no obvious pH changes upon ES were observed, cell damage was possibly caused by intracellular disturbances generated by current density and/or *E*. The impact on gene expression and on protein level will be explored in future studies.

The present study reveals that directly applied currents and electrical fields can influence cell morphology and viability. The final aim is to explore the potential of electrical stimulation to maintain or restore ECM integrity and thus provide a possible therapy approach for the treatment of DDD.

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ACKNOWLEDGEMENTS: Funded by AOSpine (Grand No. AOSBRC 07-04).

The effects of physiological compression and strain on remodelling of the cytoskeleton and extracellular matrix in bovine intervertebral disc cells

S Li, EJ Blain, VC Duance
Connective Tissue Biology Laboratories, School of Biosciences, Cardiff University, Museum Avenue, Cardiff, CF10 3AX

Introduction: Low back pain is a major public health problem in our society. Degeneration of intervertebral disc (IVD) appears to be the leading cause of chronic low-back pain[1]. Mechanical stimulations including compressive and tensional forces are directly implicated in IVD degeneration. Several studies have implicated the cytoskeleton in mechanotransduction[2,3], which is important for communication and transport between the cells and extracellular matrix (ECM). However, the potential roles of the cytoskeletal elements in the mechanotransduction pathways in IVD are largely unknown.

Methods: Outer annulus fibrosus (OAF) and nucleus pulposus (NP) cells from skeletally mature bovine IVD were either seeded onto Flexcell® type I collagen coated plates or seeded in 3% agarose gels, respectively. OAF cells were subjected to cyclic tensile strain (10%, 1Hz) and NP cells to cyclic compressive strain (10%, 1Hz) for 60 minutes. Post-loading, cells were processed for immunofluorescence microscopy and RNA extracted for quantitative PCR analysis.

Results: F-actin reorganisation was evident in OAF and NP cells subjected to tensile and compressive strain respectively and is likely due to load-induced differential mRNA expression of actin-binding proteins. The vimentin network was also more intricately organised in loaded NP cells. Compressive strain increased type II collagen and aggrecan transcription in NP cells, whereas levels decreased in OAF cells under tension. mRNA levels of ECM-degrading enzymes were significantly reduced in both cell populations after loading.

Conclusion: Tensile and compressive strains induce different mechano-responses in the organisation and expression of cytoskeletal elements and on markers of IVD metabolism. Differential mechano-regulation of anabolic and catabolic ECM components in the OAF and NP populations reflects their respective mechanical environments *in situ*.

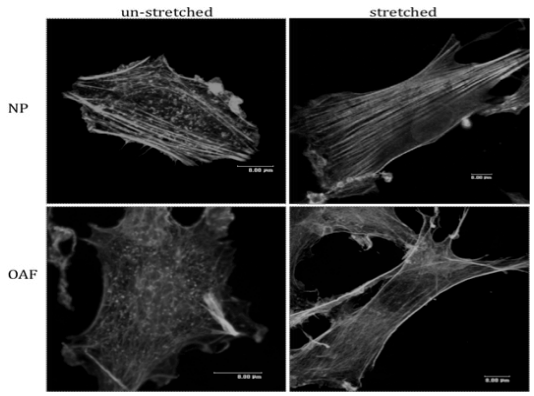

Figure 1: The effects of stretching on actin cytoskeleton of intervertebral disc cells. F-actin organization in nucleus pulposus (NP) and outer annulus fibrosus (OAF) cells before and after 10% stretching for 60 minutes. F-actin is punctate or fibre-like in both unstretched NP and OAF cells. However, the punctate-like staining of F-actin decreased or disappeared, with more fibre-like F-actin extended to the cell processes. There were no striking differences with cell types. Cells were visualized using Alexa-488™-phalloidin in conjunction with scanning laser confocal microscopy.

Reference:

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