Cell Therapy in Intervertebral Disc Degeneration

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INTRODUCTION: Cell Therapy is one of several approaches to influence the age-related progression of disc degeneration. Other approaches include the use of growth factors and cytokines sometimes referred to as molecular therapy while gene therapy provides yet another approach. Successful transplantation of cells into the intervertebral disc in animals has progressed to ongoing human clinical trials.

CURRENT STRATEGIES: With increasing age and degeneration the number and viability of intervertebral disc cells decrease particularly in the nucleus pulposus. Recent studies indicate that many cells in degenerative discs are in a senescent state. To address this researchers have tried to activate endogenous stem cells. Other research groups have transplanted cells into the intervertebral disc. Cells of different origins have been used primarily stem cells, but cartilage cells and other cells have been used as well. Transplantation experiments have been successfully performed in animal models. More recently stem cells have been transplanted into the human intervertebral disc.

MAJOR ROADBLOCKS: Transplantation of stem cells while successful in early stages of the degenerative process may not be successful at the stage when the degenerative process has accelerated. The disc hostile environment that resulted in senescence of existing cells in the first place is still present once the degenerative process reaches a more advanced staged and may prevent successful transplantation results.

Cells can differentiate in unintended directions and the development of tumors is a potential risk.

The fact that the cells have to be injected makes the cell therapy less attractive.

DISCUSSION & CONCLUSIONS: Cell therapy may have a role in the treatment of patients with intervertebral disc degeneration. Clinical trials are needed to further explore this potentially interesting approach.

Technovolution of Cartilage Repair

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INTRODUCTION: Cartilage repair is both a mature clinical practise while at the same time a challenging part of research activities. The unmet medical need comes from the considerable number of young and active patients who seek our help for pain and joint dysfunction due to trauma and early cartilage degeneration after fracture, menisectomy, ligament damage and other joint pathology. An explosion of options developed after the breakthrough of regenerative medicine through autologous chondrocyte implantation has created both opportunity for enterprise and progress as well as a need for regulation and quality assurance. The applicable concept of Technovolution encompasses both the use of technology to improve quality and length of life as well as the natural selection between technological advances.

How does this apply to current State of Art in research and clinical care for cartilage repair ?

CURRENT STRATEGIES: The use of marrow stimulation techniques has been the mainstay of treatment for many years. Microfracture treatment (MFX) creates a fibrocartilaginous scar tissue from bone marrow derived cells, surrounding cartilage, growth factors and interplay with joint homeostasis. MFX is considered by many as 'standard of care' and is frequently used in comparative clinical studies for regulatory purposes. Clinical results of MFX are good in properly selected, well informed patients. Surgical technique requires training, understanding of purpose and meticulous execution as well as intensive rehabilitation and patience from both patient and professionals. Improvement of MFX is sought in the application of various matrices to retain and improve the initial clot while steering proliferation, tissue morphology and defect biomechanics.

Cartilage Cell therapy (ACI) started with the first autologous chondrocyte implantation in Gothenburg in 1987 and is now a reliable, rigorously investigated example of orthopedic technovolution. Good clinical outcomes of ACI even at twenty year follow up has been published and after the explosion of cell therapy options regulatory requirements, natural selection between technologies has led to three (Carticell Sanofi/Genzyme, ChondroCelect TiGenix, MACI Sanofi/Genzyme) licensed/approved cartilage cell therapy options under FDA/EMA influence. Cost benefit ratio and uniform treatment algorithms need to be determined and will help us to define the ideal patient profile for successful regenerative rather than reparative technologies. Improvements are sought in arthroscopic delivery, biomaterials application and the investigation of one stage cell therapy solutions.

MAJOR ROADBLOCKS: Persistent shortcomings in our (basic)scientific understanding of intra articular biological aspects remains an important limiting factor. The complex multifactorial process of cartilage repair combined with the need for lasting solutions in active patients creates an important unmet medical need. Burden of proof is high and related costs almost unaffordable by academia. The very strict regulatory environment for cellular solutions compared to the liberal unleveled playing field in device oriented treatments is cause for concern and slows true disruptive innovation.

DISCUSSION & CONCLUSIONS: Cartilage repair is clinically successful and scientifically challenging. There is ample opportunity for teamwork between science, clinic and industry and lasting improvement will be accelerated by uniform treatment algorithms and international cartilage repair outcome documentation.

Bone repair and regeneration

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INTRODUCTION: An overview of cell therapy approaches currently being used in bone repair will be presented, outlining both the current practice and the major roadblocks hampering progress in this field.

STRATEGIES:

Cell therapy has been used for bone repair in spinal fusion, bone defects, avascular necrosis as well as early and late stages of fracture healing. This presentation will concentrate on cell therapy in the extremities. The bone repair scenarios are divided into categories: Normal fracture repair ((a) Direct and (b) Indirect), Delayed union, Hypertrophic non-union, Stiff Atrophic non-union, Mobile Atrophic non-union, Fracture at risk of non-union, segmental defect and healing in a poor host.

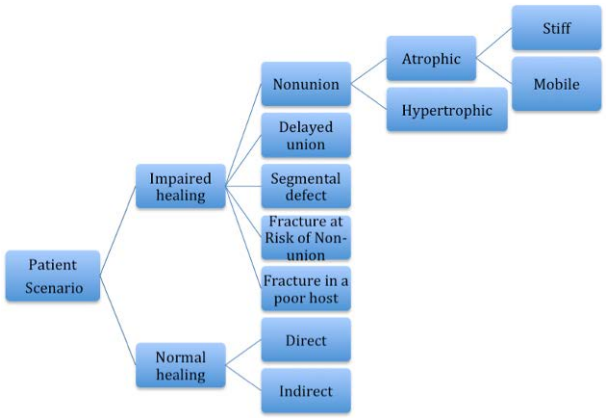

Fig. 1: Flow chart: Clinical Scenarios. The impaired healing scenarios can be complicated further by infection and the atrophic non-unions can also be complicated by a bone gap or loss of length.

In preclinical studies, it has been reported that an exogenous source of mesenchymal stem cells (MSCs) may improve fracture healing and bone regeneration[1-3], both by providing bone progenitor cells for regenerating bone and also potentially by providing trophic factors which may stimulate local progenitors to contribute to fracture healing.

In patients, bone marrow injection has been used. It contains a heterogeneous population of mononuclear cells, including MSCs. MSCs are capable of osteogenic differentiation and are involved in the normal fracture repair process. The first use of bone marrow aspirate was reported in a case of infected non-union of the tibia[4]. A subsequent prospective case series of 20 tibial non-unions had a 75% successful rate[5].

MAJOR ROADBLOCKS:

However, marrow injection is limited by volume of bone marrow aspirate that can be injected at the site. Therefore, concentrated bone marrow aspiration has been used to deliver more MSC with a lower overall volume. A success rate of approximately 90% (n=53) has been reported[6] .The number of colony forming units in those cases which failed to unite was significantly lower than in successful cases. It was concluded that the success rate of treatment was dependent on the number of MSCs. The cell therapy approaches can be divided into those with and without a scaffold. However, there is still no consensus concerning (a) the optimal type of cell or the timing of their implantation or (b) the role of a scaffold or the type of a scaffold.

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Canine notochordal cell-secreted factors protect murine and human nucleus pulposus cells from apoptosis by inhibition of activated caspases -9, and -3/7

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INTRODUCTION: Effective therapies that may stop or even reverse disc degeneration remain elusive. A minimally invasive method through which nucleus pulposus cell viability could be achieved would revolutionize the treatment of degenerative disc disease.

With the presented work, we have investigated if non chondrodystrophic (NCD) canine disc (IVD)-derived notochordal cell conditioned medium (NCCM) and chondrodystrophic (CD) canine IVD-derived conditioned medium (CDCM) are able to protect murine and human NP cells from apoptosis.

METHODS: We developed NCCM and CDCM from hypoxic culture of freshly isolated NPs from NCD and CD canines respectively. We obtained murine NP cells from 9 different C57BL/6 mice and human NP cells from 4 patients who underwent surgery for discectomy. The cells were cultured with aDMEM/F-12 (control media), NCCM or CDCM under hypoxic conditions (3.5% O2) and treated with IL-1ß+FasL or Etoposide. All media were supplemented with 2% fetal bovine serum. We then determined the expression of specific apoptotic pathways in the murine and human NP cells by recording activated caspase-8, and -9 and -3/7 activity.

RESULTS: *murine* - In the murine NP cells, NCCM inhibits IL-1ß+FasL- and Etoposide-mediated apoptosis via suppression of activated caspase-9 and caspase-3/7, CDCM demonstrated an inhibitory effect on IL-1ß+FasL mediated apoptosis via caspase-3/7 (Fig 1-A).

human - In the human NP cells, NCCM inhibits Etoposide- mediated apoptosis via suppression of activated caspase-8, caspase-9 and mainly caspase-3/7. CDCM demonstrated an inhibitory effect on Etoposide- mediated apoptosis via suppression of activated caspase-8, caspase-9 and mainly caspase-3/7, though not as effective as NCCM (Fig 1-B).

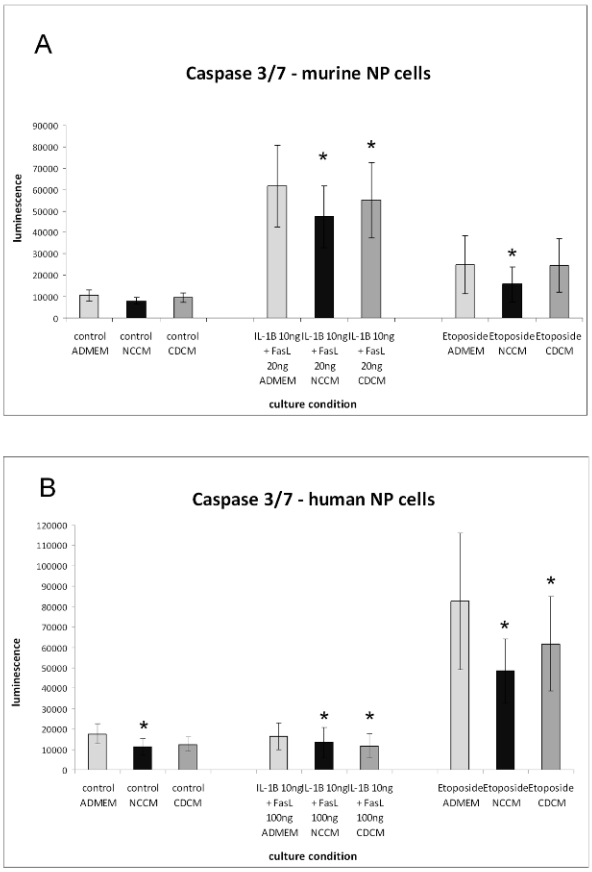

*Fig. 1: Assays for activated Caspase 3/7 for murine (A) and human (B) cells. * = significant ($p<0.01$) as compared to ADMEM in the same treatment group (control/ IL-1B+FasL/ Etoposide)*

DISCUSSION & CONCLUSIONS: Soluble factors secreted by the NCD IVD NP strongly protect murine and human NP cells from induced apoptosis via suppression of activated Caspase -9 and -3/-7.

A better understanding and harnessing of the restorative powers of the notochordal cell could lead to novel cellular and molecular strategies for the treatment of DDD.

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Mechanically induced chondrogenesis of mesenchymal stem cells can be improved by manipulating the location of cells within a tissue engineering scaffold

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INTRODUCTION: Mechanical load is increasingly being used to induce chondrogenesis in mesenchymal stem cells (MSCs). Work previously carried out by our group has demonstrated that complex multi axial load can be used to induce chondrogenesis in MSCs in the absence of exogenous growth factors through endogenous TGF-β signalling [1]. Further work demonstrated that it is the shear loading that is crucial for this chondrogenic induction [2]. This work aimed to improve chondrogenic induction by increasing the number of cells exposed to the shear component of loading. To do this a layer of MSCs was seeded onto the surface fibrin-polyurethane scaffolds.

METHODS: Different seeding patterns were used to determine whether asymmetrical seeding of cells can maximise the effects of the shear component of mechanical loading. For Group A, 4 million cells were seeded evenly into scaffolds as previously described [1]. In group B, 3.6 million MSCs were seeded into scaffolds and 400,000 MSCs were seeded onto the surface of the scaffold. For group C, 400,000 cells were seeded on otherwise acellular scaffolds. Half of the scaffolds were then exposed to multiaxial mechanical load and half were kept in free-swelling culture. Following seeding, scaffolds were cultured in a 'chondropermissive media' without any exogenous growth factors: serum-free DMEM High Glucose (4.5g/l), 1% penicillin-streptomycin, Ascorbic Acid (50μg/ml), Dexamethasone ($1x10^{-7}$M), 1% ITS+1, 1% NEAA and ε-aminocarproic acid (5μM).
Mechanical load (10-20% compression and of rotation of ±25°, both at a frequency of 1Hz) was applied for one hour a day, five days a week for the duration of loading.
After 15-20 rounds of load scaffolds were harvested for biochemical analysis by digestion in proteinase K (Roche). Histological samples were then fixed in 4% buffered formaldehyde before paraffin embedding or 100% methanol before cryosectioning. Sections were stained with Safranin O, or underwent immunohistochemical staining.

RESULTS: Biochemical analysis shows that the production of glycosaminoglycans (GAG) is increased by load in group A and B, but not group C. Histological analysis shows that the staining of scaffolds with Safranin O improves from group A to group B, but no staining is observed in group C. The same pattern of improvement is seen from group A to B with immunohistochemistry for the key cartilage components collagen type II and aggrecan. The media concentration of TGF-β when normalised to DNA content of the scaffolds is the same across all three groups.

DISCUSSION & CONCLUSIONS: Increasing the number of cells directly stimulated by the shear component of mechanical load by seeding a layer of cells on the top of a scaffold leads to an increase in GAG production and histological staining. This however only occurs if there are cells seeded within the scaffold itself, a layer of cells on top of the scaffold alone does not undergo increased chondrogenesis in response to load. The reason for this different response is likely to be due to the formation of signalling networks between cells of the surface layer (which are exposed to mechanical load) and those within the underlying scaffold, which then receive greater chondrogenic stimulus, and as a result deposit more matrix than cells in evenly seeded scaffolds.

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Optimizing cartilage extracellular matrix derived scaffolds to act as growth factor delivery platforms to promote chondrogenesis of mesenchymal stem cells

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INTRODUCTION: Cartilage ECM derived scaffolds have shown great promise for cartilage regeneration[1]. Combining MSCs and/or growth factors with such scaffolds represents a promising approach to cartilage regeneration, although the optimal scaffold characteristics to realise such aim remain to be elucidated. Challenges such as scaffold contraction and construct stability remain[2]. In this study we investigated the influence of ECM concentration and a number of different physical and chemical crosslinking methods on the migration and differentiation of human infrapatellar fat pad derived stem cells (FPSC) seeded onto ECM derived scaffolds. We then explored the use of these scaffolds as growth factor delivery systems to promote chondrogenesis of FPSCs.

METHODS Porcine articular cartilage was processed to produce slurries consisting of ECM particles. A freeze-drying procedure was used to produce scaffolds of different ECM concentrations (100–1000 mg/ml). Chondrogenesis of human FPSCs was compared on scaffolds that first underwent dehydrothermal (DHT), carbodiimide (EDAC) and/or genipin crosslinking. TGF-β3 was either first loaded into the scaffold or added to the media. Biochemical analysis was performed for glycosaminoglycan (sGAG) content. Histological sections were stained for sGAG (Alcian Blue) and collagen (Picro Sirius Red) and were immunochemically analysed for collagen type II.

RESULTS: Scaffold porosity increased as the concentration of the ECM slurry was decreased from 1000 to 100mg/ml. This led to greater cellular infiltration in scaffolds with a lower ECM concentration (data not shown). Homogenous sGAG accumulation was observed in scaffolds with an ECM concentration ≤ 250mg/ml (Fig. 1A). sGAG synthesis also increased as the ECM concentration of the scaffold decreased, however this was accompanied by an increase in scaffold contraction (for DHT only crosslinking). Contraction could be supressed by the use of additional EDAC or genipin crosslinking (Fig.1B). Genipin cross-linking was found to suppress chondrogenesis, but no suppression of chondrogenesis was observed with EDAC cross-linking for low ECM concentration scaffolds.

An ELISA analysis revealed that endogenous TGF-β3 which was loaded onto the scaffold was slowly released over 12 days of culture. Importantly, comparable levels of matrix accumulation were observed in scaffolds that were pre-loaded with TGF-β3 compared to those where TGF-β3 was added to the culture media (data not shown).

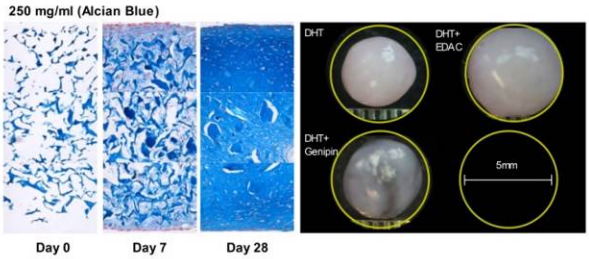

Fig. 1: (A) Left: Histology sGAG Alcian Blue staining for top, centre and bottom of the scaffold at day 0, day 7 and day 28 of culture. (B) Right: Scaffold contraction after 28 days of culture for DHT, DHT+EDAC and DHT+Genipin crosslinking.

DISCUSSION & CONCLUSIONS: The results of this study demonstrate that both the concentration of ECM in the scaffold and crosslinking mechanism regulate the migration and chondrogenic differentiation of human FPSCs. The finding that cartilage ECM derived scaffolds can be used as delivery systems for growth factors points to their use as off-the-shelf products for cartilage tissue regeneration.

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S100-based assays to determine differentiation status of human articular chondrocyte

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INTRODUCTION: Autologous Chondrocyte Implantation (ACI) represents the most promising treatment for defined cartilage lesions. Human Articular Chondrocyte (HAC) dedifferentiation, occurring during expansion to obtain sufficient cell numbers, still hampers ACI reliability and efficacy. Previously, we identified S100 as a potential intracellular marker of HAC dedifferentiation[1]. Here we present S100 flow cytometry (FACS) assay and S100 cell-based ELISA (S100-CELISA) to monitor HAC differentiation status.

METHODS: HAC were isolated, cultured in monolayer, harvested at weekly intervals and analysed for S100 expression by FACS and immunocytochemistry (ICC) using anti-S100 antibody recognizing S100A1 and S100B. Intracellular staining of S100 was combined with surface staining of CD90. S100-CELISA was established using S100-positive A2058 cell line and anti-S100A1/B antibody and applied on HAC. S100 was induced with BMP4 in passage 3, (P3) HAC and evaluated via CELISA, qRT-PCR and ICC.

RESULTS: Combining staining of CD90, continuously increasing during the first weeks of HAC monolayer culture, with intracellular S100 staining, progressively decreasing during the whole culture period, a threshold that defined S100+ HAC was established. A correlation between the decrease in percentage of S100-positive HAC, assessed via ICC or FACS, with the number of cumulative population doubling during monolayer culture was established (*Fig.1*).

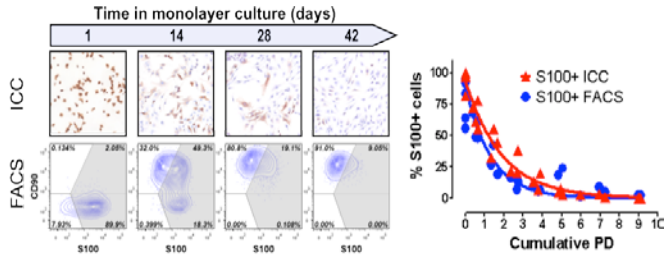
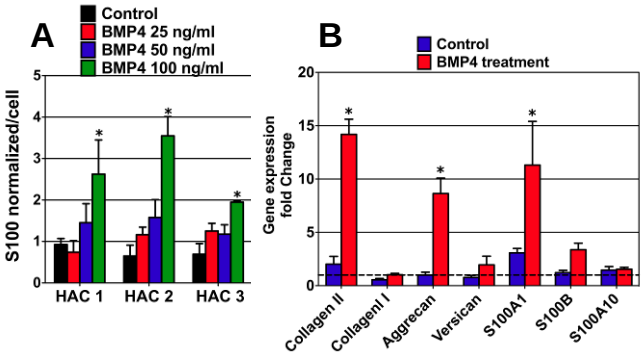


Fig. 1: Correlation in S100 expression obtained via immunocytochemistry (ICC) and FACS (n=3).

S100 CELISA showed a dose dependent S100A1/B increase in P3 HAC induced with BMP4 (*Fig. 2A*). In addition to S100A1/B increase to 100ng/ml of BMP4 in P3 HAC via CELISA, induction of S100A1 gene expression was correlated with increased levels of chondrogenic marker genes collagen II and aggrecan (*Fig. 2B*). Moreover, an increase in percentage of S100A1 positive HAC was obtained via ICC, confirming qRT-PCR data, thereby validating S100A1 as a chondrogenic marker at the gene and protein levels.

*Fig. 2: Induction of S100A1/B in P3 HAC with BMP4 via CELISA (A) and qRT-PCR (B) (n=3). * statistically*



significant

DISCUSSION & CONCLUSIONS: S100 FACS was established. The correlation between the number of cumulative population doublings and the percentage of S100-positive HAC via FACS and ICC was demonstrated. S100-CELISA was established as an assay to monitor HAC redifferentiation in monolayer. The S100-CELISA could offer an advantageous alternative to 3D pellet culture for testing factors/conditions to induce HAC redifferentiation. Some of these factors/conditions may improve HAC quality and find clinical application for cell-based cartilage repair treatments.

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Supplementation of fresh bone marrow preparation with cultured mesenchymal stem cells enhances ectopic bone formation

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Introduction: Repair of large bone defects is a major clinical challenge. Stem cell-based biomaterial therapies have shown promise in enhancing bone healing. Marrow-derived, multipotent mesenchymal stem cells (MSC) are typically expanded *in vitro* to obtain large quantities, but the process is lengthy and costly. Potential advantages of using fresh, uncultured bone marrow progenitor cells include the heterotypic cell and paracrine interactions between MSC and other marrow-derived cells (including HSC/HPC and EPC). In this study, we tested and compared the *in vivo* bone formation capacity of fresh bone marrow mononuclear cells (BMMC), culture-expanded MSC, as well as a combination of BMMC supplemented with MSC. All cell preparations were encapsulated in 3D collagen-chitosan microbeads that were designed as an injectable therapy.

Methods: Freshly isolated BMMC (25×10^6/ml, Fischer rats, 3-6 wks), or culture-expanded rat bone marrow-derived MSC (5×10^5/ml, passage 4), or both BMMC (3×10^6/ml) and MSCs (5×10^5/ml) were added to a collagen-chitosan (65/35 wt%) hydrogel mixture and fabricated into 3D microbeads by emulsification in PDMS and thermal gelation. All microbeads were cultured in control MSC growth medium for an initial 3 days, and subsequently, samples were cultured in growth medium or osteogenic medium (supplemented with ascorbate, β-glycerolphosphate, and dexamethasone) for 17 days. At day 14, cell viability within microbeads was assessed. On day 17, microbeads (n=6) were mixed with 500 µl of fibrin gel carrier (fibrinogen = 4 mg/ml, thrombin = 50 U/ml) and injected subcutaneously in rat dorsum (**Fig. 1**, where **A**=acellular; **F**=freshly isolated BMMC; **C**=culture-expanded MSC; **G**=growth medium, **O**=osteogenic medium). 5-week ectopic implants were harvested, and analyzed by microCT. All data are reported as mean ± SEM and analyzed by one-way ANOVA with Tukey's multiple comparisons test. For all tests, statistical significance was defined as $p<0.05$.

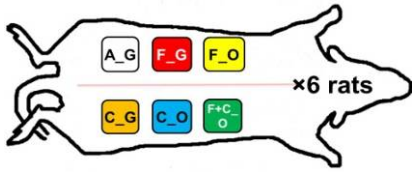

Fig. 1. Schematic of microbead implants in subQ rat dorsum.

Results: At day 14, F_O microbeads contained fewer live and more dead cells, compared to C_O microbeads and F+C_O microbeads, which predominantly contained live cells with a spread morphology (**Fig. 2**). Some of the microbeads cultured for 14-days in osteogenic medium exhibited a dark and opaque structure, suggesting pre-mineralization prior to implantation.

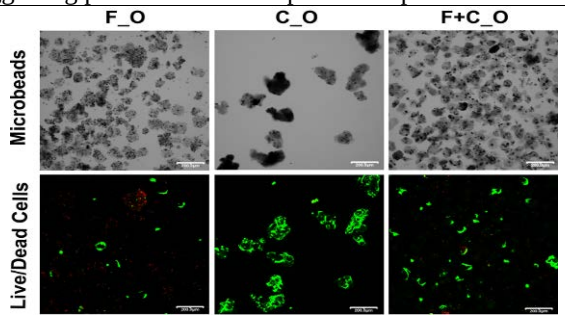

Fig. 2. Cell viability in pre-mineralized microbeads at day 14. Green=Live, Red=Dead. Scale bar = 200 µm.

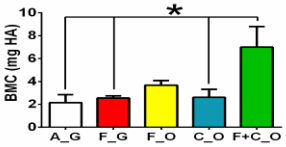

Fig. 3. Bone mineral content (BMC). n=6 for all samples, except n=5 for F+C_O. *p<0.05.

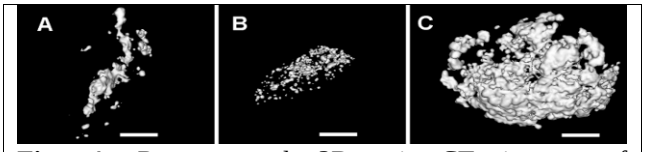

Fig. 4. Reconstructed 3D microCT images of representative 5-week implants F_O **(A)**, C_O **(B)**, and F+C_O **(C)**. Scale bar = 1 mm

Compared to all other samples except F_O microbeads, F+C_O microbeads resulted in a statistically significant stimulatory effect on BMC (**Fig. 3**), as assessed by microCT analysis. The most notable amounts of mineralized tissue were observed in F+C_O microbeads (**Fig. 4c**), compared to lesser amounts in F_O (**Fig. 4a**) and C_O (**Fig. 4b**) microbeads. There was a lack of bone formation detected in C_G implants.

Conclusions: Freshly isolated BMMC and culture-expanded MSC were encapsulated and viable within pre-mineralized 3D collagen-chitosan microbeads for 14 days, prior to use as an injectable implant. The combination of freshly isolated marrow progenitor cells and culture-expanded MSC within pre-mineralized microbeads has a strong synergistic effect on newly formed mineralized bone *in vivo*, compared to either cell source alone. This type of combined cell therapy holds promise as an injectable treatment for the repair of bone defects.

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Chemotaxis of neutrophils towards the fracture hematoma in vitro

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INTRODUCTION: Neutrophils infiltrate the sterile fracture hematoma within 24 hours after trauma. Animal studies show that systemic depletion of neutrophils improves fracture healing, suggesting that neutrophils have a negative effect on bone repair and thereby contribute to the high incidence of nonunion in severely injured patients. Selectively blocking neutrophil migration towards the fracture hematoma may become future focus of therapies to prevent nonunion in multitrauma patients. Since it is unknown which factors in the fracture hematoma induce rapid neutrophil infiltration to the fracture, we have investigated whether chemotaxis could be demonstrated in vivo, and which pathways are involved in the chemotaxis of neutrophils towards the fracture hematoma serum in vitro.

METHODS: Human fracture hematomas were isolated during an Open Reduction Internal Fixation (ORIF) procedure between 12-48 hours after trauma and spun down to obtain the fracture hematoma serum (FHS). Using IBIDI µ-Slide Chemotaxis 3D slides, we studied whether chemotaxis of neutrophils towards FHS could be inhibited by heat-inactivated denaturation of the complement system, simultaneous or individual blocking of the CXCR1 and CXCR2 receptors or using neutralizing antibodies against NAP-2.

RESULTS Neutrophils significantly migrated towards the FHS ($p<0.0001$), irrespective of duration of the fracture. This migration starts early and is seen in all experiments. Neutrophil migration was not significantly affected by heat-denaturation of the complement system. Although results show some variability, migration of neutrophils seemed to be inhibited by simultaneously blocking the CXCR1 and CXCR2 receptor and by neutralizing anti-NAP2 antibodies.

DISCUSSION & CONCLUSIONS: Neutrophil migration towards the FH is not dependent on the complement system, as heat inactivation does not influence this chemotaxis. Our study suggests that migration of neutrophils towards the fracture hematoma is dependent on CXCR1 and CXCR2 neutrophil receptors, although some variation in the experimental results was seen. This suggests that chemotaxis towards the fracture hematoma is multifactorial.

Blocking the CXCR1/CXCR2 pathway in vivo could be a target of future therapies to prevent impaired fracture healing in multitrauma patients.

Merging micro/nanoscale technologies and advanced biomaterials for tissue regeneration and stem cell bioengineering

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Tissue engineering is an interdisciplinary field, aimed at maintaining, restoring and enhancing normal tissue and organ functions by merging of engineering, biological sciences and medicine[1]. One of the central themes in the field of tissue engineering is to develop the body's architectural and geometric intricacies, through proper cell-cell and cell-matrix interactions. To date, tissue engineering has been successfully applied to engineer many types of tissues including bone[2,3], cartilage[4,5] and vasculature systems[6]. Despite the significant progress in this field, many challenges still remain to develop fully functional and vascularized engineered tissue constructs. Recent advancements in micro- and nanofabrication technologies have paved the way to address the current difficulties in the fields of tissue engineering and regenerative medicine[1]. In the past few years, our lab has been actively involved in merging micro and nano-engineering techniques with advanced biomaterials for tissue engineering and stem cell bioengineering applications. Our work encompasses a wide range of scientific subjects from engineering to materials science and biology. On the biomaterials design aspect, we have developed three dimensional (3D) hydrogel-based biomaterials to mimic the native *in vivo* microenvironment. Our extensive studies have demonstrated that hydrogels are excellent scaffolding materials for bone tissue engineering and osteogenic differentiation of human mesenchymal stem cells (hMSCs). In particular, we have used "top-down" and "bottom-up" approaches to fabricate microscale cell laden hydrogels with desired material characteristics and geometrical features to create functional osteon units. Furthermore, we have performed extensive studies related to hMSC differentiation towards osteogenic lineage using 3D cellular microarray platforms. We have demonstrated that the miniaturized cellular microarrays are suitable platforms to rapidly investigate the effects of various combinatorial factors within 3D microenvironments for bone tissue engineering applications. We have also developed bioactive and cytocompatible synthetic silicate nanoparticles to enhance *in vitro* osteogenic differentiation of hMSCs in the absence of osteoinductive factors (i.e. BMP-2) or dexamethasone[7]. In this talk, I will outline our major findings related to bone tissue engineering and stem cell differentiation using miniaturized systems and advanced biomaterials.

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Molecular strategies for adaptive materials

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INTRODUCTION: Significant advances in biomaterial science and engineering have occurred over the last several decades, leading to widespread clinical use of medical and dental devices. However, current- generation implants, and the materials from which they are fabricated, remain poor substitutes for the tissues they are intended to replace. The ability to adapt to changes in environmental conditions and to repair damage are properties that most living tissues possess, but are lacking in synthetic biomaterials. In this talk I will describe some emerging adaptive materials strategies that have the potential to significantly improve medical device performance in the future.

CURRENT AND EMERGING STRATEGIES: Although the idea of adaptive and self-healing materials has been embraced by the materials community for some time, very little activity has been devoted to materials for tissue repair and regeneration. Self-healing mechanisms are inherent to most (but not all) biological tissues, with an obvious example being that of long bone fracture repair via the initial formation of a fracture callous followed by formation of cancellous and ultimately cortical bone. Inspired by biological systems, materials of the future will benefit from self- healing concepts.

The most commonly explored self-healing mechanism involves the incorporation of extrinsic factors within the polymer matrix, for example in the form of microencapsulated monomers, which can mediate the formation of new structural material as a consequence of defect formation (e.g. a crack). Some groups in the biomaterials field are now actively pursuing the development of self- healing bone cement by encapsulating a healing agent within a self-setting acrylic bone cement matrix; fracture by crack propagation releases the healing agent that spontaneously polymerizes to fill the crack with newly formed (hopefully load-bearing) polymer matrix.

A second approach involves the design and development of novel materials with intrinsic capacity to self-heal through inherent molecular phenomena. In the case of polymers, examples of intrinsic phenomena that can give rise to self-healing include diffusion of polymer chains across a fracture interface above the glass transition, or chemical bond formation between transient reactive species created as a direct result of molecular scission during fracture. A number of research groups are developing novel bulk polymer systems that are constructed of 'sacrificial' noncovalent bonds that rupture under applied force. Such molecular linkers dissipate significant energy upon rupture but have the ability to dynamically re-form in the absence of load. Many of these strategies are inspired by biological systems that exploit hydrogen bonds, hydrophobic interactions, or coordination bonds between metal ions and organic proteins to toughen the tissue and produce a self-repairing response after deformation.

Finally, in the future more attention must be paid to self-healing strategies that operate at the interface between phases in a material. One motivation for this is the increasing evidence in mineralized biological tissues of molecular components (proteins) that are present at the inorganic-organic interface and which appear to play important roles in dissipating energy by breaking and then remaking sacrificial bonds in the presence of applied loads. Such organic molecules can be considered as "glues" in highly filled systems, and the molecular features of these systems that give rise to macroscopic self-healing properties can provide clues to making synthetic composite materials with similar characteristics. Guiding concepts provided by studies of bone, teeth and nacre include the use of a relatively small amount of organic "glue" in an otherwise primarily inorganic hierarchical structure, the use of entropic elasticity in the organic glue molecules, and significant energy dissipation through breaking of weak bonds rather than non-recoverable covalent bonds.

How mechanobiology inspires new approaches on the path from basic sciences to the clinics

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INTRODUCTION: Investigations of the mechanisms how the extracellular microenvironment directs cell fate and tissue repair has gained major momentum only very recently. While it is well recognized that fibrosis is the most typical tissue response of the body to biomedical implants, little is known how to effectively prevent it. In a first step, we try to gain new insights into the mechanisms how cells recognize implant surfaces and how this directs their ability to assemble and remodel extracellular matrix. A second major hurdle is the sparse knowledge how environmental factors direct cell fate, knowledge that is urgently needed to advance (stem) cell-based regenerative therapies. Here, we will discuss some fundamental processes that cells exploit to sense the physical properties of their environments, including rigidity and topography.

CURRENT STRATEGIES: Major advances of this field have been made possible by novel micro- and nanofabrication techniques to engineer precisely controlled cell environments, combined with novel nanoanalytical techniques to probe the mechanics of single molecules, cells and tissues, as well as of advanced imaging.

DISCUSSION & CONCLUSIONS: Cells exploit mechanical forces generated by their motors to pull on distal extracellular anchoring points. Integrin-based junctions thereby connect the actin cytoskeleton to the extracellular matrix or to protein coated materials (1). Topography sensing is one of the most fundamental processes that cells exploit to interact with their environments, either during development, wound healing or metastatic invasion. Since little was known about the role of filopodia in topography sensing, especially within nanofibrillar environments, we have grown highly flexible hairy silicon nanowires on micropatterned islands on otherwise flat glass surfaces and coated them both with the extracellular matrix protein fibronectin (2). This allowed us to visualize how filopodia steer fundamental cell functions such as cell adhesion, spreading, migration and division. What cells locally feel furthermore depends not only on the displacement of a material, but also on the stability of molecular interactions, on the conversion of mechanical forces to biochemical signals by stretching proteins into structural intermediates (mechano-chemical signal conversion), and on the micro- and nanoscopic features of the extracellular protein coating (1-4). Our most recent findings will be discussed. Gaining insights into mechanotransduction mechanisms (1,4) is crucial to learn how to properly design biomaterials that have the ability to steer stem cell differentiation (3), (micro)tissue growth (5), healing and tissue regeneration.

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Repair of cranial defects in humans with bioactive implant

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INTRODUCTION: A number of bioactive compounds, including bioceramics and osteoinductive cytokines, have shown bone-healing effects but their usages are limited to smaller defects at orthothopic sites [1,2]. We report the outcomes of using a scaffold comprising ceramic tiles with specific composition of calcium phosphate phases used for the repair of large cranial defects in humans.

METHODS: Implants: Implants comprising ceramic tiles interconnected by titanium mesh were manufactured by moulding technique. Sterilised ceramics were analysed for phase composition using X-ray diffraction (XRD, Bruker D8 Diffractometer). **Patients:** Thirteen patients with various cranial defects were treated with customized ceramic implants. The cranial defect sizes measured from 20 to 320 cm^2. The causes of cranial defects included traumatic injuries, decompressive hemi-craniectomies, and previously failed implants. The follow-up times were from 2 to 39 months. **PET/CT examination:** Two patients were examined with PET/CT 27 and 29 months after surgery, respectively, using Biograph 64 TruePoint PET/CT scanner. 60 min after i.v. administration of 300 MBq [^{18}F]-fluoride a PET/CT examination of the calvaria was initiated.

RESULTS: Analysis of the hardened ceramic compositions revealed the phases monetite (86%), β-calcium pyrophosphate (β-PPi, 7%), β-tricalciumphosphate (β-TCP, 6%), and brushite (1%). Ten out of 13 patients displayed excellent results without signs of acute or long-term complications. A functional analysis on bone growth was performed using [^{18}F]-fluoride PET-CT scans in two patients showing uptake within the entire implants providing evidence of bone formation. Notable was that in two patients complete resorption of the ceramics was evident by CT after 6 months. This outcome was associated with difference in phase composition with absence of β-PPi (<0,1%) as compared to composition used in the other patients without resorption. One patient was unsatisfied with the aesthetical result with flat contour of the reconstructed frontal bone.

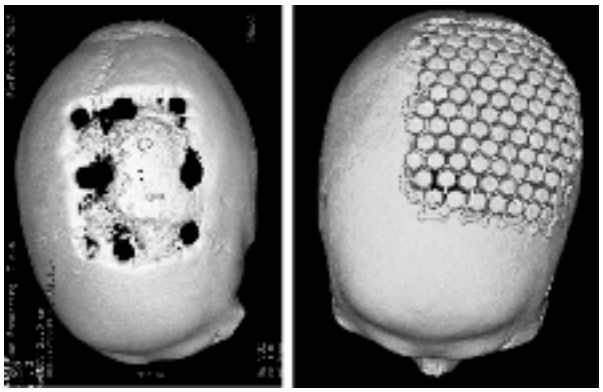

Fig. 1:Osteoradionecrosis of the cranial bone (left) caused extrusion and infection. After revision, the defect was repaired with ceramic implant (right) as demonstrated by 3D-CT.

DISCUSSION & CONCLUSIONS: We demonstrate a bioactive scaffold designed for large cranial defect repair. The implant was shown to stimulate bone regeneration. Importantly, We show that the addition of β-PPi is critical for prolonging resorption rate, allowing sufficient time for coupled bone formation to happen and thereby realizing the implant´s potential for bioactivity. [4]

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Effect of mechanical environment on the BMP-2 dose-response of large segmental defect healing

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INTRODUCTION: Several treatments exist to aid the healing of large osseous defects, including recombinant human bone morphogenetic protein-2 (BMP-2). Although BMP-2 has shown preclinical efficacy in animal models, the clinical effectiveness in healing long bone defects has been disappointing. In our previous studies we demonstrated that the local mechanical environment influenced the healing of large bone defects in response to a standard dose of BMP-2[1]. The main goal of the present study is to determine if the mechanical environment can reduce the dose of BMP-2 needed for boney union. In preparation for this, it is necessary to establish a dose-response curve for BMP-2 using fixators of different stiffnesses. The preliminary data from this study are presented in this abstract.

METHODS: A rat, femoral, 5 mm defect was stabilized with different degrees of stiffness[2]. Defects were treated with 11 (standard dose), 5.5 or 1.1 μg BMP-2 delivered on an absorbable collagen sponge in the same way as the INFUSE® product used clinically to enhance bone healing. The rat groups healed under conditions of low, medium and high stiffness with various doses of BMP-2. Healing was monitored by weekly X-ray. At 8 weeks, the rats were euthanized. Mechanical testing, μCT, and histology are still in progress.

RESULTS: Under conditions of constant stiffness, the lowest dose of BMP-2 caused the deposition of only small amounts of bone, which failed to fill the defect no matter which stiffness external fixator was used (data not shown). In contrast, defects treated with the two higher doses of BMP-2 showed clear evidence of intra-lesional mineralization by 3 weeks (Fig.1). However, defects treated with a dose of 5.5 μg appeared to be less uniform, and at 8 weeks there was still presence of radiopaque line in all groups (Fig.1). Likewise, evidence of healing in these defects seem to be delayed by one week occurring at 3 weeks instead of 2 weeks post-op when compared to the standard dose, except in the group with highest stiffness fixator (Fig.1). Furthermore, the callus size appeared to be smaller during healing with all stiffness fixators when 5.5μg dose was used. Additionally, this data did confirm our previous findings that lower stiffness fixators had bigger callus formation as compared to the more rigid fixators[1].

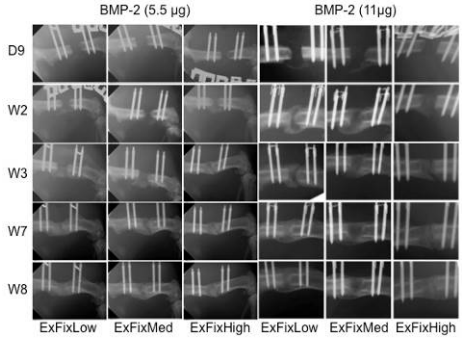

Fig. 1: Representative X-ray images with different stiffness fixators and 2 different doses of BMP-2.

DISCUSSION & CONCLUSIONS: Defect healing was influenced by the dose of BMP-2. Although the full study is still in progress, the initial results suggest that a dose of 5.5 μg BMP-2 has the ability to drive the healing of segmental defects, but healing was delayed and the quality of healed bone was inferior when compared to 11μg of BMP-2. Although at this point it is not clear if the mechanical environment plays a role using 5.5 μg dose of BMP-2, but the presence of the radiopaque line at the end of treatment might be a consequence of prolonged movements in the defect during early stages of healing. A dose of 5.5 μg BMP-2 is thus appropriate for studying the effects of different mechanical regimens, such as reverse dynamization [1], on the healing of large bone defects. The results of this study will have significant consequences on the fixation stability used in order to maximise the regenerative capacity of bone healing while minimising the dose of BMP-2 required clinically.

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Annular repair using high-density collagen. An in vivo study.

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INTRODUCTION: Unrepaired annular defects potentially increase the reherniation rate after lumbar discectomies[1]. This is the first study to test an injectable biological substance to repair annular defects *in vivo*. We used high-density collagen gel in a needle-punctured rat-tail model. Needle puncturing leads to extrusion of NP tissue and subsequent degenerative changes[2]. Restoring annular integrity will help to retain the NP material and therefore inhibit these changes.

METHODS: We punctured the S3/S4 intervertebral disc (IVD) of 42 athymic rats using an 18-gauge needle. Subsequently high-density collagen (HDC) gel was injected to seal the defect. Riboflavin (RF) was added to increase the stiffness of the collagen gel by inducing cross-link formation. The animals were subdivided into four groups. The first group was injected with uncross-linked HDC gel, the second with collagen cross-linked using 0.25mM RF, the third using 0.5mM RF, and the fourth control group was punctured and left untreated. The animals were followed up for five weeks with X-ray measurements to assess the disc heights and MR imaging to evaluate degenerative changes of the IVD according to the modified Pfirrmann grade. We developed an algorithm based on T2-relaxation time measurements to assess the size of the nucleus by the number of voxels that composed it. Tails were collected for histological analysis to assess disc degeneration and the cross-sectional area of the NP.

RESULTS: After five weeks, the 0.5mM RF cross-linked group showed only a minimal decrease of nuclear tissue compared to healthy discs with no obvious signs of IVD degeneration (Fig.1). The AF appeared partially repaired by a "fibrous cap" bridging the defect (Fig.2). The 0.25mM RF group showed signs of moderate degeneration with extrusion of nuclear tissue. After five weeks, the control group as well as the uncross-linked group displayed no significant difference from each other. Both showed signs of progressive degenerative changes and no residual NP tissue in the disc space.

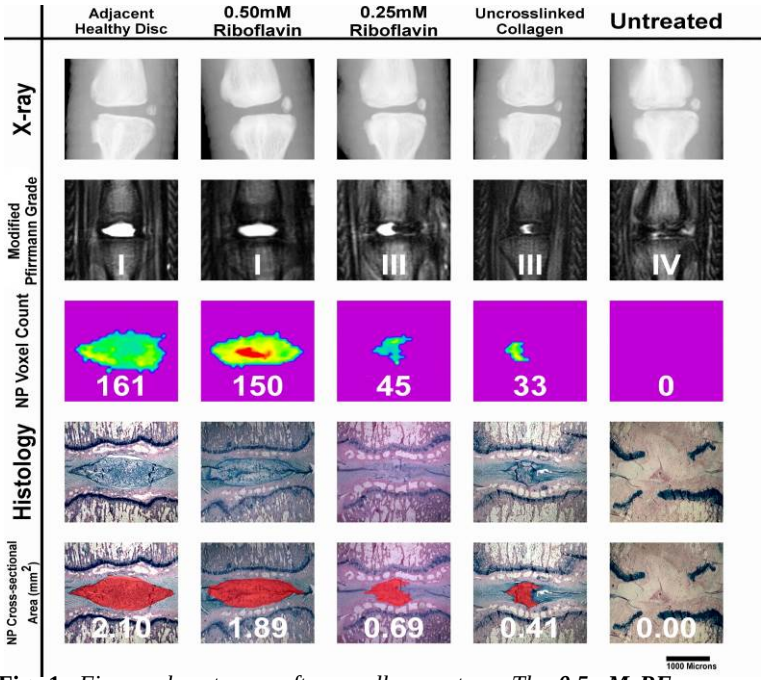

Fig. 1: *Five-week outcome after needle puncture. The **0.5mM RF group** only showed a slightly reduced NP size according to the NP voxel count and histological cross-section measurements compared to healthy discs. The **0.25mM RF group** shows a reduced nuclear size. On T2 weighted images (second row from the top), the NP appears hyperintense but lost its oval shape. The decreased disc height, results in a Pfirrmann grade of III. The **uncross-linked** group showed a further decrease of NP size. The NP appears more heterogeneous. The disc height is slightly decreased compared to healthy discs. The untreated disc shows terminal degenerative changes with a black disc sign combined with collapsed disc space seen on the X-Ray image. The NP tissue has been completely replaced by connective tissue.*

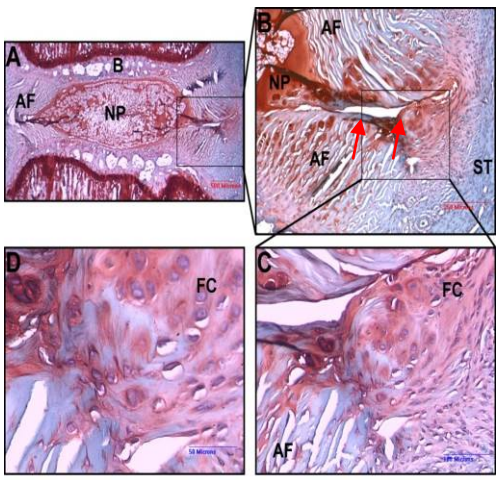

***Fig. 2**: Punctured IVD, 0.5mM RF group after 5 weeks. **A**, Low magnification. The NP displays a standard size and a clear border between the AF and the endplate bone, B. The box marks the needle puncture defect. **B**, Higher magnification displays the needle puncture defect piercing through all AF layers (red arrow). Marked by the box, there is a fibrous cap visible, bridging the annular defect at the outer portion of the AF. **C, D** Higher magnifications of the fibrous cap.*

DISCUSSION & CONCLUSIONS: HDC is capable of repairing annular defects induced by needle puncture in a rat-tail model. The stiffness of HDC appears to influence the repair mechanism. Longer-term data as well as experiments in larger animals will be necessary to evaluate the clinical applicability.

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Shape memory scaffolds for minimally invasive disc repair

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INTRODUCTION: At present there is a lack of viable treatment options to repair damaged annulus fibrosus (AF) tissue. Recently, devices have been developed to seal and prevent the nucleus pulposus from protruding the AF but are limited in their inability to integrate and promote biological repair and prevent further degeneration[1]. Developing biomaterials that can be delivered in a minimally invasive manner to the damaged AF and promote neo-tissue formation is a key challenge.

In this work we developed a porous alginate scaffold with shape memory properties which can be delivered using minimally invasive approaches and recover its original geometry once hydrated. In vitro experiments demonstrated the ability of the scaffold to support tissue formation using AF cells over 21 days with significant elaboration of new matrix. This approach may offer potential in regenerating damaged AF tissue of the IVD.

METHODS: Porous shape memory scaffolds scaffolds were fabricated using sodium alginate (2%, ProNova, Norway) covalently cross-linked through standard carbodiimide chemistry [2] with the bifunctional cross-linker adipic acid dihydrazide (AAD). A freeze drying step (-30°C) was subsequently performed to impart porosity.

Porcine derived AF cells were seeded on porous scaffolds (diameter:5mm, height:3mm) at a density of 8 x10^6 cells/ml and maintained in normal (20%) or low (5%) oxygen (O_2) conditions with or without TGF-β3 supplementation for 21 days. Constructs were assessed at days 0 and 21 in terms of mechanical properties, cell viability, histology and biochemical analysis (DNA, sulfated-glycosaminoglycan (sGAG) and collagen content).

RESULTS: Covalently crosslinked porous scaffolds demonstrated high shape memory capabilities (Fig 1A) and could be easily delivered through a catheter to a defect site (Fig 1B). In vitro experiments demonstrated high cell viability when seeded with AF derived cells (Fig 1C). After 21 days of culture, the porous network of the scaffold contained a high proportion of ECM (Fig. 1D) and sGAG.

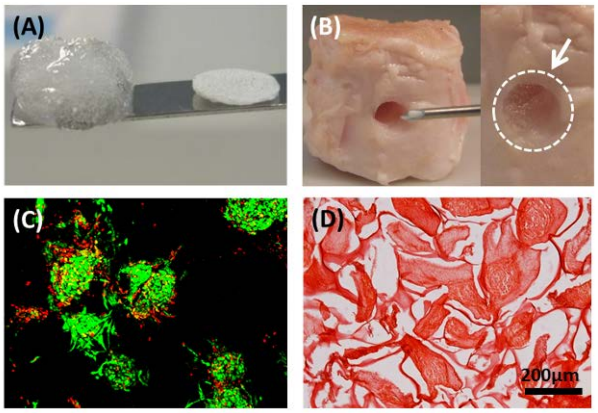

Fig. 1: (A) Swelling behaviour (B) Minimal invasive delivery of porous scaffold (C) Cell viability and (D) collagen deposition after 21 days of culture.

DISCUSSION & CONCLUSIONS: Developing alternative strategies to seal, promote biological repair and integration of ruptured annulus fibrosus is of significant importance and will be a key step towards translating such therapies into clinical practice. In this work we developed porous shape memory scaffolds which could be delivered in a minimally invasive fashion.

In vitro experiments demonstrated that, after seeding, cells colonized the scaffold and synthesised ECM similar to healthy IVD tissue (collagen and sGAG). Current work is focused on increasing the bioactivity of these scaffolds through the incorporation of collagen and assessing their ability in a preclinical ex-vivo organ culture model to assess the biological integration with host tissue.

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Osteoblastic differentiation, mineralization and bone regeneration by a bioactive protein-based membrane

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INTRODUCTION: The periosteum plays a critical role during bone fracture healing [1]. Here we report a nanotechnology approach to engineer bioactive membrane scaffolds based on elastin-like recombinamers (ELRs) exhibiting multiple physical and biomolecular signals for applications in bone regeneration.

METHODS: Four ELRs exhibiting the bioactive epitopes REDV for endothelial cell adhesion, RGDS for mesenchymal stem cell adhesion, DDDEEKFLRRIGRFG for nucleation of mineralization (HAP), and a combination of the later two (RGDS-HAP) were synthesized using standard recombinant protein techniques. In addition, an ELR without bioactive sequence (IK) was used as control. The materials were then used to fabricate ELR membranes by a recently reported drop-casting/evaporation technique [2,3], comprising either a smooth surface or channel microtopographies. Bioactivity of the membranes was assessed *in vitro* by quantifying mineralization, and primary rat mesenchymal stem cell (rMSC) adhesion, proliferation, and differentiation. Furthermore, the membranes were tested *in vivo* in a critical-size rat cranial defect model and analysed for new bone formation at 36 days after surgery.

RESULTS: Quantification of cell morphology and adhesion revealed minor differences depending on the tested membrane. Cell proliferation was clearly affected on all HAP membranes, initially slowing down their growth from day 0 to day 2, but then significantly increasing it by day 5. In these membranes, an early expression of the osteoblastic transcription factor osterix at day 5 in non-osteogenic differentiation medium was strongly induced, and to a lesser degree on HAP-RGDS membranes, independently of topography (Fig. 1a). In addition, smooth HAP membranes exhibited the highest quantity of calcium phosphate (Ca/P in 1.76) deposition with and without cells. Finally, animals implanted with smooth HAP membranes exhibited a significantly higher bone volume within the defect as measured by micro-computed tomography (µCT) and histology compared to animals implanted with a non-bioactive ELR membrane and untreated animals (Fig. 1b-f).

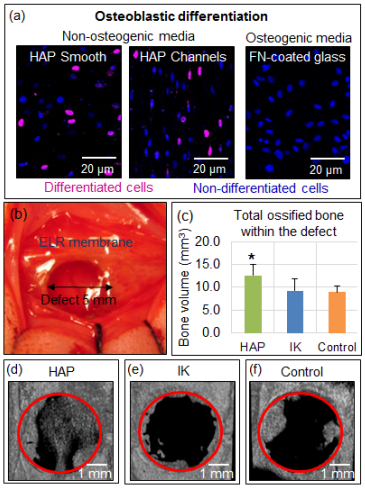

Figure 1. (a) Confocal images of cells growing without osteogenic medium on HAP Smooth and HAP Channels expressing osterix compared to cells growing in osteogenic medium on the positive control Glass-FN at day 5. (b) Representative image of a rat cranial critical-size defect covered with an ELR membrane. (c) Quantification of bone volume within the defect and 3D µCT image reconstruction of the cranial defect covered with (d) HAP membrane, (e) IK membrane and (f) control (untreated) at day 36. (Red circles indicate the cranial defect edges).

DISCUSSION & CONCLUSIONS: Membranes exhibiting selective bioactivity capable of eliciting specific biological responses may offer a more efficient periosteal graft alternative towards improving bone regeneration. ELR membranes displaying the bioactive sequence DDDEEKFLRRIGRFG (HAP) exhibited the highest capacity *in vitro* to induce osteoblastic differentiation and mineralization and significantly improved bone regeneration *in vivo*.

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In vivo implantation of microsphere-incorporeated cartilage sheets

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Introduction: Human bone marrow-derived mesenchymal stem cells (hMSCs) are a commonly utilized cell source for cartilage tissue engineering strategies. hMSCs can be cultured in high-density systems that enable cell-cell interactions important for chondrogenic differentiation. These cellular constructs often require several weeks of in vitro culture in growth factor-containing medium in order to form implantable, engineered cartilage tissues. Alternatively, cartilage sheets can be formed via the incorporation of growth factor releasing microspheres within high-density hMSC constructs, which eliminates the need for supplementing the medium with chondrogenic growth factor during extended in vitro culture [1]. Within this system, the microspheres also increase the thickness and mechanical integrity of the resultant cartilage, enabling handling and implantation after in vitro culture periods of as little as 2 weeks. Here, we demonstrate the production of self-assembling hMSC sheets incorporated with TGF-β1 loaded gelatin microspheres for early implantation and formation of cartilage tissue in a rabbit osteochondral defect model.

Materials and Methods: Gelatin microsphere synthesis. Gelatin microspheres were synthesized by a water-in-oil emulsion technique. Microspheres were crosslinked in a 1% genipin solution for 2 hours, lyophilized and UV sterilized, and then loaded with 400 ng TGF-β1 per mg microspheres or PBS only for "empty" microspheres by adding a small volume of solution. Microsphere-incorporated cell sheet production. 1.5 mg crosslinked microspheres and 2×106 P3 hMSCs were suspended in a chemically defined medium and allowed to settle onto the membranes of 12 mm cell culture inserts. Control sheets and sheets containing TGF-β1 loaded microspheres were treated every other day with medium with 10 ng/ml TGF-β1 or without growth factor, respectively. In vivo implantation. Rabbit osteochondral defects (3 mm diameter×1.5 mm depth) were made under an IACUC-approved protocol as previously described [2]. Sheets were harvested after 2 or 3 weeks of culture and 2 to 4 punches (3 mm diameter) from experimental or control sheets were placed into each defect (N≥6). Empty defects without implanted sheets served as a negative control (N≥6). Osteochondral tissues were harvested for immunohistochemical evaluation as well as gross morphological and histological scoring after up to 3 months.

Results and Discussion: Three rabbits were harvested for early evaluation of implant retention and cartilage formation. After 5 weeks in vivo, the defects implanted with 3-week sheets containing TGF-β1 microspheres were filled with shiny white tissue (Fig. 1A). Upon histological evaluation, implanted sheets were visible within the osteochondral defects (Fig. 1B) and exhibited no apparent inflammation or immune response. At this early time point, the defects contained cartilaginous tissue which stained positively for glycosaminoglycans (GAG) via Toluidine blue (Fig. 1C) and type II collagen (not shown). The empty control defects were partially filled with a fibrocartilage tissue (not shown).

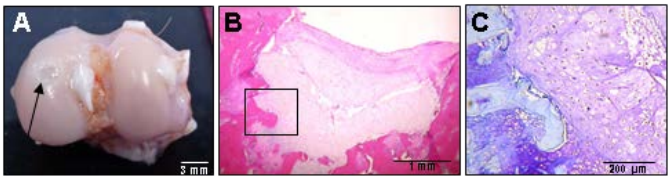

Figure 1. 5-week osteochondral defect implanted with hMSC sheets incorporated with TGF-β1-loaded gelatin microspheres. (A) Defect site with implant on medial condyle (arrow); (B) H&E stained image of repair tissue; (C) Toluidine blue staining for GAG (inset from C).

Conclusions: We have demonstrated that self-assembled hMSC sheets incorporated with TGF-β1 loaded microspheres can be implanted after only 2 to 3 weeks of in vitro culture, and are capable of forming early neocartilage after 5 weeks in a rabbit osteochondral defect model. Samples from later time points are currently undergoing analysis to determine the progression of longer-term cartilage repair in the rabbit knee defects. The ability to implant engineered cartilage constructs without extended prior culture could allow for accelerated implementation as part of therapeutic strategies for the repair and regeneration of damaged articular cartilage.

Acknowledgements: The authors gratefully acknowledge funding from the National Institutes of Health (AR007505 for LDS training support and AR063194 to EA).

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Influence of hypoxia on co-culture spheroids consisting of endothelial cells and osteoblasts

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INTRODUCTION: Co-culture spheroids are a useful tool to study angiogenic phenomena both, in vitro and in vivo. Furthermore, they are a promising strategy which might help to improve tissue engineered bone grafts in the future. In this study we investigated the influence of several hypoxic culture conditions on apoptosis as well as on secretion of vascular endothelial growth factor (VEGF). We aimed to find hypoxic conditions which are able to increase VEGF-secretion and guarantee sufficient cellular viability.

METHODS: We generated co-culture spheroids (50.000 cells) consisting of 50% human osteoblasts (HOB, PromoCell, Heidelberg, Germany) and 50% human dermal mircovascular endothelial cells (HDMEC, PromoCell)[1]. Twenty-four hours after generation, spheroids were cultured under hypoxic conditions[2] (pO_2 < 5 mmHg) for 6, 12 and 24 hours, respectively, followed by different times of reoxygenation. Control spheroids were cultured under normoxic conditions. Spheroids were embedded in paraffin and sectioned. The number of apoptotic cells for each cell type was quantified by immunofluorescence staining of Caspase-3 and CD31. Furthermore the secretion of VEGF in the cell culture medium was measured by means of ELISA. A one-way ANOVA or a t-test were used for statistical testing ($p<0.05$).

RESULTS: As expected, the rate of apoptotic cells correlates with the length of hypoxia. After twelve hours of hypoxical preconditioning followed by 24 hours of reoxygenation, the number of apoptotic endothelial cells trends to decrease. Under the same conditions we detected a maximum release of VEGF (Fig.1). In general, the number of apoptotic HOB were not affected by long time hypoxic conditions. In the immunofluorescence stained histological sections we detected tube like structures consisting of endothelial cells (Fig. 2).

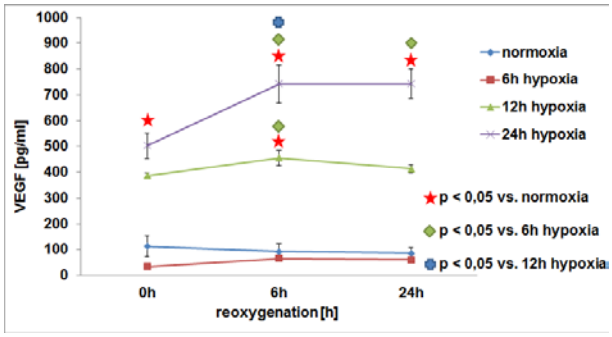

Fig. 1: ELISA-quantification of VEGF release depending on different hypoxic conditions.

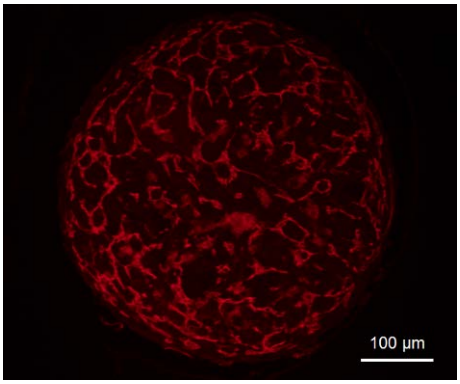

Fig. 2: Photomicrograph of CD31 positive tube like structures in a co-culture spheroid.

DISCUSSION & CONCLUSIONS: An appropriate length of hypoxia followed by reoxygenation trends to decrease apoptosis while the release of VEGF is increased. The positive effect of these conditions may be caused by VEGF as well as by further cytokines. In further studies spheroids will be cultured under these optimal conditions and placed in the dorsal skinfold chamber in mice to investigate the effects under dynamic processes using intravital microscopy.

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Angiogenesis and osteogenesis are impaired in type 2 diabetic long bone regeneration.

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INTRODUCTION: Although bone regeneration is typically a reliable process, patients with the endocrine disease type 2 diabetes face an impaired or delayed healing process. In addition, neovascularization, a crucial step in bone regeneration, is often impaired in diabetic patients. While bone regeneration in type 1 diabetes has been studied in animal models, markedly less is known for the more prevalent type 2 diabetes.

METHODS: In this study, different stages of bone regeneration, including neovascularization, were characterized in an unicortical long bone model comparing *db⁻/db⁻*, resembling type 2 diabetes, and wild type mice *in vivo.* 1 mm defects were placed in mice tibia and harvested at different time points to investigate angiogenesis, callus formation and remodeling by means of histomorphometry, gene expression analysis and immunohistochemistry as previously described [1].

RESULTS: Histomorphometry of aniline blue stained slides indicated that bone regeneration was significantly impaired in *db⁻/db⁻* as opposed to wild type mice on day 5 and 7 (62% and 84% impairment respectively) (Fig. 1). Moreover, immunohisto-chemistry and gene expression analysis revealed decreased levels of Runx2, PCNA, Osteocalcin, Vegfa and Pecam-1 (Fig. 2) in *db⁻/db⁻* defects, indicating impairments in both, angiogenesis and osteogenesis.

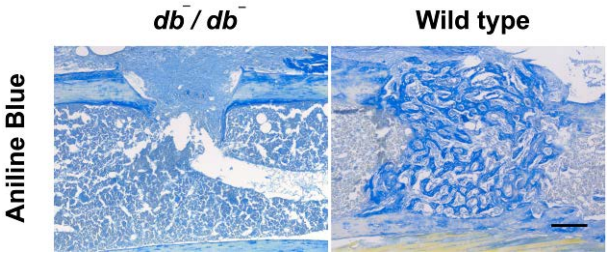

Fig. 1: Aniline blue staining shows dramatic impairment of bone regeneration in db⁻/db⁻ mice (left) vs. wildtype mice *(right)* at day 7. *Scale bar: 200 µm*

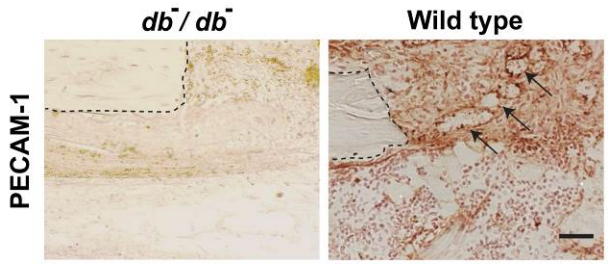

Fig. 2: Immunohistochemistry shows impairment of angiogenesis in db⁻/db⁻ mice (left) vs. wildtype mice *(right)* at day 3. *Scale bar: 50µm*

DISCUSSION & CONCLUSIONS: In conclusion, our study suggests that type 2 diabetes impairs bone regeneration by affecting both angiogenesis and osteogenesis. These data serve as a basis for future therapeutic applications aiming at improving bone regeneration in the type 2 diabetic patient population.

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Responses of the intervertebral disc when compressed and twisted dynamically in an organ culture model

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INTRODUCTION: Complex loading affects the intervertebral disc (IVD) mechanical behavior and its ability to withstand various loading.[1] Previous researches on IVD mechanobiology have been focus on axial compression, whereas research analyzing the role of torsion and complex loading on disc degeneration is very limited. This study aims to evaluate the responses of the IVD cells under complex dynamic loading. It was hypothesize that the complex loading (dynamic torsion - compression) accelerates IVD degeneration including increased cells death and activation of matrix destruction gene expression than pure dynamic torsion or pure dynamic compression. The hypothesis is tested using bovine caudal disc culturing in our 2 degree of freedom (DoF) loading bioreactor.

METHODS: Bovine caudal IVDs with endplates were isolated as previously described[2] and mechanical loading was applied using a 2 DoF loading bioreactor for 14 days.[3] Discs were assigned to different loading groups: 1) No loading (NL), 2) cyclic compression (CC) [8h: axial compression of 0.6 ± 0.2 MPa, 0.2 Hz], 3) cyclic torsion (CT) [8h: ± 2° torsion, 0.2 Hz, 0.2 MPa compression], 4) cyclic compression & torsion (CCT) [8h: 0.6 ± 0.2 MPa, 0.2 Hz & ± 20 torsion, 0.2 Hz]. Disc tissue was dissected for various molecular analyses after the last day of loading. Tissue was stained with live/dead stain and imaged with a confocal microscope for cell viability assessment. Real-time RT- PCR was performed to evaluate the activation of anabolic and catabolic genes. Histology was performed to access morphology of the IVD cells at different regions.

RESULTS: Result showed significant drop in nucleus pulposus (NP) cell viability in the CCT group to 10 % ($p < 0.001$), while cell viabilities were maintained above 60% in both the NP and the annulus fibrosus (AF) of all the other groups. Gene expression showed the strongest expression in both anabolic and catabolic genes on CCT AF region. (Fig 1)

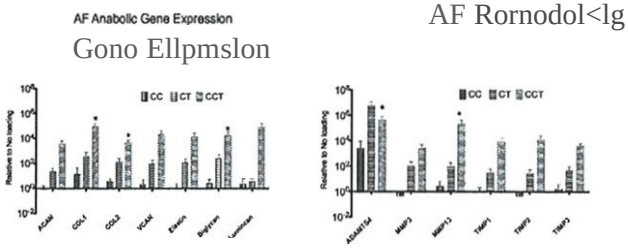

Fig. 1: Annulus fibrosus gene expression after 15 days of loading. CC: cyclic compression, CT: cyclic torsion, CCT: cyclic compression & torsion.

DISCUSSION & CONCLUSIONS: Results showed that cyclic complex loading consisting of compression and torsion applied to IVD has caused deleterious effect on the NP cell viability. There was a region specific response of the disc to complex loading, while NP was eradicated, AF responded with active matrix remodeling as seen by high anabolic and catabolic gene expression.

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ACKNOWLEDGEMENTS: This project was supported by the SNSF 310030_127586/1.

Cell surface glycosylation and glycosaminoglycan composition profiles in immature and mature intervertebral discs

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Back pain is a predominant cause of disability, and the major cause is intervertebral disc (IVD) degeneration [1]. Proteoglycan (PG) content of the IVD is decreased during degeneration, and glycosaminoglycan (GAG) composition is altered [2]. In this study, the cell surface glycosylation and GAG composition profiles of immature and mature ovine IVD tissue were profiled in an effort to understand potential signals involved in cell-extracellular matrix crosstalk during maturation and degeneration.

METHODS: IVD tissue from L3-L4 and L4-L5 lumbar segments were harvested from 3 and 11-month-old animals. A portion of the tissue was fixed for histochemical profiling with a panel of lectins. The remaining portion was digested with proteinase K and the sulfated GAG (s-GAG) content was quantified by dimethylmethylene blue assay. The digested tissue was treated with chondroitinase ABC (ChABC) and analysed by HPLC for s-GAG constituents.

RESULTS: DMMB assay showed that nucleus pulposus (NP) tissue contained 3-fold more s-GAGs than annulus fibrosus (AF) and cartilage in 3 month-old samples. In the 11 month-old samples, no difference was observed between AF and NP, but overall content of s-GAG was higher than cartilage. The glycosylation expression level differed between AF and NP tissue. For studying this glycosylation expression, panels of lectins were used. All lectins stained cells and ECM of the IVD independently of the tissue-type. However, a higher fluorescence was measured on the cells compared to the ECM showing a high glycosylation of NP and AF cells. Intensity of staining differed by the tissue-type. With maturity, the sugar expression profiles changed on the surface of the cells. For example, Neu-□-(2→6)-Gal(NAc) (SNA staining) and GalNAc (GalNAc-□-(1→6)-Gal>GalNAc-□-(1→3)-GalNAc (WFA staining) were more expressed on the cell surface 10 times and 2 times respectively in 11 month-old NP tissues compared with 3 month-old NP tissues. HPLC analysis of the ChABC-digested IVD tissue revealed that the quantity and ratio of s-GAG components of the NP tissue differed from AF tissue. Moreover, a change in sulfation pattern was observed with the maturity of the disc (Figure 1). An inversion of sulfation pattern was observed. Indeed, a significant increase of 4S disaccharides associated to a significant decrease of 6S disaccharides was noted within the AF tissue in 11 month-old IVD compared to 3 month-old. A similar pattern, but not significant, was observed for the NP tissue. Cartilage tissue did not present a significant difference in percentage of sulfated disaccharides with maturity.

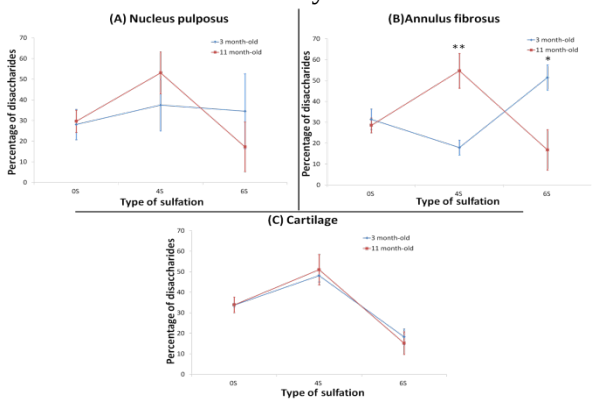

Figure 1: Percentage of non sulphated and sulphated chondroitin sulphate in ovine nucleus pulposus (A), annulus fibrosus (B) and cartilage (C) in 3 and 11 month-old. Data represented relative to the total sGAG as mean ± sem*, ** represent significant differences at $p<0.05$, $p<0.01$, respectively.

GAG sulfation pattern was altered with maturation. Sulfation is critical for maintaining water content and specific patterns are influential in cellular signaling and differentiation. Changes in glycosylation profiles at a cellular and extracellular level were observed. An understanding of these phenomena (sulfation and glycosylation profiles) will help understand the IVD microenvironment.

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ACKNOWLEDGEMENTS: AO Foundation (S-09-7P), Science Foundation Ireland, Research Frontiers Programme (07/RFP/ENMF482).

*indicates equal contribution

Intervertebral disc regeneration by mesenchymal stem cells and fibrin.

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INTRODUCTION: It has been shown that whole bovine caudal intervertebral discs (IVDs) remain viable after culture in a bioreactor system under simulated physiological load and degenerative changes can be induced in the discs already after one week of high magnitude/high frequency loading [1]. The goal of this work is to investigate (i) whether degenerative changes can be induced by a low magnitude - high frequency loading and (ii) whether the disc can be regenerated by delivering fibrin gel and/or bone-marrow derived mesenchymal stem cells (MSCs) to nucleotomized IVDs. Disc height changes and histology were assessed to obtain indications for degeneration and recovery.

METHODS: Intervertebral discs with endplates were harvested from calf tails [1]. Discs were loaded 3 hr/day in a bioreactor under either physiological (loading at 0.1 Hz and 4.5 g/L glucose medium) or degenerative (loading at 10 Hz and 2 g/L glucose medium) conditions for one week. Then, a core (diameter = 4 mm) of the nucleus pulposus was removed from all discs and cavities were filled with: (1) fibrin gel, (2) MSCs in fibrin gel (3) MSCs in PBS, (4) left empty (as control). Approximately $6x10^5$ cells and 150 µl of carrier were used for each disc. The final fibrinogen concentration was 60 mg/ml. Discs were loaded under physiological conditions for one more week; disc height was measured at selected time points after loading (days 9 and 15) and after recovery (days 10 and 16). At the end of the study, discs were snap-frozen in cryocompound. Formalin-fixed cryosections were stained with Safranin O/Fast-Green.

RESULTS: After one week of loading, degenerative culture conditions induced a slightly higher disc height loss than physiological conditions (~3% higher). During the second week of culture, fibrin had a positive influence on the disc height recovery after repeated loading. In fact, degenerating discs filled with fibrin showed a better disc height recovery after free swelling than empty controls (~6% and ~1%, respectively) (Figure). Safranin-O/Fast Green stained sections showed that fibrin gel fully occupied the nucleotomized region and MSCs were homogenously distributed in the fibrin. No differences among groups were observed in the tissue surrounding the defect.

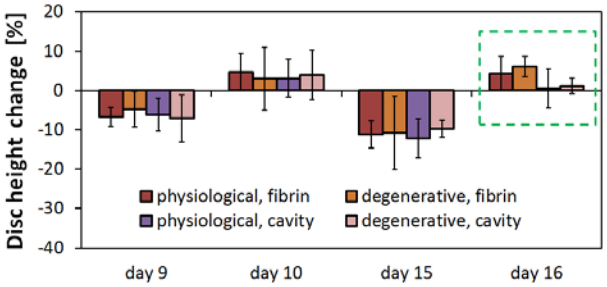

***Figure**: Disc height change (compared to disc height after dissection) in nucleotomized IVDs after loading and recovery. Red and orange are fibrin groups, purple and pink the groups without fibrin. Dashed rectangle highlights that fibrin promotes recovering of disc height.*

DISCUSSION & CONCLUSIONS: If a nucleotomy is required, the maintenance of disc height is of primary importance to avoid pain reoccurrence due to vertebral contact or re-herniation. The chosen fibrin gel (60 mg/ml fibrinogen) was able to withstand repeated loading without loss of disc height and may be a valid alternative to bare nucletomy. While the stem cells did not have an effect on the disc height, they could play a role on the gene level. Analysis of gene expression and histology as well as experiments with similar settings, but with an observation period of 2-3 weeks after nucleotomy, may add important information in the future.

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Engineering sticky surfaces for cartilage repair

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INTRODUCTION: Cell-based cartilage repair strategies such as autologous chondrocyte transplantation (ACT) suffer variable clinical outcome partly because there is little control over the retention of the transplanted cells and the microenvironment in which they reside. We utilize covalent interactions, based on the formation of Schiff-base linkages, to rapidly adhere cells to their neighbors and/or to damaged tissue. By positioning of the amino and aldehyde bearing polymers on surfaces, high density microtissues were formed. The chondrogenic potential of these engineered microtissues was compared to conventional pellet culture.

METHODS: Chitosan (95.7% deacetylation, Heppe Medical Chitosan GmbH) was succinylated to be soluble at pH 7 (1) and LVG alginate (NovaMatrix) was oxidized with sodium periodate (2). Aldehyde content was measured using an infrared spectrophotometer in attenuated total internal reflection mode (Frontier Spectrometer ATR-FTIR, Perkin Elmer). Nanofilm buildup of oxAlginate and succinylated chitosan was followed by quartz crystal microbalance with dissipation (QCM-D). To form microtissues, human mesenchymal stem cells (Lonza) and adipose-derived stromal cells coated by a 2% chitosan solution were dropped into 5uL volumes of oxAlginate. Microtissues were gelled for 10 min and were transferred to agarose coated-wells and cultured in chondrogenic media for up to 21 days.

RESULTS:
The high density microtissues made via Schiff base linkages formed very rapidly (within 3 min). During the 21 day culture, they were visibly larger than pellets made by conventional centrifugation. The engineered microtissues also stained much stronger for alcian blue (Figure 1), suggesting that glycosaminoglycan production was strongly induced, a result confirmed by RT-PCR.

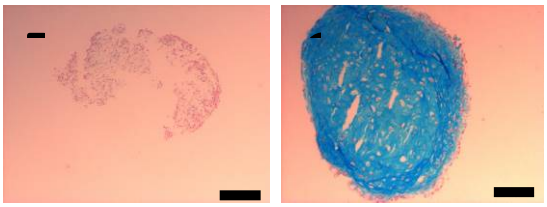

Fig. 1: Human mesenchymal stem cells formed by convention centrifugation (left) and by Schiff based-mediated adhesion (right).

The FTIR results revealed the presence of an additional peak at wavenumber 1735 cm-1 which confirmed the formation of the aldehyde group. QCM results showed that nanofilm buildup was dependent on the oxidation of the alginate and no buildup could be detected when unmodified alginate was used.

DISCUSSION & CONCLUSIONS: The transplantation of 3D microtissues instead of single cells has many clinical advantages for regenerative medicine. Many types of cells better retain their phenotype in 3D culture, however, conventional pellet formation often fails or takes several days. Here we introduce a novel technique to form almost instantaneous high cell density microtissues. These microtissues can also be formed adherent to a tissue surface by simply "painting" the desired substrate with the oxidized alginate component.

Functionally, the microtissues produced by this technique promoted superior chondrogenesis and could be used to improve the retention and microenvironment of chondrocytes and/or stem cells in cell-based treatments of cartilage lesions.

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Bioactive and biomimetic scaffolds for cartilage regeneration

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In the biomedical field interest is increasing for the development of self-assembling materials with new biological and biomechanical properties to be used in cellular therapy for cartilage regeneration. During this project we have developed of a new class of material by molecular self-assembling in order to obtain 3-dimensional (3D) scaffolds which could provide an early microenvironment to foster adult stem cells to undergo chondrogenesis. The material platform obtained, basically made by the simple combination of two components: a self-assembling peptide RAD16-I (Puramatrix) and a polysaccharide molecule, such as hyaluronic acid (HA) or heparin; lead to a hierarchical self-assembling scaffold with newly structural and mechanical properties. Finally, combination of self-assembling peptide hydrogels and woven PLC scaffolds was also studied in order to obtain 3D-constructs to study stem cell maintenance and differentiation. Adult stem cells (from bone marrow as well as adipose tissue) were used to test the capacity of these scaffolds to support chondrogenesis. In presence of specific chemical inducers the 3D-constructs acquired biological and biomechanical properties of cartilaginous tissue, by assessing the presence of specific biological markers (by western blots, real time PCR, and GAGs staining) as well as global mechanical properties measured by dynamic mechanical analysis (DMA). These results indicate that the scaffolds obtained by molecular self-assembling and in combination with woven PLC can be used in future combined cellular therapies to promote cartilage repair/regeneration.

Advanced tissue engineering scaffold designs based on TPMS structures prepared by stereolithography

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INTRODUCTION: In tissue engineering, the architecture and the complexity of the porous scaffold may be crucial for the successful regeneration of tissues and organs. More sophisticated porous structures with advanced microarchitectures of the pore network and scaffolding structures that mimic the intricate architecture and complexity of native organs and tissues are then required. Hence, scaffold designs must allow the creation of a variety of porous structures to attain appropriate mechanical functions, suitable mass transport, promote efficient cell adhesion, -proliferation and -differentiation and subsequently reconstruction of a desired tissue. Stereolithography (SL) is a high precision rapid prototyping process that can produce 3D objects with specific predetermined geometries, porosities and morphologies that can be utilized to generate the complex structures required for tissue engineering. The potential benefits of specific architecture based on minimal surface energy such as triply-periodic constant mean curvature surfaces (triply periodic minimal surfaces, TPMS) as tissue engineering scaffold have been investigated recently.[1,2] It was shown that the sophisticated substrate geometry of a TPMS enhances cell migration and vitalization, while retaining a high degree of structural stiffness. The aim of this work is to prepare a series of different tissue engineering scaffolds with TPMS pore network architectures by SL using photocurable resins based on poly(trimethylene carbonate) (PTMC) and poly(D,L-lactide) (PDLLA).

METHODS: Different TPMS structures were first designed using a 3D mathematical drawing functions software (K3Dsurf), after which conversion to the .STL file format was done using CAD software (Rhinoceros 3D). The designed scaffolds were then built with a SL apparatus (EnvisionTec Perfactory) using PTMC and PDLLA dimethacrylate (5 kg/mol) resins as previously described.[2] The mechanical properties of the designed scaffolds were determined in compression. The scaffolds were analyzed in terms of their porosity, pore size and pore size distribution using µCT and SEM

RESULTS: Based on trigonometric functions, a series of TPMS structures (8 different microstructures) with 70 % porosity were successfully prepared by SL (Figure 1). The SEM images illustrate the precision of the microstructures built by SL. The µCT analyses allowed making accurate reconstructions of the scaffold.

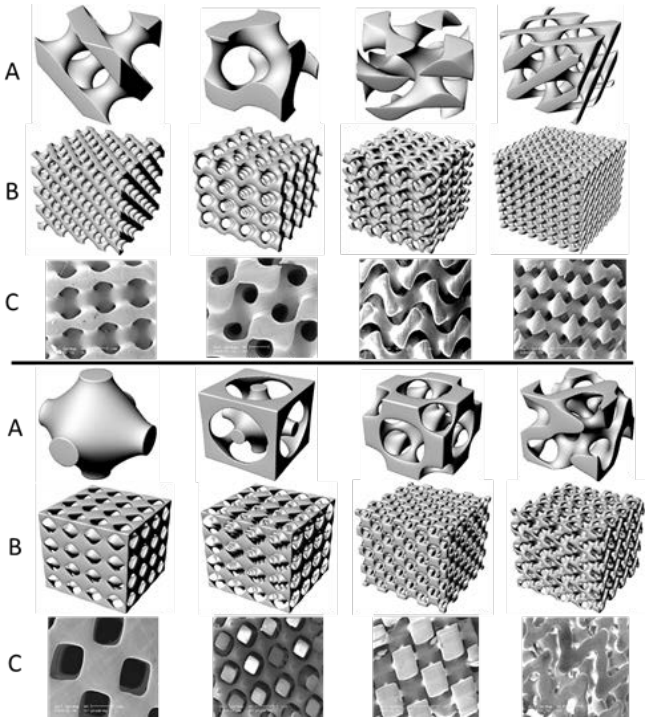

Figure 1: Visualization of eight different pore network architectures. The CAD-designs represent repeating unit cells (A) and assemblies of 4x4x4 unit cells (B). SEM images of the built structures prepared from the PTMC resin are shown in (C).

The results showed that all scaffolds have perfect interconnectivity with a high surface areas.

Compression testing of these TPMS scaffolds showed that in addition to the type of resin used (PTMC- or PDLLA- based resin), the specific microarchitecture also has a pronounced effect on the mechanical properties of the scaffold. **DISCUSSION & CONCLUSIONS:** This work shows the suitability of SL to prepare sophisticated biodegradable scaffolds with TPMS pore architectures. The diversity of the TPMS designs and consequently the diversity of the properties of the prepared scaffolds, allow them to be used for specific tissue engineering applications.

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ACKNOWLEDGEMENTS: Financial supported by AO foundation is acknowledged.

Vertical osteoconductivity and early bone formation of different implants in a subperiosteal model

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INTRODUCTION:

Osteoconductive characteristics of different implant surface coatings are in the focus of current interest. The aim of the present study was to compare the vertical osteoconductivity at the implant shoulder of supracrestal inserted calcium-phosphate coated implants (CaP), Titanium-Zirconium (TiZr), Titanium (Ti) with conventional sand-blasted/acid-etched (SLA) implants in a rabbit model.

METHODS:

There were used implants with a similar geometrical design: Bioactive (CaP) and SLA implants (both Alfa Gate Implant System, Israel), Titanium (Ti) and Roxolid (TiZr) dental implants (both Straumann" Bone Level System; Institut Straumann, Switzerland) were used. The Ti and TiZr implants had the modified sand blasted, large grit and acid etched, hydrophilic (SLActive) surface. Implant length (8 mm) and diameter (3.3 mm) were the same as well. The study was planned in accordance with the ARRIVE guidelines and performed on 12, 9-month-old, 4–5 kg, New Zealand white rabbits as an experimental animal model. Each rabbit received 1 TiZr implant, 1 CaP implant on the right tibia bone; 1 Ti and 1 SLA implant on the left tibia. Though, implants were inserted only 5 mm into the bone and 3 mm remained above the bone level (supracortical) to examine possible vertical osteoconductive properties (Fig. 1). The animals were randomly allocated to three groups regarding the observation periods: 10, 20 and 30 days.

RESULTS:

The quality of bone and vertical bone apposition was measured. The following parameters were evaluated at an original magnification of x 10:

1. Percentage of linear bone fill (PLF; %): this was determined at the mesial and distal implants' shoulders. In brief, the relation between the total volume and the new-formed bone in a 3 x 3 mm region of interest above the cortical bone was evaluated. Total mean values were calculated.

2. Marginal vertical bone height (VBH; mm): within the chosen 3 x 3 mm region, the height of new mineralized bone was measured on five, arbitrary chosen and equally distributed points at the mesial and distal side. Mean values were calculated for each sample.

3. Percentage of vertical bone-to-implant contact (vBIC; %): this value was calculated as the length of the implant surface along the 3 mm of exposed implant surface that was in direct apposition to (new) bone x 100%.

Fig. 1: Clinical picture of the inserted implant. The cortical bone stayed intact during the cleaning procedure.

DISCUSSION & CONCLUSIONS:

A significant delay in vertical osteoconductivity at the earliest time point under examination was seen for TiZr implants when compared to their Ti counterparts. For the later points, TiZr as well as Ti implants demonstrated comparable values in this animal model. The results showed for the first time that calcium-phosphate coated surfaces on supracrestal inserted implants have vertical osteoconductive characteristics and increase the bone-implant contact at the implant shoulder. PLF was significantly higher in SLA-CaP implants (11.2%). BIC-D was significantly increased in the SLA-CaP implants (13.0%) as well.

Role of a microporosity on bone integration

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INTRODUCTION

β-tricalcium phosphate (β-TCP) is an excellent degradable bone graft and has been successfully used as scaffold for bone tissue engineering substitute. Many scientific studies have investigated the influence of material parameters such as porosity, crystallinity, and geometry on degradation behaviour and integration into bone. Interest continues to focus on macroporous β-TCP ceramics with pores over 100 µm in diameter. Little attention has been paid to micropores with diameters between 0.5 µm and 10 µm. Only in recent years has it become clear that microporosity has a considerable effect on osteointegration and neoformation of bone. Habibovic et al. [1] reported that microporosity elicits osteoinductive effects in both ectopic sites and critical size bone defects. There is some direct evidence and also speculation as to why this occurs. Some authors found cells inside micropores [2, 3]. These cells are likely to retain access to nutrients and expel waste by the same mechanism as osteocytes do. The lacunar–canalicular network, is used for nutrient and waste transfer, as well as for communication between the osteocytes via gap junctions. Hypothesis: Cells can occupy micropores. The interconnectivity of ceramic scaffold build a network and this is used for nutrient and waste transfer.

EXPERIMENTAL METHODS

Microporous (5 µm) cylindrical plugs of ß-TCP (diameter, 7 mm; length, 25 mm, porosity 40%, axial failure load 7200 N / cm ²) with interconnecting pores were used. Sheep studies were conducted to test applicability of microporous pure ß–tricalcium phosphate (ß-TCP) ceramic for anterior cruciate ligament (ACL) reconstructions and tissue engineering of bone and osteochondral defects. Scaffolds were placed in medial femoral condyles [4], [5] or in the area of origin of the anterior cruciate ligament [6]. After 6, 12, 26 (all studies), and 52 weeks (only one study), 7 animals from each group were euthanized and investigated. The healing process was examined by histology, histomorphometry, immunohistochemistry, and electron microscopy. Histological grading scales were used for assessment of bony integration. Statistical analysis: t-test, Mann-Whitney-U-test, Wilcoxon-test with statistical significance $p < 0.05$.

RESULTS AND DISCUSSION

We showed the degradation of TCP starts always at surface of the ceramic. Inflammatory cells were rarely seen. Scanning electron microscopy revealed persistence of the interconnecting porosity of the TCP implant, incorporation of TCP into bone and connective tissue surrounding TCP particles. Degradation went hand in hand with bone neoformation. The proportion of lamellar bone in the TCP implant at the bone-implant interface increased significantly ($p<0.001$) between 6 and 52 weeks. After 52 weeks the mean proportion of the resorbed TCP implant was 81% and bone structure looked like native bone. The degradation process involved osteoclast-type giant cells that were demonstrated at every time point. Cells and tissue were found within the ceramic. The tissue was distributed uniformly throughout the ceramic and was positive for collagen I and TGF beta. Bone formation, and thus mineralisation began around the edge of the ceramic; this was confirmed by TEM and ESEM. The typical fibrillary structure of collagen was seen in the border zone between bone and ceramic. The cells within the ceramic behaved like osteocytes in spongious bone. Cells expressed markers (CD68, CD45) that show a phenotype similar to monocyte–derived mesenchymal progenitor cells. This cell type has been shown to differentiate into osteoblastic cells, producing mineraized matrix and expressing bone markers [7]. In 2010, Levengood et al. [2] investigated the osteointegration behaviour of a biphasic ceramic (87% HA and 13% β-TCP) that possessed both macropores and micropores. They found cells, soft tissue, osteoid or partially mineralised bone in the micropores, comparable to the present study. Levengood et al. spoke of migration of osteogenic precursor cells into the micropores and their subsequent differentiation to osteoblasts, osteoid, and mineralised tissue. The structure of the ceramic and the interconnecting micropores favoured this process.

CONCLUSION

We showed that microporosity (5 µm) positively influence bone regeneration. Cells can migrate and reside in the micropores and differentiate to osteoblasts and later transformate to osteocytes.

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Interaction of rhBMP-2 and TG-VEGF on osteo- and angiogenesis in a novel rabbit model

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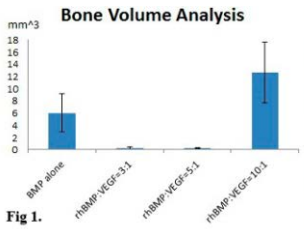
INTRODUCTION: It was already shown in vivo that tissue engineering of large amounts of heterotopic bone can be achieved [1]. Further on it was detected that suitable delivery systems such as fibrin gel provide a prolonged retention and release of bone morphogenetic proteins (BMPs) and vascular endothelial growth factor (VEGF) may continuously stimulate bone formation [2]. This novel animal model investigated interactions between different dosage relations of recombinant human BMP-2 (rhBMP-2) and transgenic vascular endothelial growth factor (TG-VEGF) on heterotopic bone formation around vessels deprived from skeletal muscle contact using a membrane.

METHODS: Twelve adult New Zealand white rabbits were used. Preoperatively, they were anesthetized by a veterinarian. Skin incision was performed on the medial side of the right upper thigh in supine position under general anaesthesia. After dissection and mobilisation of the femoral vascular bundle, an Inion® membrane cylinder was wrapped around it and filled with fibrin sealant carrying different dosage relations of rhBMP-2 and TG-VEGF. Twelve rabbits were assigned to 4 groups each with 3 animals; Group A: rhBMP-2 alone; Group B: rhBMP-2: TG-VEGF=3: 1; Group C: rhBMP-2: TG-VEGF=5: 1; Group D: rhBMP-2: TG-VEGF=10:1.

Wound closure was performed in layers. Eight weeks postoperatively the animals were sacrificed and the specimens harvested. Micro-computed tomography (micro-CT) was used for quantitative evaluation of the bone formation in the cylinder. Histology and immunohistochemistry were applied to evaluate new vessels formation within the cylinder.

RESULTS: Intra- and postoperative course presented uneventful in all animals. Micro-CT and histological examination demonstrated new bone formation within the cylinders. Higher ratio of rhBMP to VEGF increased the bone volume (Fig. 1.). Immunohistochemistry staining of the specimens with CD31 showed new vessel formation within the cylinder (Fig. 2.).



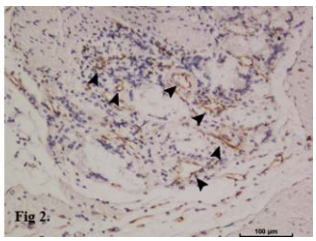


Fig. 1: Quantitative micro-CT analysis of bone formation. Fig. 2: Immunohistochemical staining of CD31 (arrows).

DISCUSSION & CONCLUSIONS: Heterotopic bone formation was found around vessels without direct contact to the surrounding skeletal muscles due to the membrane. The 10:1 ratio of rhBMP-2 to TG-VEGF increased bone formation within the cylinder. Ongoing investigation will clarify eventual dependence between new vessel formation and different rhBMP-2 : VEGF ratios.

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Medial collateral ligament regeneration using the recombinant human amelogenin protein (rHAM$^+$), in a rat model

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INTRODUCTION: Ligaments play an essential role in mediating normal movement and stability of joints. Injuries to ligaments are common, painful and debilitating, causing significant joint instability, which may lead to the development of degenerative joint disease. In most cases, healing of a torn ligament fails to take place, and replacement tissues or grafts are required. When healing does occur the mechanical properties do not return to normal. To date, no treatment enables regeneration of injured ligaments. The medial collateral ligament (MCL) of the knee is a relatively superficial ligament, rendering it a good model for ligament regeneration studies. Previously we showed that *in vivo* application of the recombinant human amelogenin protein (rHAM$^+$) alone caused significant and progressive regeneration of all three tooth supporting tissues; alveolar bone, periodontal ligament and cementum, after induction of chronic periodontitis in the dog model, through recruitment of CD105-positive mesenchymal stem cells (MSCs). Hence, we assumed that amelogenin might have a role also in skeletal ligament function and regeneration.

METHODS: The rat full thickness MCL tear model was used. 71 Sabra rats were operated. Various concentrations of rHAM$^+$ dissolved in its propylene glycol alginate (PGA) carrier (experimental) or PGA carrier alone (control) were applied to the transected MCL of the right knee. 12 weeks after transection, the force to failure (mechanical strength) and stiffness of the ligaments treated with 0.1-5µg/µl rHAM$^+$ was measured and compared to the PGA-control. Ligament structure and composition was studied using histology, electron microscopy and immuno-histochemical reactions using antibodies against collagen I and III. The number of mesenchymal stem cells (MSC) was measured four days after the transection using immuno-fluorescence reactions with antibodies against CD105, STRO-1 and CD90. The number of proliferating cells was also measured using anti-Ki67 antibody.

RESULTS: 12 weeks after full transection, the transacted MCL treated with rHAM$^+$ (dose dependently) were stronger and stiffer than the control- PGA treated MCL, and on average equal to the normal untransected MCL. The collagen fibbers arrangements of the regenerated MCL were similar to the normal untransected MCL, while the control PGA treated MCL seemed to arrange like a scar tissue. Four days after the transection significantly more cells expressing MSC markers were found in the granulation tissue between the stumps of the experimental MCL, compared to the control. Furthermore, the number of proliferating cells was significantly higher in the experimental MCL compared to the control, after four days.

DISCUSSION & CONCLUSIONS: rHAM$^+$ caused significant regeneration of the mechanical properties and composition of fully transected rat MCL. Applications of rHAM+ induced directly or indirectly increase in MSC amount at the injured site. The increased proliferation at the injured site four days after the injury may explain the increase in MSC amount, though our previous tissue culture experiments showed that rHAM$^+$ induced also cell recruitment.

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An academic biologist's perspective on research translation

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Research translation has traditionally been viewed as a linear activity where the laboratory scientist makes fundamental discoveries that are taken into animal models which, if successful, lead to testing in human subjects participating in clinical trials. Different experts serve as links in the chain (figure 1).

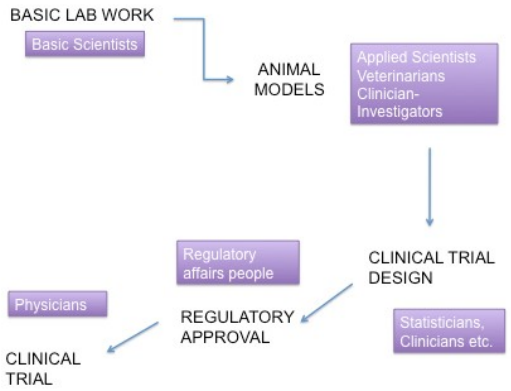

Figure 1 A linear model of research translation

The problem with this model is that it ignores the reality that research translation is a field unto itself and not the mere transfer of promising laboratory findings into the hands of a clinician investigator. Indeed, the laboratory scientist is often only dimly aware of the constraints imposed by regulators, financial considerations and clinical expediency. Under these conditions there is the danger of devoting years to the development of beautiful laboratory artifacts that will never see the light of clinical day. Similar observations could be made concerning the other links in the chain [1].

A better model is one in which the participants are in full communication from the beginning (figure 2), so that a scientist with a hot idea can discuss its clinical viability before devoting too much time, money and energy to a flawed enterprise.

Achieving such a translational environment is difficult in academia – certainly in most US departments of orthopaedic surgery that I am familiar with, where there is increasingly a powerful financial imperative for surgeons to perform as much surgery as possible. The decline of the clinician-investigator is widely lamented. The PhD scientist, on the other hand, is often on short, soft money contracts that are not well suited to the lengthy, publication-poor process of research translation. Moreover, it is difficult to identify individuals within academia with whom to spend quality time in addressing the translational process. Most academic institutions lack a core facility, or equivalent, that provides guidance on this issue [2].

Under these conditions, the investigator may hand the project off to an agency that is skilled in this area, typically a drug company. For obvious reasons, pharmaceutical companies have much experience in clinical translation, especially for traditional small molecule drugs. Biologics are a more recent development and the roadmap to the clinic is less clear and still evolving. In the absence of industrial support of this nature, the investigator is obliged to give up and return to the bench, or go it alone. The latter is possible, but slow, inefficient and frustrating, especially in the field of gene therapy, arguably the most problematic of the biological therapies from the regulatory point of view [3].

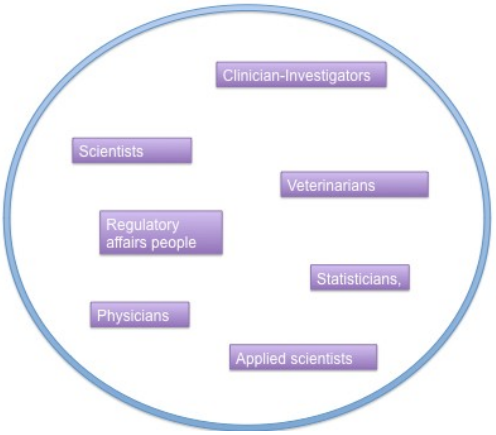

Figure 2 A translational research environment

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Cartilage repair; barriers and strategies for translation clinical perspectives

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INTRODUCTION: The research field of cartilage repair has experienced a tremendous growth in the last twenty five years since the first human autologous chondrocyte implantation in 1987[1]. With first promising results in pre-clinical animal studies, that first example of clinical cartilage tissue engineering became ready quite fast to be translated from the bench top to the bedside[1]. However, still the translation from basic science to clinical practice is slow and difficult to perform.

CURRENT STRATEGIES: In the scientific literature, there has been many published research discoveries that, for the most part, have not been successfully brought into the clinical use. One main problem with translational research is explained by an academic situation that hinders collaboration between clinical and laboratory-based investigators[3]. The ideal situation is basic scientists and clinical doctors working together with the ultimate goal to deliver a product for clinical direct use. The basic science is a fast process when testing cells in vitro models, little more time consuming when testing the theories in animals models but far too more complicated when finally transferring the technology to the human trials with many patients and long-time follow ups in years to follow. Traditionally, the ones of bridging the gap between laboratory and clinic have been M.D./Ph.D.'s or other physician-scientists with training in both the clinical and laboratory areas[3]. Many of the PhD-students go into purely clinical practice after their exams. They are then pressured to see more patients and spend less time in the laboratory. At the same time, laboratory researchers who obtain salary support from research institutions and other grants are pressed to follow a more traditional, basic science career that is better suited to earning grant support to keep the laboratories alive, reducing the possibilities form clinical connections[3].

With increasing costs for the development of new methods of cartilage repair, we need to restructure traditional approaches to basic and clinical research in order to facilitate the rapid, efficient integration and translation of new technologies $

into novel effective therapeutics. A continuous collaboration between basic scientists and clinical researchers with flow of knowledge going in both directions including education, seminars, workshops very close to the cells in manipulation and to the daily clinical reality[2].

MAJOR ROADBLOCKS: The roadblocks are between the bench side to bedside but also from bedside to the community when transferring the novelties to a broad clinical road. Today, the largest roadblocks are the new cell regulations, complicated and very expensive to fulfil to bring a product to the market. A re-evaluation of how to regulate cells used in cartilage repair is warranted to ease up the clinical translatation

DISCUSSION & CONCLUSIONS: How to transfer new technologies in cartilage repair to the clinical side is presented including a discussion on regulatory aspects, economy and multicentre randomised trials **REFERENCES:**

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Translating concept to product for articular cartilage repair: barriers and strategies

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INTRODUCTION: The development of a tissue engineered product for articular cartilage repair or replacement represents a major goal for many academic and industry groups, and has the potential to have a major clinical impact and commercial success. This field has been active for more than 20 years, however the limited number of products on the market demonstrates the difficultly in achieving the required effectiveness, safety and commercial feasibility. The pre-clinical and clinical studies that must be successfully completed to demonstrate safety and effectiveness are accompanied with meeting manufacturing and regulatory needs, and will included fundraising, patent applications and business plans.

CURRENT STRATEGIES: The design of products for articular cartilage repair and regeneration may be simple or complex, and each concept may have specific advantages and disadvantages. They may range from materials, soluble mediators, cells and tissues, and multiple combinations of these. They may be regulated as a device or biologic, and may involve allogeneic or autologous approaches.

The complete project should first be planned using representation from all major programs (for example, research, product development, manufacturing, business development, lawyers, clinicians, clinical trials, regulatory pathways, etc), ensuring that all requirements can be met, and identifying potential challenges. This process can provide great insight into the validity of the proposed product, and may be able to identify insurmountable challenges or new opportunities. The technical hurdles and clinical effectiveness must also be accompanied by a realistic understanding of the size of the addressable market and an economic rationale.

The design of a product must meet the clinical need, but it must also be possible to manufacture the product in a uniform and efficient process capable of generating the number of units to meet the predicted need. Early design activities should minimize complexity in the product design, ensuring the key features are necessary and participate in the mechanism of action. The early design phase should encompass necessary safety features including sterility or aseptic manufacture. The design should also consider cost to manufacture (cost of goods) and ensure that this will fit into the business model.

Appropriate animal models to test safety and effectiveness should be used and agreed upon with regulatory agencies before performing critical animal trials. The clinical trial design must test effectiveness and safety, but be achievable. For articular cartilage trials with stringent inclusion and exclusion criteria there is a risk of not being able to complete a trial due to lack of patient recruitment.

Understanding the market is critical. The addressable market size, and needs of the surgeon, patient, hospitals and health systems (payers) must all be understood. While the general field of articular cartilage repair represents an extremely large clinical need, the addressable market for a specific product regulated for a specific application may be much smaller, and can be influenced by the particular articular joint, size of the defect to be repaired, age of the patient, etc. Frequently the market size projections are excessively optimistic, but given the costs of developing such products a realistic market size projection and a positive return on the investment made are obvious requirements. This understanding can therefore substantially influence the design of a cartilage repair product for a specific application.

DISCUSSION: Repair of articular cartilage remains a major technical challenge and clinical opportunity. Success of a product will depend many factors and the clinical effectiveness of a particular technology is only one of those factors. A comprehensive and coordinated approach that addresses the complete research and development, clinical and business programs is necessary for a compelling technology to be clinically and economically successful.

Regulatory perspectives on the translation of chondrocyte implantation products

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INTRODUCTION: There is a need to better support the transfer of cell therapy and tissue engineered products to the EU market. EMA and its committee for advanced therapies (CAT) has therefore identified the needs for a more flexible approach for ATMPs once nationally approved and that since the end of 2012 need to be authorised in the centralised procedure within EU. The main objectives for a success have been highlighted in the CAT work programme 2010 - 2015 (1).

CURRENT STRATEGIES: One of the actions made within the CAT work programme was a survey addressed to developer of ATMPs and whose products were legally on the national market. From that survey one could identify several developers of chondrocyte implantation products with different clinical experience but with a few of them having been used for treatment of approximately 15000 patients over the last 10 to 15 years. These products were used for repair of cartilage defects and used mainly in patients with cartilage defects of the knee.

The assessment of a product within the centralised MA follows a multidisciplinary approach where quality data, non-clinical data, clinical data, and published data complement each other. The technical requirements for quality of an ATMP are defined in Annex I (Dir. 2009/120/EC) and more specifically for chondrocyte implantation products can be found in the CAT Reflection paper on in-vitro cultured chondrocytes (2). Although, based on a risk-based approach in accordance with Dir.2009/120/EC, some specific considerations to address these requirements may be applicable to the products, taking into account scientific knowledge generated so far and the experience gained by the applicant during the time its use was nationally approved. The main objective when filing the Quality part of the MAA dossier should be to document the current quality profile and consistency of both the product and the production process, using all possible data and evidence gained all along the lifecycle of the product as supportive data to justify the development strategy and the current set of specifications proposed (3).

MAJOR ROADBLOCKS: Any flexibility in relation to agreed quality requirements according to applicable CAT guidance can only be justified in cases where the benefit/risk profile remains positive and the safety of the patient is not compromised. For some ATMPs with clinical experience at the national level, documentation of pharma-ceutical development and of quality data might not be fully in accordance with standard pharmaceutical approaches and current regulatory guidance as expected for MA within EU. It may be acceptable to have reduced testing at one level of manufacturing provided an exhaustive control is performed at the other.

Lack of proof-of-concept studies may be substituted by sufficient clinical data. The relevance of the clinical data submitted to substitute for non-clinical data should be justified and will depend on the quality of the submitted data.

DISCUSSION & CONCLUSIONS: The technical requirements for cell-based medicinal products may be seen as hurdles hard to overcome. It is too early to say if a more flexible approach as discussed in the CAT work programme will give support to the entrance of new innovative products within the field of tissue engineering and more specifically for chondrocyte implantation products on the EU market. The numbers of ATMPs that have reached the stage of the MA process are still relatively few.

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Engineered coral, an optimal scaffold for tissue engineering of bone.

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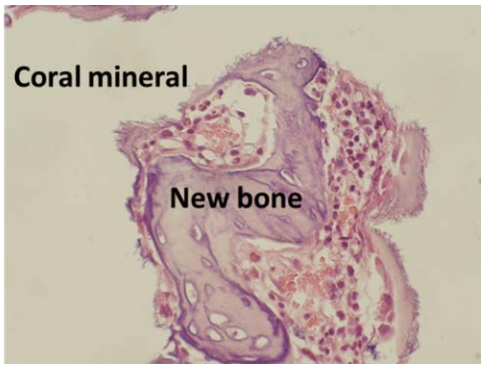
INTRODUCTION: Generation of bone is the challenge faced by current technologies in tissue engineering, depending very much on bioactive scaffolds and on osteoprogenitors. The ultimate goal is to regenerate and replace structural and functional deficits of tissue, beyond its natural healing capacity. Repair or engineering of bone depends on accurate sequence of wound healing and the ability of the grafting material to interfere minimally in the development of provisional matrix and in the development of the bone phenotype. An early cell-scaffold interaction is a crucial determinate to initiate a sequence of cellular and molecular events activating osteogenic pathway. Histogenesis of bone can be predicted as early as day 3 in ectopic rat model (Bahar et al, 2010).The presence of organic bone components are known to activate generation of bone, consisting of ligands adhering to cell integrins. Also, it is accepted that mesenchymal cells in the bone vicinity are interacting with tricalcium phosphate mineral to form bone. Calcium carbonate in the form of Aragonite crystals, the exoskeleton of coral was shown to be a passive scaffold allowing bone to form in close proximity to this mineral. In the present study we have modified the exoskeleton mineral of coral grown in aquariums by incorporating calcium silicate in its intrinsic structure. We tested the modified coral mineral scaffold for its ability to attract mesenchymal cells to adhere and transform into active osteoblasts that produce bone directly on the coral mineral surface.

Methods: Our DA rat model was employed to test osteogenesis when modified coral was mixed with fresh marrow from same strain animals(Bahar et al, 2010).

Our results revealed that by enrichment of the coral aragonite mineral with calcium silicate the modified coral mineral acquired properties to attract mesenchymal cells to adhere to its surface and to exhibit the osteoblast phenotype.

In conclusion, coral aragonite mineral can be enriched by calcium silicate that modifies its ability to interact with mesenchymal cells, transforming them into active osteoblasts.



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Inhibition of osteogenic differentiation of MSCs by neutrophils

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INTRODUCTION: Multipotent stromal cells (MSCs) and their differentiated progeny contribute to fracture healing by synthesizing collagens and inducing mineralization of extracellular matrix (ECM). The process of healing includes an inflammatory phase, and the influence of this inflammatory phase on the activity of the MSCs in not yet clear [1]. The current literature suggests that neutrophils, the most prevalent inflammatory cells, negatively affect fracture healing and contribute to the high incidence of nonunion in severely injured patients [2]. Based on these studies, we hypothesized that neutrophils inhibit proliferation and osteogenic differentiation of MSCs, as well as ECM mineralization by MSCs. To test this hypothesis we have co-cultured MSCs with different neutrophil concentrations and studied the effect of neutrophil co-culture on 1) proliferation and osteogenic differentiation of MSCs and 2) on ECM mineralization by MSCs in vitro.

METHODS: Human multipotent stromal cells were isolated from Reamer Irrigator Aspirator (RIA) drilling residues by plastic adherence. Neutrophils were isolated from peripheral blood of healthy donors and co-cultured with MSCs. The effect of different neutrophil concentrations on MSC proliferation and osteogenic differentiation was measured after 1 week using the number of nuclei and Vector Red for quantification of Alkaline Phosphatase activity, respectively. The effect of co-culture on ECM mineralization was measured after 2 weeks using Xylenol Orange staining in an array scanner.

RESULTS Neutrophils exerted a concentration-dependent effect on differentiation of MSCs in vitro. Low concentrations did not inhibit differentiation, whereas high neutrophil concentrations inhibited proliferation and osteogenic differentiation of MSCs in vitro, as well as the ability of MSCs to induce ECM mineralization.

DISCUSSION & CONCLUSIONS: Our data suggests that high neutrophil concentrations inhibit proliferation and osteogenic differentiation of MSCs, as well as the ability of MSCs to induce ECM mineralization. Systemic inflammation as seen in severely injured patients is associated with a high influx of neutrophils in to the fracture hematoma. High neutrophil concentrations within the fracture hematoma after major trauma may impair fracture healing through a negative effect on MSCs.

Acknowledgements: This study was supported by an AO Start Up Grant

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RUNX2 is a potential transcriptional regulator of ST2 in growth plate chondrocyte

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INTRODUCTION:

For many years, the interleukin-1 receptor family member ST2 was an orphan receptor that was studied in the context of inflammatory and autoimmune disease. However, in 2005, a new cytokine — interleukin-33 was identified as a functional ligand for ST2.[1] IL-33/ST2 was shown as an intercellular signalling which participates mainly in immune system–related disorders such as pulmonary inflammation, asthma and rheumatoid arthritis.[2] Recent studies showed that this signalling is also an important regulator of osteoclastogenesis.[3] However little is known about the role of ST2 signalling in skeletal metabolism especially cartilaginous tissue. In this study we aimed to investigate the role of the ST2/IL-33 signalling pathway in the growth plate. The growth plate is the cartilaginous organ at the end of long bones where endochondral ossification occurs, the process leading to longitudinal bone growth. One of the most important transcription factors regulating growth plate chondrocyte differentiation and maturation is RUNX2. As we found several putative RUNX2 binding sites (PuACCPuCA or TGTGGT or [T/A]CCCACA) on the ST2 promoter we hypothesized that RUNX2 is capable of regulating the ST2 gene at a transcriptional level within the growth plate. To conduct our study we used the murine ATDC5-chondrocyte cell line and tibial bones from euthanized Wild-type Swiss mice.

METHODS: Gene silencing using siRNA, Immunoblotting, RT-PCR, Real Time RT-PCR, and Immunohistochemistry.

RESULTS:

RT-PCR results showed the endogenous expression of the ST2 signalling pathway components ST2 (both sST2 and ST2L) and IL-33 in ATDC5-chondrocytes. While the ST2 transcript was highly expressed, the IL-33 transcript was expressed to a lower extent. Furthermore we were able to detect the expression of ST2 and IL-33 at protein level by Immunoblotting. Immunohistochemistry performed in murine tibiae showed a moderate to strong expression of ST2 (depending on stages of chondrocyte differentiation) and a moderate expression of IL-33 in the growth plate. Runx2 was highly expressed on mRNA and protein level in ATDC5 chondrocytes and also in the growth plate of murine tibiae, especially in chondrocytes in later stages of differentiation. The silencing of RUNX2 led to a highly significant downregulation of ST2 transcripts in ATDC5 chondrocytes.

DISCUSSION & CONCLUSIONS:

In our study we were able to show for the first time that ST2/IL-33 signalling, i.e. its components are present in the growth plate. We detected a strong expression of ST2 and a moderate to low expression of IL-33 in prehypertrophic and hypertrophic murine growth plate chondrocyte. Interestingly, silencing of RUNX2 led to a significant decrease of ST2 expression. Based on this result and given the fact that ST2 and RUNX2 are concomitantly expressed in prehypertrophic and hypertrophic growth plate of murine tibiae we conclude that RUNX2 might be able to regulate ST2 expression on a transcriptional level.

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Guiding chondrogenesis and osteogenesis with hydroxyapatite and BMP-2 incorporated within high-density hMSC cultures for bone and cartilage regeneration

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INTRODUCTION: Tissue engineering (TE) solutions are necessary to meet the great clinical need for bone and cartilage repair. Multipotent and easily accessible, human bone marrow-derived stem cells (hMSCs) are an attractive cell source for these applications. Hydroxyapatite (HA) has been widely used for bone TE due to its osteoconductivity and chemical resemblance to bone mineral, and has also been reported to promote cartilage repair in vivo [1]. While BMP-2 is a well-known osteogenic inducer and has been shown to enhance chondrogenesis when combined with TGF-β1 or -β3, it is unclear whether BMP-2 alone can reliably induce cartilage formation by hMSCs. To explore the roles of HA and BMP-2 on chondrogenesis and osteogenesis in high-density hMSC cultures, a system of aggregates incorporated with mineral coated hydroxyapatite microparticles (MCHA) capable of sustained BMP-2 release was developed. The inclusion of BMP-2-loaded MCHA may overcome the transport limitations and time/cost inefficiencies of exogenous supplementation.

METHODS: *MCHA synthesis.* MCHA were produced by incubating HA microparticles at 37°C for 7 days in modified simulated body fluid. Size was determined via light microscopy and Image J. BMP-2 was loaded by incubating in PBS solution of MCHA at 37°C for 4 hours. ^{125}I-BMP-2 release was characterized by measuring radioactivity of supernatant collected during incubation in TBS at 37°C. *Aggregate production.* MCHA (0.05 mg/aggregate), with or without BMP-2 (1400 ng/mg MCHA), were suspended with P3 hMSCs ($1.25x10^6$ cells/ml). Aliquots were centrifuged in multiwell plates to form aggregates [2]. In conditions exposed to exogenous ("exo.") BMP-2, 100 ng/ml BMP-2 was added to the media. At 2 and 5 weeks, aggregates (N=4) were assayed for glycosaminoglycan (GAG) and calcium content using dimethyl-methylene blue and o-Cresophthalein complexome assays, respectively. One-way ANOVA with Tukey's *post hoc* tests was used for statistical analysis.

RESULTS: Sustained release of BMP-2 from MCHA (3.41±1.04 μm) was achieved (Fig. 1A). MCHA-incorporated aggregates exhibited significantly higher GAG content than hMSC-only aggregates at week 5 (Fig. 1B) and calcium content increased from week 2 to 5 (Fig. 1C). Significant increases in GAG and calcium content in "MCHA, no BMP-2" indicate that MCHA may have induced both chondrogenesis and osteogenesis as shown by GAG production and matrix mineralization, respectively. "MCHA, exo. BMP-2" and "BMP-2-loaded MCHA" aggregates exhibited significantly higher GAG content than other groups at both time points, signifying that BMP-2 may have enhanced chondrogenesis induced by MCHA. Taken together, cartilage formation occurred prior to mineralization, suggesting that the presence of MCHA and BMP-2 may have promoted endochondral ossification. Furthermore, "BMP-2-loaded MCHA" had significantly higher GAG content at both time points and a larger increase in calcium content between 2 and 5 weeks compared to "MCHA, exo. BMP-2", which may signify the importance of spatial distribution of BMP-2 on chondrogenesis and mineralization. In contrast, "hMSC-only, exo. BMP-2" had no GAG but a significant increase in calcium content, indicating that intramembranous ossification may have occurred and confirming the role of BMP-2 as an osteogenic inducer.

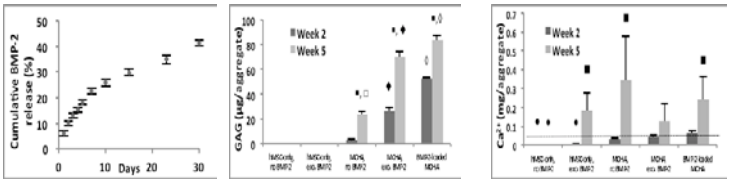

Fig. 1: (A) BMP-2 release profile from MCHA. (B) GAG and (C) calcium (Ca^{2+}) content of aggregates at weeks 2 and 5. Line denotes amount of calcium initially incorporated within each MCHA-containing aggregate. Significantly higher than: ■ week 2, □ hMSC-only groups at time point, ◆ all except "BMP-2-loaded MCHA" at time point, ◊ all groups at time point. Significantly lower than: ● MCHA groups at time point. p<0.05 considered significant.

DISCUSSION & CONCLUSIONS: This study explored the effects of HA and BMP-2 in high-density hMSC aggregates. The inductive role of BMP-2 on osteogenesis was confirmed in hMSC-only group treated with BMP-2. MCHA appears to be a chondrogenic inducer as shown by GAG production, and this effect is enhanced with BMP-2. Localized delivery of BMP-2 from incorporated MCHA resulted in enhanced GAG production and calcium deposition compared to MCHA-incorporated aggregates that were treated with exogenous BMP-2. Current studies are investigating the effects of varying concentrations of MCHA and BMP-2, exogenous and loaded, and release rates of BMP-2.

ACKNOWLEDGEMENTS: The authors gratefully acknowledge funding from AO Foundation and a National Science Foundation (NSF) Graduate Research Fellowship.

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Notochordal cells activate nucleus pulposus cells more strongly after stimulation with serum in 3D cross-species co-cultures

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INTRODUCTION: Notochordal Cells (NC) are shifted back into focus due to their apparent action of activating other disc cells via indirect release of yet unknown factors into the medium (conditioned medium = CM).[1,2] Recent evidence confirms the results from the late 90ies.[3,4] Here, we test porcine (p) NC cultured in 3D and the influence of adding serum or using serum-free medium onto the culture on NC cells and its stimulating effects for subsequent co-culture with primary bovine (b) nucleus pulposus (bNPC) and annulus fibrous cells (bAFC).

METHODS: Primary pNC, bNPC and bAFC were isolated from fresh porcine tails (< 6-12 months age) or bovine tails (~1 yr age), which were obtained from the food chain. All Cells were seeded into 1.2% alginate, each with a density of 4 x 10^6 /mL. NC were then either cultured for 7 days in serum free medium (SFM = Dulbecco's Modified Eagle Medium DMEM supplied with ITS+, 50µg/mL vitamin C and non-essential amino acids) or DMEM + 10% fetal calf serum (FCS). CM was produced from NC collecting 4mL SFM and keeping ~30 beads for 7 days. Then, a co-culture was set-up in SFM for 14 days using indirect cell-cell contact (culture insert, high density pore, 0.4 µm) using a 50:50% ratio[5] of pNC:bNP or bAF, or by addition of CM, respectively. The Glycosaminoglycan per DNA (GAG/DNA) ratio, real-time RT-PCR of IVD relevant genes and cell activity was monitored.

RESULTS: GAG/DNA ratio was slightly increased in hypoxia relative to day 0 and relative to normoxia (Fig. 1). bNPC tended to be more strongly activated in hypoxia but in co-culture under a 50:50 ratio with pNC that were kept for 7 days in DMEM + 10% FCS the GAG/DNA ratio was up-regulated by almost 300% (Fig. 1). CM did not show any stimulating effects on bNPC nor on bAFC. Furthermore, cell activity as measured by resazurin red tended to be increased in pNC even after 14 days post-switch to SFM for co-culture experiments.

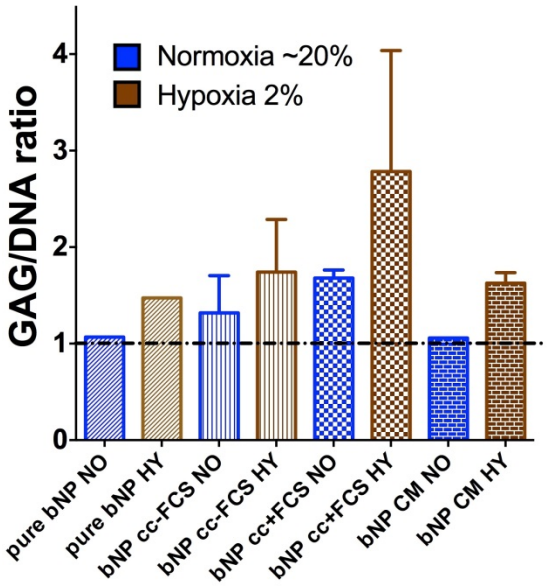

Fig. 1 Barplot of GAG/DNA ratio after 14 days of bNPC in 3D alginate in 1:1 coculture (CC) with porcine NC which were in kept in DMEM +FCS or in SFM (-FCS) for 7 days prior CC. Note the activation of the bNPC in CC with pNC previously cultured in DMEM containing (+) FCS in hypoxia.

DISCUSSION & CONCLUSIONS:

Our results showed a trend that GAG/DNA ratio of the hypoxic condition was higher than in normoxia[6] and that FCS has a stimulating effect to pNC for subsequent co-culture.

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Mechanical testing of locked plate fracture fixation constructs: limitations of previous methodologies

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INTRODUCTION: Clinical studies of locked plated constructs (LP) have reported non-union rates of up to 17%, with additional late implant failures suggesting non-unions. This has raised the concern that perhaps LPs are too stiff to reliably promote healing [1].

It has been suggested that the cause of the non-union is excessive stiffness, leading to mechanical stimulation below the threshold required to produce callus formation. The 'ideal' amplitude of movement within the fracture gap has been reported as being 0.2-1.0 mm. It is unclear however, what range of motion is achieved in LP.

While many LPs have been tested mechanically the methodology used is often not representative of natural deformation. Over constraint of the sample dramatically shortens the bending length resulting in a vast overestimate of the stiffness of the construct. Replacing constraints with spherical bearings allows free bending to occur over the entire length of the sample, resulting in a closer approximation of natural bending behavior (Figure 1).

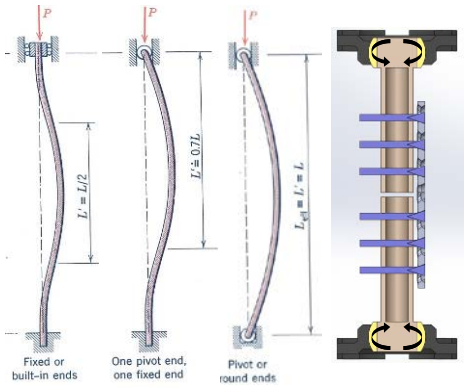

Fig. 1: Effect of constraint on free bending length, double spherical bearing design used, shown on the far right (adapted from [2]).

METHODS: Mechanical testing a 9 hole stainless steel LCP (Synthes, Switzerland), with central 3 hole working length, was conducted in a test rig utilising two spherical bearing constraints (Figure 1).

Sawbones (Pacific Research Laboratories, USA) 4th generation cylinders with an OD of 20mm, wall thickness of 3mm and length of 250mm were used as a tibial analogue. A 3mm osteotomy was created centrally in each sample and the LCP applied with three empty holes centred over the osteotomy. A gap of 2mm gap was left between the underside of the plate and the cylinder.

The Optotrak (Northern Digital Inc, Canada) infrared optical tracking system was used in combination with two Orthopaedic Research Pins, positioned proximally and distally to the osteotomy. This allows the calculation of the translations and rotations of the two fragments independently. From this data the near (closest to the plate) and far (furthest from the plate) cortex deformations are reported. Cyclic loads of 500N were applied to each sample, and the fifth cycle used for analysis.

RESULTS: At a load of 500N, a movement of 0.35 (0.08) mm (mean(SD)), was recorded at the near cortex and 2.43 (0.44) mm at the far cortex.

DISCUSSION & CONCLUSIONS: Rather than supporting the proposition that insufficient movement occurs on the near cortex with LPs, this data suggests that the described ideal range of motion is easily achievable on both the near and far cortex of the fracture gap under physiological load levels. With recent advances made to screw design to increase flexibility and movement there is now potential for generating excessive motion on the far cortex of the fracture.

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ACKNOWLEDGEMENTS: This work was funded by the Osteosynthese and Trauma Care Foundation and the Australian Research Council Discovery Project.

Cortical bone mimetic scaffolds for regeneration of load-bearing defects

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INTRODUCTION: In this work, we combine the bottom-up and top-down approaches to produce scaffolds that mimic the complex structure of cortical bone at the nano- and microscale to address the issues of scaffold stiffness and uniform nutrient transport to the entire scaffold volume.

METHODS: First, layers of resorbable aligned electrospun nanofibers was functionalized with a sequence of glutamic acid (GLU) residues, derived from acidic proteins of the bone matrix, to nucleate and grow CaP nanocrystals on the fibers.[1] Second, a layer-by-layer assembly approach was used to produce a microsheet, mimicking the lamellar layer of the osteons. Third, the microsheets were perforated with a regular array of circular holes and wrapped around a rod to form a laminated microtube with circular holes in well-defined intervals in the transverse direction. Fourth, a set of the microtubes was shrink-bonded by shape-memory effect of aligned nanofibers, to form a macroscale scaffold. The microtubular network was seeded with mesenchymal stem cells and cultured in osteogenic medium to assess the extent of differentiation and mineralization.

RESULTS: A typical image of GLU-conjugated PLGA fibers nucleated with CaP crystals is shown in Figure 1a. Insignificant CaP nucleation was observed on fibers without GLU conjugation. The average CaP crystal size was <100nm. The extent of mineralization of MSCs seeded on the fibers as a function of CaP nucleation is shown in Figure 1b. Extent of MSC mineralization was a string function of CaP nucleation. At 200% CaP to fiber content, extent of mineralization was 2.5-fold higher than no CaP nucleation (compare purple with navy blue line, the light blue is MSC seeded fibers incubated in basal media). Expression of osteocalcin and osteopontin was consistent with the extent of mineralization. The CaP-nucleated fiber sheets were perforated with a regular array of circular holes and wrapped into a tubular shape. Figure 2a shows the regular array of circular pores in the transverse direction of the tube. A microtube with wall thickness to diameter ratio is shown in Figure 2b, resembling the structure of osteons in cortical bone. Figure 2c shows a bundle of microtubes fused by shrink-bonding, resembling the microstructure of cortical bone.

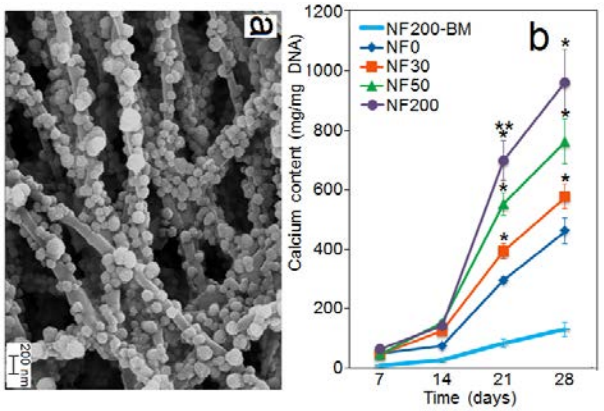

Fig. 1: (a) micrograph of the fibers nucleated with CaP crystals, (b) effect of CaP nucleation on mineralization of MSCs in osteogenic medium with incubation time.

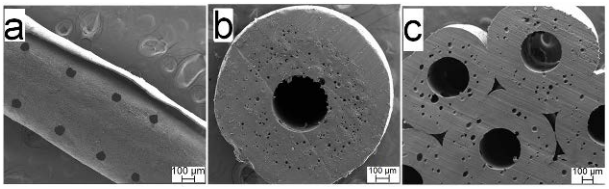

Fig. 2: (a) image of perforated microtube, (b) microtube mimicking an osteon, (c) fused bundle of microtubes mimicking cortical bone.

DISCUSSION & CONCLUSIONS: The fused microtubes with 50% CaP-nucleation had compressive modulus of 50 GPa the permeability of perforated microtubes was significantly higher than solid microtubes. Fused microtubes seeded with MSCs had higher ECM than those on microsheets.

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Wnt3a re-establishes osteogenic capacity to bone grafts from aged animals

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INTRODUCTION: Age-related fatty degeneration of the bone marrow is strongly associated with delayed fracture healing and osteoporosis-related fractures in the elderly. The mechanisms underlying this fatty change are unknown, but may relate to the level of Wnt signaling within the aged marrow cavity.

METHODS: Transgenic mice were used in conjunction with a syngeneic bone graft model to follow the fates of cells involved in the engraftment. Immunohistochemistry along with quantitative assays were used to evaluate Wnt signaling and adipogenic and osteogenic gene expression in bone grafts from young and aged mice. Liposomal Wnt3a protein (L-Wnt3a) was tested for its ability to restore osteogenic potential to aged bone grafts in critical size defect models created in mice and in rabbits. Radiography, micro-CT reconstruction, histology, and histomorphometric measurements were used to quantify bone healing resulting from L-Wnt3a or control, L-PBS treatment.

RESULTS: Gene expression profiling of bone grafts demonstrated that aging was associated with a shift away from an osteogenic profile and towards an adipogenic one. This age-related adipogenic shift was accompanied by significantly reduced Wnt expression and Wnt activity ($p<0.05$) in bone grafts from aged animals. In both large and small animal models, osteogenic competence was restored to aged bone grafts by a brief incubation with the stem cell factor, Wnt3a. Initially, liposomal Wnt3a treatment significantly reduced cell death in aged bone grafts in rabbits. Later, at 8 weeks, bone grafts treated with liposomal Wnt3a gave rise to significantly more bone ($p<0.05$) (Fig. 1), whereas aged bone grafts treated with liposomal PBS exhibited fatty and fibrous tissue in the defect site.

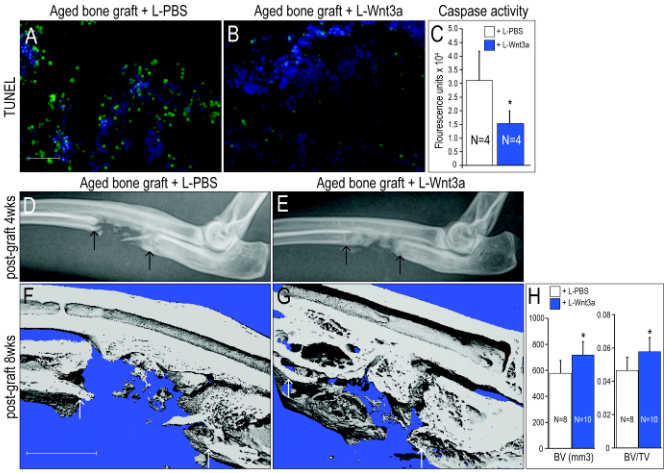

Fig. 1: L-Wnt3a treatment restores osteogenic potential to bone grafts from aged animals. (A) TUNEL staining demonstrates the extent of apoptosis in aged bone marrow treated with L-PBS (10μL), compared with (B) L-Wnt3a treatment. (C) A measurement of caspase activity in aged bone graft samples treated with L-PBS (white bars) or L-Wnt3a (blue bars). (D) Radiographic assessment at 4 weeks following bone grafting. Compare (D) L-PBS treatment with (E) L-Wnt3a treatment. (F) Micro-CT reconstruction at 8 weeks following bone grafting. Compare L-PBS treatment with (G) L-Wnt3a treatment. (H) Bone volume and bone volume/total volume. Single asterisk denotes $P<0.05$.

DISCUSSION & CONCLUSIONS: Liposomal Wnt3a treatment enhanced cell survival in the graft and re-established the bone-forming ability of grafts from aged animals. We developed a safe, effective, and clinically applicable regenerative medicine-based strategy for revitalizing bone grafts from aged patients.

ACKNOWLEDGEMENTS:

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Polyurethane membrane for intervertebral disc annulus rupture closure - feasibility under dynamic loading in an organ culture study

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INTRODUCTION: The incidence of reherniation through ruptured annulus fibrosus (AF) ranges from 10% to 15% in patients with herniated discs treated with discectomy. In this study, we produced polyurethane (PU) membranes containing leachable poly(ethylene glycol) (PEG) for their application in AF rupture closure. An organ culture study was conducted to investigate the capability of the PU membrane to retain a Tissue Engineering (TE) construct and reduce the reherniation risk.

METHODS: Segmented PU was synthesized as already reported [1]. Preparation of PU membranes with/without PEG (Mn 0.6 kDa) consisted in the solubilisation of PU 10 w:v % with addition of 0 and 1 w:v % of PEG in a mixture of solvents. The solutions were poured into a flat tray and left evaporating for 1 week before drying under vacuum. Half of the samples were washed repeatedly with water for 1 week to remove leachable PEG. Tensile tests were conducted on dry samples using an Instron mechanical testing system model 4302 (High Wycombe, Bucks, UK). A feasibility study was conducted in organ culture of bovine caudal discs under physiological loading conditions. A defect through the AF of the discs was created with a biopsy punch. The AF cavity was refilled with a TE construct consisting of poly(trimethylene carbonate). The PU membrane without PEG was sutured (4 point-suture) with the surrounding AF tissue to close the AF defect. After one week of pre-culture, discs were loaded with 4 hours sinusoidal dynamic load per day for 7 continuous days, at 0.06±0.02 MPa, 0.2 Hz. Disc dimensions were measured with a calliper after dynamic loading and after overnight free swelling. Stability of the membrane was macroscopically assessed. Safranin O/Fast Green staining was performed.

RESULTS: PU membranes with PEG showed 17% increase of elongation at break and 40% decrease of strength. The original property of the PU membrane was recovered after leaching of the PEG.

In organ culture study, compared with the initial height, disc height decreased by 5-7% after 4 hours of dynamic loading, and recovered after overnight free swelling. Safranin O/Fast Green staining and macroscopic observation showed that the PU membrane sutured with adjacent AF tissue maintained the TE construct inside the AF cavity under dynamic loading (Fig. 1). No protrusion of nucleus pulposus tissue was observed after one week of pre-culture and a second week of dynamic load (Fig. 1).

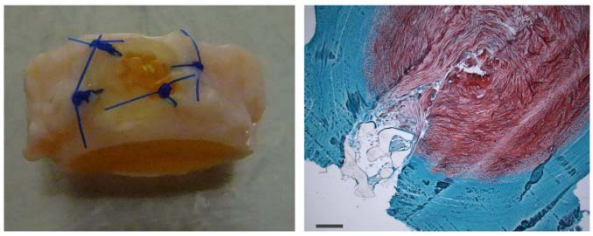

Fig. 1. Discs with AF defect implanted with TE construct and covered with PU membrane after 2 weeks of culture with dynamic load during the second week. Left: macroscopic view. Right: Safranin O/Fast Green stained section, scale bar 1000 µm.

DISCUSSION & CONCLUSIONS: Temporal modulation of PU membrane mechanical properties can be achieved by addition and leaching of PEG. Sutured PU membrane retained an AF TE construct in a disc under mechanical load. PU membranes may be suitable as an AF rupture closure device to reduce disc reherniation risk.

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ACKNOWLEDGEMENTS: Funded by the AF Rupture Collaborative Research Program from AO Exploratory Research Board.

Condylar reconstruction using hydroxyapatite/collagen with platelet rich plasma

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INTRODUCTION: The aim of this prospective clinical experimental study was to evaluate the results for condylar reconstruction using preshaped hydroxyapatite/collagen condyles soaked in platelet-rich plasma in patients with temporomandibular joint (TMJ) ankylosis

METHODS: Experimental group comprised of 32 patients where condyle was reconstructed using preshaped hydroxyapatite/collagen condyles soaked in platelet-rich plasma and fixed to ramus with titanium plates and screws, control group had 11 patients where reconstruction was done with sternoclavicular graft. All patients were followed up for a minimum period of 12 months and functional /radiographic assessment were made to infer the treatment outcomes.

Micro CT evaluation of an ankylotic chunk, sternoclavicular graft in a recurrence case and hydroxyapatite collagen scaffold was done to observe the microarchitecture.

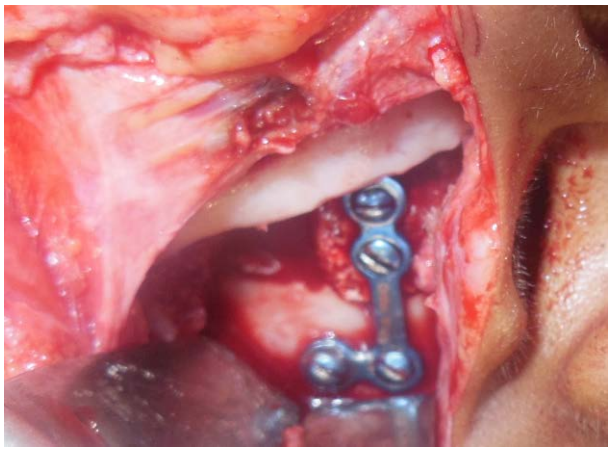

Fig 1: Preshaped hydroxyapatite/collagen condyles soaked in platelet-rich plasma fixed with titanium L plate and screws.

RESULTS: In experimental group radiographic evaluation at 3 months showed a less opaque condyle, but the opacity at 18 months was more defined, suggesting bone formation and ossification. In control group too there was maintenance of function, and radiographic evidence of a good condyle. Recurrence of ankylosis was observed in two cases where sternoclavicular graft was used for reconstruction.

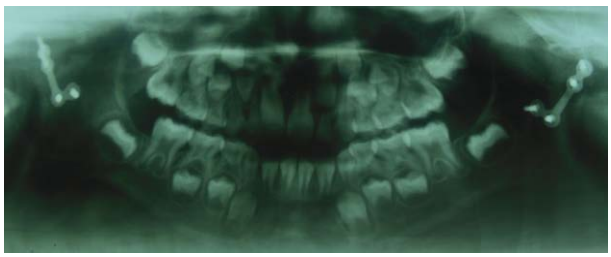

Fig 2: Orthopantomogram showing bilateral reconstruction using hydroxyapatite/collagen condyles soaked in platelet-rich plasma fixed with titanium L plate and screws at 6 months.

DISCUSSION & CONCLUSIONS: The preshaped hydroxyapatite/collagen condyle with platelet-rich plasma is a promising alternative to sternoclavicular graft with reduced donor site morbidity. A long term study, to analyze the growth potential of the two condyles, should be planned.

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Alginate sulfate hydrogels promote 3D expansion of primary chondrocytes: ionic versus covalent crosslinking

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INTRODUCTION: Current cell-based cartilage repair requires millions of autologous chondrocytes for transplantation. To achieve these cell numbers, in vitro 2D expansion of the cells is necessary, but leads unavoidably to a loss of chondrogenic phenotype. Here we introduced a novel 3D system to expand freshly isolated chondrocytes in vitro while maintaining the natural phenotype. This system is based on the natural polysaccharide alginate modified with sulfate groups to resemble the cartilage tissue enriched in highly sulfated glycosaminoglycans. Moreover we compared ionic and covalent cross-linking to study the influence of the gelation method on the effective action of the biopolymer.

METHODS: Alginate (Novamatrix) was reacted with SO_3/Pyridine to achieve 0.8-1 degree of sulfation. Freshly isolated bovine chondrocytes (P0) were encapsulated in sulfate or unmodified alginate either by immersion in 100 mM $CaCl_2$ or by ultraviolet (UV) cross-linking in the presence of the photoinitiator lithium acylphosphinate (LAP). Samples were evaluated for cell proliferation (light microscopy) and expression of chondrogenic markers (RT-qPCR).

RESULTS: Chondrocyte proliferation and spreading were highly induced in alginate sulfate hydrogels which were cross-linked with calcium chloride (Fig. 1). Gene expression analysis showed that the chondrogenic marker collagen 2 was maintained and aggregan was even increased in alginate sulfate compared to 2D culture, though the cells were highly proliferative (Fig. 2).

Maintenance of the chondrogenic, proliferative phenotype of P0 chondrocytes was dependent on the presence of sulfate groups to which the cells could attach, and the presence of a physically cross-linked network which could be displaced by the cells. If the attachment sites were missing (unmodified alginate) or if the cross-linking was covalent (alginate methacrylate), the phenotype was lost.

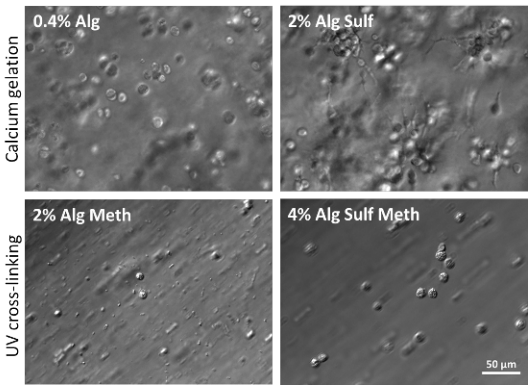

Fig. 1: Chondrocytes proliferate exclusively in alginate sulfate gelled with Ca^{2+} ions.

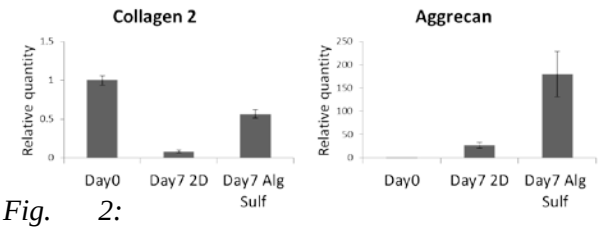

Fig. 2: Chondrogenic markers are expressed by chondrocytes encapsulated for 7 days in Ca^{2+} gelled alginate sulfate.

DISCUSSION & CONCLUSIONS: Here we show that alginate sulfate stimulates cell proliferation of freshly isolated chondrocytes while preventing the de-differentiation which accompanies expansion in monolayer culture. Moreover, the cross-linking method was crucial in determining cell behavior. Ionically cross-linked hydrogels contain reversible bonds that can be continuously remodelled while covalently cross-linked hydrogels constrain the cells in a rigid and un-modifiable environment. Overall, alginate sulfate with calcium gelation represents a cartilage mimetic hydrogel with high potential for improving the outcome of cell-based procedures like autologous chondrocyte implantation (ACI).

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Immunological response to biodegradable magnesium implants *in vivo*

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INTRODUCTION: Using biodegadable implants in pediatric trauma surgery would render surgical interventions for implant removal after tissue healing unnecessary, thereby preventing patient's stress and reducing therapy costs. The aim of this study was to investigate the systemic immunological response to two different biodegradable magnesium implants in a growing rat model.

METHODS: Magnesium pins, made out either of the faster degrading alloy ZX50 or the slower degrading alloy WZ21 were implanted into the femoral bones of five-week old rats. Rats that underwent surgery but had no pin implanted served as controls. Blood samples were collected immediately before operation, weekly in the first four postoperative weeks and then every 4 weeks up to the 24th postoperative week. Phagocytic ability of neutrophile granulocytes was determined using the Phagotest® kit.

RESULTS: Animals that had biodegradable magnesium pins implanted showed a higher phagocytic ability compared to the preoperative level one week after operation, indicating that these implants do not provoke a foreign body reaction, and also after 8 weeks of operation, showing good bone healing. The phagocytic ability of the WZ21 group was reduced after 12 weeks of operation. This might be attributed to a delayed release of the rare earth material yttrium out of this implant. The phagocytic ability of the control group was decreased at several postoperative time points compared to the preoperative level and also compared to the implant groups. This means that magnesium implants do not only have no negative effect on the immune system, but they are even able to overcome the negative immunological effects of anesthesia and surgery.

DISCUSSION & CONCLUSIONS: This study confirms magnesium alloys as being promising candidate materials for use in biodegrdable implant application in pediatric trauma patients.

Bimolecular-based self-assembling hydrogel for application in cartilage repair

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INTRODUCTION: Adult articular cartilage has a very limited capacity of regeneration. Therefore, treatments to assist cartilage repair is a challenge topic in regenerative medicine. This work aims to mimic cartilage extracellular matrix using a new bi-component scaffold produced by a simple combination of oligosaccharide molecules of heparin and the commercial available self-assembling peptide RAD16-I (PuraMatrixTM). This bi-molecular hydrogel was used to foster chondrogenic differentiation in vitro culturing Mesenchymal Stem Cells (MSCs). Particularly, human Adipose Derived Stem Cells (ADSC), a population of adult stem cells able to differentiate into mesenchymal derived tissues such as bone, cartilage and fat, were used to study the potential properties of the new material. First, we obtained an optimal working mix range of heparin and RAD16-I to ensure the self-assembling process and the most homogenous mixture of the two components. Then, ADSC were cultured using different RAD16-I/heparin combinations and good cell viability was observed after four weeks of culture. Interestingly, during the first days of culture the three-dimensional (3D) constructs underwent a pronounced diameter reduction that ended in a small compact structure with an increased storage modulus (G'). This morphological change was prompted partly due to the chondrogenic induction media which contains: recombinant human transforming growth factor-β1 (TGF-β1), dexamethasone and L-Ascorbic Acid 2-phosphate (AA2P). In addition, the induced constructs become highly stained with toluidine blue dye, indicating the presence of synthetized proteoglycans. Moreover, specific markers of mature cartilage such as collagen type II and aggrecan were detected by Reverse Transcription Polymerase Chain Reaction (RT-PCR). These results suggest that this simple approach to obtain three-dimensional (3D) scaffolds seems to recreate the required microenvironment for the cells to differentiate to chondrogenic lineage