Stemness as emergence

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Recent evidence for induction of stem cell characteristics in somatic cells by epithelial–mesenchymal transition, by induced activation of a small number of transcription factors, or by modification of the microenvironment has invalidated the once held notion of differentiation as a strictly unidirectional deterministic process. Together with the broad interest elicited by current clinical trials testing different cellular preparations dubbed "stem cells," this necessitates reconsideration of the very definition of the stem cell [1]. As a result, the focus has shifted from consideration of "stem cells" as a class of cells, to "stemness" as a property that can be acquired, conferred and/or retained by cells. What is stemness that allows a cell (population) to divide, maintain the size of its pool and differentiate at the same time? How is stemness defined, induced, maintained? A recent view posits "stemness as a cell default state" [2]. It is based on experimental evidence compatible with a deterministic model of stemness maintained as long as maintained is the intrinsic/inherent inhibition to differentiation. Another model invokes stochastic gene and protein expression that drives transitions among deterministic attractors characterizing stemness and the possible differentiation states [3]. Most recent developments interrogate the factors that define all levels of cell differentiation (*i.e.*, attractors) as properties emergent within complex biological systems.

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Skeletal stem cell based strategies for bone regeneration

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INTRODUCTION: Regenerative medicine strategies have sought to repair skeletal defects resulting from trauma and disease with the application of cells, typically isolated from the patients themselves, in combination with porous biomaterials or scaffolds. Skeletal stem cells, commonly referred to as Mesenchymal stem cells, or human bone marrow stromal stem cells are defined as multipotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament and fat. To date, technologies to facilitate the identification and isolation of specific skeletal stem cells and development of scaffolds that address issues of growth factor delivery and angiogenic support to aid de novo tissue formation remains a significant unmet clinical need.

We have developed protocols for the isolation, expansion and translational application of skeletal stem cell populations with cues from developmental biology, nanotopography and nanoscale architecture as well as biomimetic niche development informing our skeletal tissue engineering. Central are translational studies to examine the efficacy of skeletal stem and cell populations for orthopaedic application.

This talk will describe: i) isolation and characterisation strategies for fetal and adult skeletal populations, ii) derivation of niche environments through combination of progenitor cells with tailored nanotopographical strategies / architecture to modulate the osteogenic and angiogenic repair process and iii) translational studies to examine the efficacy of skeletal populations for orthopaedic application.

Advances in our understanding of skeletal stem cells and their role in bone development and repair, offer the potential to open new frontiers in bone regeneration and offer exciting opportunities to improve the quality of life of many.

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Wnt3a enhances self renewal and chondrogenic potential of adult human mesenchymal stem cells

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INTRODUCTION: Adult human mesenchymal stem cells (ahMSCs) have an excellent capacity to repair tissues since they can easily proliferate and differentiate into various cell types. However, ahMSCs are a heterogeneous population with different differentiation capacity and, unfortunately, the in-vitro expansion of ahMSCs leads to a gradual loss of stem cell characteristics. Fibroblast growth factor-2 (FGF2) is known to enhance the ahMSC chondrogenic potential but it can not prevent the gradual loss of multipotency or the formation of hypertrophic cartilage [1]. Since Wnt and FGF signals cooperate to maintain the mesoderm cells in a proliferative and undifferentiated state during limb development [2], we investigated the possibility to use Wnt and FGF proteins during the expansion of ahMSC to improve their cartilage repair capacity.

METHODS: We expanded ahMSCs from bone marrow (ahBMSCs; n=4 donors) in 4 conditions: alphaMEM + 10% fetal calf serum (FCS), +Wnt3a (250ng/ml), +FGF2 (1ng/ml) or +Wnt3a+FGF2. After one passage (P1), we counted the cells and we analyzed them by FACS. Next, we transferred the ahBMSCs for 5 weeks to a pellet culture system to assess the chondrogenic potential by immunohistochemistry, mRNA and glycosaminoglycans (GAGs) analysis.

RESULTS: After expansion, FACS analysis revealed that CD105 was strongly down-regulated by Wnt3a administration (~95% vs ~35%, $p<0.01$ compared to the two conditions without Wnt3a). Wnt3a and FGF2 were equally capable of enhancing ahBMSC proliferation (~1.4-fold compared to FCS-only, $p<0.05$) and the combination of the two factors enhanced the cell growth even further (~1.7-fold compared to FCS-only, $p<0.01$). After chondrogenic induction, pellets formed with cells expanded with Wnt3a and FGF2 showed a higher content of collagen type-II and GAGs than normally obtained with FGF2-only expansion and, in the same condition, the mRNA expression of the hypertrophic markers collagen type-X, and MMP-13 was decreased (~0.3-fold and ~0.5-fold respectively; $p<0.01$ compared to the condition FGF2-only). Additionally, in the presence of Wnt3a and FGF2 during the expansion, ahBMSCs can be expanded more than 350,000-fold (*Fig1a*), while maintaining their chondrogenic potential (*Fig1b*).

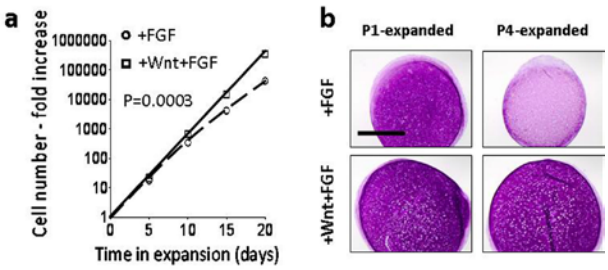

Fig.1: (a) Cell growth of ahBMSC expanded up to P4 (20 days) with +FGF2 or +Wnt3a+FGF2 (b) Thionine staining (glycosaminoglycans) on pellet cultures formed by ahBMSCs P1- or P4-expanded with FGF2, with or without Wnt3a. Scale bar, 0.5 mm.

DISCUSSION & CONCLUSIONS: Combination of Wnt3a and FGF2 during the expansion seems to "select" a specific sub-population of cells with a unique phenotype characterized by high proliferation capacity, high chondrogenic potential and low hypertrophy. The combination of these aspects makes the administration of Wnt3a to FGF2 during the expansion a promising tool for ahBMSCs-based cartilage tissue engineering.

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Natural History of Mesenchymal Stem Cells Uncovered for Improved Therapeutic Use

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INTRODUCTION:

Mesenchymal stem (or stromal-) cells (MSCs) are elusive, ubiquitous progenitors that can differentiate into mesodermal cell lineages but also indirectly contribute to tissue repair via regenerative factor secretion. MSCs have long been separated on their ability to grow selectively in long-term culture of total cell isolates. Only recently was MSC native perivascular identity uncovered, permitting prospective purification by flow cytometry.

We shall successively discuss 1- the identification, purification and functional characterization of MSC native ancestors, the perivascular stem cells (PSCs); 2- rising evidence that PSCs are naturally involved, in vivo, in the development and repair of mesodermal tissues; 3- PSC secretory activity; 4- the molecular control exerted by neighbouring endothelial cells on the progenitor cell potential of PSCs.

Finally, we shall describe PSC preclinical validation, and advanced projects to use these cells for the therapy of the musculoskeletal system. In the latter respect, we shall stress advantages of using thoroughly purified PSCs in place of conventional culture derived MSCs.

Mesenchymal stem cell niches in joint health and disease

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The synovial membrane covers the inner surface of the capsule of synovial joints such as the knee. It is a tissue that has the ability to self-regenerate in patients with rheumatoid arthritis undergoing synovectomy. In addition, during osteoarthritis the joint undergoes significant remodelling with attempts at repair that can result in the formation of aberrant cartilage and bone, the so-called chondro-osteophytes. Altogether, these considerations made us believe that the synovium postnatally would nurse stem cells in the joint.

In 2001 we reported the isolation and characterisation from the adult human synovium of mesenchymal stem cells (MSCs) with multipotency inherent at the single cell level (1). In a comparative study of MSCs from multiple tissue sources including the synovium, bone marrow, adipose tissue, periosteum and skeletal muscle, the synovium-derived MSCs displayed superiority in cartilage forming-potency (2). It is thought that the MSCs from the synovium could possibly be the ideal chondroprogenitors for the repair of the articular cartilage.

We postulated that postnatally the synovium would function as a reservoir of stem cells for the regeneration/repair of joint tissues such as articular cartilage and menisci that are known to have poor intrinsic capacity for regeneration. Of note, the synovium shares with the joint surface a common embryonic derivation from the developmental joint interzone (3, 4), while bone marrow and periosteum which, like synovium, are known to contain MSCs in the adult life, seem to have distinct developmental origins.

Postnatally, stem cells safeguard tissues and organs by replacing those mature cells that are lost because of physiologic turnover, injury or disease. Stem cells are quiescent slow-cycling cells which, following injury, become activated and undergo proliferation followed by differentiation into mature cell type(s) (such as chondrocytes) to maintain and restore tissue anatomy and function. With this notion in mind, my lab recently provided data on the identification and characterization of endogenous resident MSCs in the adult mouse knee joint synovium in vivo. We used a double nucleoside labelling scheme in a validated mouse model of knee joint surface injury (5) to identify in the adult knee synovium, long-term label-retaining slow-cycling cells which, following injury to the articular cartilage, proliferated and differentiated into chondrocytes to form chondrophytes, thereby displaying typical features of adult stem cells (6). These slow-cycling cells were non-haematopoietic, non-endothelial stromal cells with a phenotype compatible with MSCs, and were located in the lining layer and in perivascular areas, where they were distinct from pericytes.

The existence of functional MSC niches in the adult joint will offer opportunities to develop novel medications that target MSC niches and related reparative signalling pathways to activate and modulate intrinsic joint tissue regeneration/repair. We are also studying the roles of MSC niches in the pathogenesis of osteoarthritis and rheumatoid arthritis. Such studies will put forward the endogenous stem cells as possible therapeutic target to influence outcomes of joint disorders, in order to prevent progression, stimulate joint tissue repair and ultimately restore a functional joint homeostasis.

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Engineering a functional hematopoietic microenvironment with human MSCs through endochondral ossification

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INTRODUCTION: We demonstrated previously that adult human bone marrow-derived mesenchymal stem/stromal cells (hMSC) can execute an endochondral program and ectopically generate mature bone[1,2]. In the current work, we hypothesized that hMSC, primed in vitro in order to undergo the endochondral ossification process, can generate an ossicle with features of a "bone organ", including a fully functional haematopoietic compartment together with physiologically remodeled bone and mature vasculature.

METHODS: Human MSC were expanded for 2 passages, seeded onto 8mm diameter, 2 mm thick collagen sponges (UltrafoamTM), cultured for 5 weeks in vitro under chondrogenic and hypertrophic conditions, implanted subcutaneously in nude mice and then retrieved after 5 and 12 weeks in vivo. Samples were analyzed by histology (Safranin-o; Alizarin red; H&E; TRAP), IHC (Collagen I, -II, -X, BSP; MMP9, MMP13, DIPEN), ISH for human Alu sequences and quantitative μCT. Bone marrow was extracted from the samples after 12 weeks in vivo by mechanical crushing/collagenase digestion and cells were characterized by culture in methylcellulose (MethoCult®) and flow cytometry for phenotype. Total BM cells, obtained from long bones of control or treated mice (CD45.2+) or from the engineered ossicles, were treated with red blood cell lysis buffer and transplanted into lethally (9.5cGy)-irradiated congenic animals (CD45.1/2+). Blood samples were collected from the transplants and analyzed by flow cytometer after staining with antibodies against CD45.1/2 allotype, B- (CD19), T- (CD3ε) and myeloid lineage markers (CD11b/Gr-1).

RESULTS: Frequencies of LT-HSC (LKS+CD34-CD135-CD150+), megakaryocyte-erythroid progenitors (LKS-CD34-), and common myeloid /granulocyte-macrophage progenitors (LKS-CD34+) in the ossicles had a similar distribution compared to femurs from control mice. One month after transplantation, peripheral blood analysis revealed that more than 80% of cells were ossicle-derived. Most importantly, bleeding at 1, 2, and 3.5 months post transplantation confirmed the long term self-renewing capacity of the ossicle-derived HSC, with stable engraftment and multilineage reconstitution. An equivalent functionality of HSC derived from the ectopically engineered ossicles compared to the femurs was demonstrated by the similar relative frequencies of the different lineages. BM cells, harvested from the femurs were cytofluorimetrically analyzed 3.5 months after transplantation, showing a relevant contribution of donor-derived cells within the compartments of phenotypic HSC and progenitors.

DISCUSSION & CONCLUSIONS: We established an ectopic model of endochondral bone formation that is characterized by large bone marrow spaces, hosting and maintaining fully functional HSC. This was achieved by activating hMSC towards an endochondral ossification route, invoking a 'developmental engineering' paradigm. The system can be used in fundamental investigations on the biology of HSC niches, as well as in translational studies on normal and malignant haematopoiesis.

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Mesenchymal Stem Cells (MSC) induce the homing of endogenous stem/progenitor cells through the activation of alternatively activated macrophages in an ectopic bone formation model

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INTRODUCTION: MSC are effective therapeutic agents in a variety of clinical situations of tissue injury acting as cellular modulators[1]. The *in vivo* therapeutic effects of MSC depend on their anti-inflammatory potential, as well as on their capacity to stimulate functional mobilization of host cells [2]. Using an ectopic model of bone regeneration, we demonstrated that macrophage populations were initially mobilized as a consequence of the scaffold implantation. Macrophages with a pro-inflammatory (M1) phenotype were recruited into the implanted scaffolds either seeded or not seeded with cells, but alternative activated macrophages with an anti-inflammatory profile (M2) were present only in the MSC-seeded scaffolds, suggesting that the presence of MSC could induce a functional switch of the macrophage profile. To investigate the mechanisms by which MSC could influence macrophage polarization, we mimicked *in vitro* the inflammatory environment surrounding the implanted MSC and we studied the cell-released factors possibly involved in the macrophage functional switch. Moreover, we described the presence of migrated resident mature endothelial cells, after 7 days from implantation, in both MSC-seeded and non-seeded scaffolds. However, bone marrow (BM)-derived endothelial progenitor cells (EPC) $CD133^{pos}$ $VEGFR2^{pos}$ $TLR2^{pos}$ were observed only in scaffolds seeded with MSC. After 11 days, BM-derived $CD105^{pos}CD146^{pos}$ populations were present within the host cells recovered from the MSC-seeded scaffolds, but not from the non-seeded empty scaffolds. No local precursors expressing the same cell surface antigens and possibly residing within the tissues surrounding the graft site were detected in the cell-seeded implants.

METHODS: Combinations of wild-type (WT) MSC/scaffold were implanted in syngenic WT mice that were lethally irradiated and reconstituted with a Green Fluorescent Protein-positive (GFP+) bone marrow (chimeric mice). Implants were extracted at different times and endogenous cells, harvested through enzymatic digestions, were characterized.

RESULTS: Implanted MSC are able to mobilize inflammatory macrophages that progressively acquire an alternative activation profile. Macrophage polarization promotes angiogenesis and tissue repair skewing the secretion of molecules involved in the regulation of cell migration. Polarized macrophages were effective at inducing endothelial progenitor cells and pericytes migration within the scaffold, leading to the development of the engineered tissue.

Fig. 1: Images of cells in this case – please use the

DISCUSSION & CONCLUSIONS: Our results proved the potential of uncommitted MSC to functionallly mobilize host cells into an induced bone regenerative niche *in vivo*. In particular, we demonstrated a cross-talk between implanted MSC and host cells, such as macrophages as well as bone marrow-derived endothelial and mesenchymal precursors, leading to their recruitment toward the bone regenerative niche. Moreover, our *in vitro* and *in vivo* data demonstrated that the MSC-mediated secretion of PGE_2 is responsible for the macrophage switch from a pro-inflammatory to a pro-resolving phenotype. The activation of endogenous stem cells from either the blood or a tissue-specific niche is a promising approach for therapeutic success.

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Regulation of MSCs ultimate phenotype: repercussions for cartilage tissue engineering

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As a relatively flat, non-vascularized and non-innervated tissue, articular cartilage was once thought to be a "simple" target for tissue engineering. Yet, after decades of exhaustive research, the creation of a stable implant to repair cartilage defects, one that mimics native tissue properties, has eluded the scientific community. In general, we have been unable to reconstruct a tissue with similar biophysical, biochemical, and ultrastructural characteristics as true hyaline articular cartilage. These limitations are highly related with the difficulties associated with the control of the phenotype of the tissue-forming cell.

Mesenchymal Stem Cells (MSCs) have been proposed as an alternative to chondrocytes to repair cartilage damage, due to their ease of isolation, high expansion capacity and established chondrogenic differentiation potential. It is becoming clear that MSCs reside as perivascular cells around the vasculature in multiple tissues including the bone marrow (BM).[1, 2] Furthermore, inside the BM there are distinct subpopulations of MSCs with different localizations, functions and phenotypic expressions, determined by the presence of specific cell surface markers such as CD146 and CD271.[3] These subpopulations render the BM-harvested cells highly heterogeneous, potentially accounting for the high donor-to-donor variability in their differentiation capacity. In addition, it has been shown that MSCs, under current protocols, follow an intrinsic program of endochondral bone development, which involves terminal differentiation into hypertrophic chondrocytes with specific undesired changes in the secreted extracellular matrix (ECM).[4]

Various stimulatory regimes have been designed to regulate MSCs phenotype, from early to late differentiation, including cell co-cultures and multi-factor formulations with strict temporal specifications. Four critical points are proposed **(Fig. 1)**, where interventions can be made in order to modulate the phenotype and function of MSC-derived chondrocytes. First, pre-selecting a population of cells with greatest chondrogenic potential, based on CD146 and Sox9 expression, via expansion of MSCs with FGF2; second, the effect of FGF2 on chondrogenic differentiation of MSCs; third and fourth, stimulation of differentiating MSC-derived chondrocytes at specific timepoints, with other members of the FGF family (FGF9 and FGF18), to increase ECM production (anabolic effect - third) and to delay the appearance of hypertrophy-related changes in both the cells and the resulting ECM (anti-hypertrophy effect - fourth).

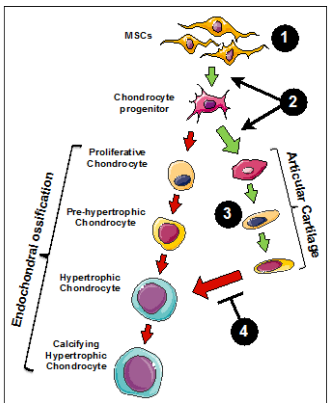

Fig. 1: Points of intervention to control the MSC-derived chondrocyte phenotype: *1) Pre-selection of a "pure" chondrogenic cell population; 2) Chondrogenic induction; 3) Anabolic effect, and 4) Delay of hypertrophic differentiation.*

In conclusion, novel approaches to control the ultimate differentiation phenotype of MSC-derived chondrocytes are discussed, including an *in vitro* stimulatory protocol based on the selection of MSCs with highest differentiation potential and their further sequential stimulation with specific growth factors that controls ECM secretion and prevents terminal differentiation. These type of protocols have critical repercussions for cartilage tissue engineering, as they provide the means to modulate the phenotypic conversion of differentiating cells, ultimate responsible for the synthesis of the tissue.

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Novel therapeutic approaches in regenerative medicine – potential application of very small embryonic like stem cells and harnessing adult stem cells paracrine signals.

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INTRODUCTION: Pluripotent very small embryonic like stem cells (VSELs) as we hypothesize are deposited at begin of gastrulation in developing tissues and play an important role as backup population for tissue committed stem cells. Molecular analysis of adult murine bone marrow (BM)-derived purified VSELs revealed that they share several markers characteristic for epiblast as well as migratory primordial germ cells (PGCs), and possess a unique pattern of genomic imprinting (e.g., erasure of differently methylated regions at Igf2-H19 and Rasgrf1 loci and hypermethylation at KCNQ1 and Igf2R loci).

We hypothesize that these pluripotent stem cells play an important role in tissue/organ rejuvenation, and their proliferation and potentially premature depletion is negatively controlled by epigenetic changes of imprinted genes that regulate insulin factor signaling (Igf2-H19 locus, Igf2R and RasGRF1). On other hand the same epigenetic changes of imprinted genes keep these cells quiescent in adult tissues and prevent them from efficient ex vivo expansion in vitro. Efficient expansion of these cells will lead to their broad clinical applications. In meantime regenerative medicine employs various types of more differentiated adult stem and progenitor cells to regenerate damaged organs (e.g., heart, kidney, or neural tissues). It is striking that, for a variety of these cells (HSCs, MSCs), the currently observed final outcomes of cellular therapies are often similar. This fact and the lack of convincing documentation for donor-recipient chimerism in treated tissues in most of the studies indicates that a mechanism other than transdifferentiation of cells infused systemically into peripheral blood or injected directly into damaged organs may play an important role.

It will be discussed the role of i) growth factors, cytokines, chemokines, and bioactive lipids and ii) microvesicles (MVs) and exosomes released from cells employed as cellular therapeutics in regenerative medicine. In particular, stem cells are a rich source of these soluble factors and MVs that released from their surface may deliver RNA and miRNA into damaged organs. Based on these phenomena, paracrine effects make major contributions in most of the currently reported positive results in clinical trials employing adult stem cells and different possibilities will be presented how these paracrine mechanisms could be exploited in regenerative medicine to achieve better therapeutic outcomes.

This may yield critical improvements in current cell therapies before true pluripotent stem cells such as VSELs isolated in sufficient quantities from adult tissues and successfully expanded *ex vivo* will be employed in the clinic.

Hypertrophic chondrogenic differentiated MSC pellets stimulate bone regeneration in segmental bone defects

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INTRODUCTION: Extensive segmental bone defects are usually stabilized by internal or external fixation. However, upon rigid fixation bone regeneration becomes more reliant on primary (intramembranous) bone formation as essential micromovements are diminished, thereby reducing fracture callus and subsequent secondary (endochondral) bone formation. Fracture callus is consists of chondrocytes that form a cartilage template and undergo terminal hypertrophic differentiation[1]. Mesenchymal stromal cells (MSCs) can differentiate into hypertrophic chondrocytes forming a cartilage template similar to a fracture callus[2]. The aim of this study was to stimulate secondary (endochondral) bone formation in segmental bone defect by implanting terminal chondrogenically differentiated MSC pellets.

METHODS: *Cell culture.* Human MSCs from three donors were cultured in pellets and differentiated into hypertrophic chondrocytes (HC) or maintained undifferentiated (UD, control) as described previously[2]. *Animal experiment.* Athymic nude rats (n = 27) were used to create a 6 mm segmental femoral bone defect that was stabilized by internal fixation with a PEEK plate and six bicortical screws (RatFix, AO Foundation). After 6 weeks, when the initial bone healing response subsided, HC pellets (n = 15) or UD pellets (n = 12) were implanted. Bone formation in the 6 mm defect was measured with *in vivo* micro-CT scans at 4 and 8 weeks. Histological evaluation at 3 days, 1, 2 and 8 weeks was done on decalcified paraffin embedded sections stained with hematoxylin/eosin, thionine, Col-II, Col-X, TRAP, vWF and CD31.

RESULTS: Implantation of HC pellets, six weeks after establishing the bone defect, resulted in almost complete regeneration of cortical bone and restoration of the medullary canal of the segmental defect within eight weeks. Bone regeneration upon implantation of HC pellets was seen for two donors, as HC pellets of one donor did not do better than UD pellets. UD pellets resulted in minimal bone regeneration, not differing much from the bone response observed six weeks after establishing the bone defect (Fig. 1).

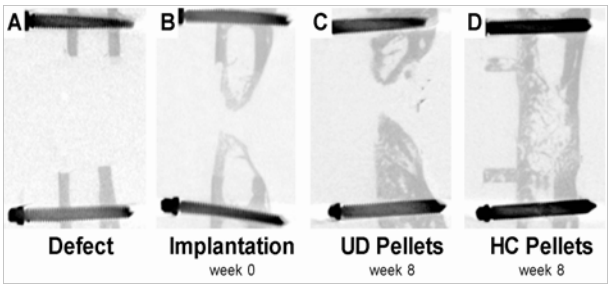

Fig. 1: Micro-CT images of 6 mm bone defects.

HC pellets, forming a cartilage template inside the defect, started a mineralization response at the outside of the pellets after 14 days, whereas UD pellets could not be traced back nor started mineralization after 14 days (Fig. 2).

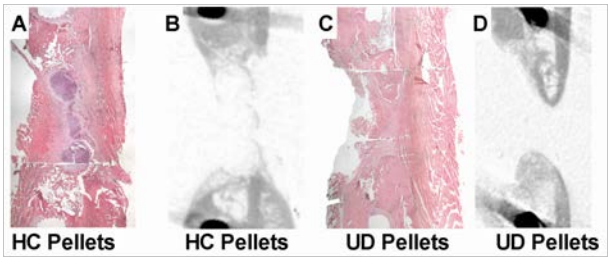

Fig. 2: Histology (H&E staining) and corresponding micro-CT images after 14 days.

DISCUSSION & CONCLUSIONS: Implanting terminal chondrogenically differentiated MSCs pellets into segmental bone defects resulted in extensive bone regeneration. Bone regeneration started around the cartilage template within 2 weeks. Angiogenic growth factors secreted by terminal chondrogenically differentiated MSCs might be primarily responsible for this effect, and might also explain the donor variability. This will be further elucidated through histological and molecular analysis.

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Priming 3D cultures of human mesenchymal stromal cells towards cartilage formation via developmental pathways

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INTRODUCTION: Tissue engineering has increasingly recognized the importance to study developmental biology models and to gain mechanistic insights into key signaling pathways, crucial for 3D organogenesis and, thus, for tissue regeneration. In the context of the so-called "developmental engineering"[1], we aimed at recapitulating the first events occurring during limb development (i.e., cell condensation, expansion of an undifferentiated mesenchymal cell population and chondrogenesis) as crucial steps to prime human bone marrow derived mesenchymal stromal cells (hBM-MSC) towards the endochondral route. Thus, we hypothesized that Wnt and FGF, as key morphogens at these stages of development[2], may induce hBM-MSC to expand in 3D micromasses as undifferentiated pool of progenitors, able to self-establish chondrogenic pathways upon withdrawal of initial signals.

METHODS: Expanded hBM-MSC were cultured as 3D micromasses in media supplemented with Wnt3a and/or FGF2 up to 7 days. Cells were tested for responsiveness to the morphogens and for proliferation by molecular biology (RT-PCR), cytofluorimetry (FACS), immunofluorescence (IF) and biochemical analyses. Upon withdrawal of initial morphogens, 3D micromasses underwent chondrogenic differentiation by using a TGFβ supplemented medium for further 14 days and were assessed for glycosaminoglycans (GAG) deposition via histological and biochemical analyses.

RESULTS: First, we verified that hBM-MSC are responsive to Wnt signaling in 3D pellet culture by significant upregulation of main target genes and increase of active β-catenin (β-cat). Then, we observed that Wnt3a is able to promote hBM-MSC proliferation in 3D (Fig.1A), although total DNA content decreases over time (Fig.1B). Preconditioning with Wnt3a had positive effects on TGF-β1 mediated chondrogenesis (up to 30% more GAG produced per cell compared to vehicle, Fig1C). FGF2 antagonized these Wnt-mediated effects (Fig.1A,C). Interestingly, the $CD146^+$ subpopulation was found to be more responsive to Wnt3a (Fig.1D).

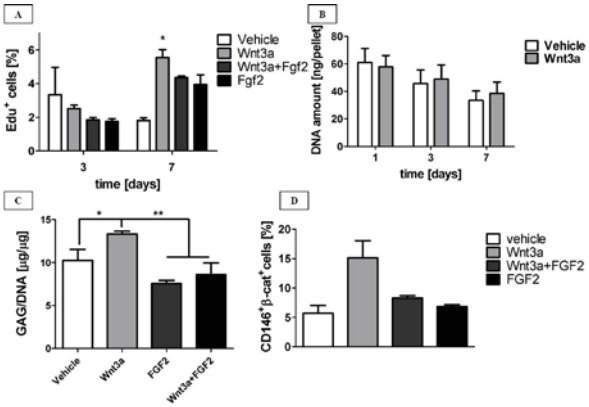

Fig. 1: A) Quantification of Edu^+ cells from IF (% Edu^+ cells respect to the total number of $Hoechst^+$ cells); B) Total DNA quantification; C) GAG content normalized per DNA; D) FACS analysis for co-expression of CD146 and β-catenin markers.

DISCUSSION & CONCLUSIONS: In this study we showed that Wnt3a is capable to stimulate 3D proliferation of hBM-MSC, meanwhile improving chondrogenic capacity and selecting a specific $CD146^+$ *bona fide* stem cell subpopulation[3]. However, Wnt3a, alone or in combination with FGF2, is not sufficient to induce a self-maintained 3D tissue growth. This study exemplifies that certain developmental paradigms can be recapitulated with adult MSC, though with significant differences. The translation from developmental models to human systems still requires new insights. Next investigations will focus on detecting crucial nodes to control and manipulate hBM-MSC fate, to ultimately improve osteochondral graft generation.

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Articular cartilage-specific progenitor cells: a frank assessment of progress

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INTRODUCTION: Articular cartilage is an enigmatic tissue; it is isolated from both vascular and neural supply and appears glacial compared to other tissues in the body. It is composed of chondrocytes that inhabit, at a low density, an extracellular matrix that is composed principally of collagen and proteoglycans. The extracellular matrix is exquisitely adapted to allow synovial joints to display almost frictionless articulation, and also to efficiently transmit large forces through the skeleton.

We have know for some ten years that articular cartilage contains multipotent tissue-specific progenitor cells which can be isolated and expanded many generations in vitro which can subsequently differentiate to produce cartilage. Despite these discoveries we know little about what happens to articular cartilage-derived progenitors during development, growth and disease. The main hurdles to further progress are; a lack of cell-specific markers for progenitors, a lack of suitable model systems in which to study progenitor cell dynamics over a short time period and the absence of in vitro disease models.

Understanding the dynamics of progenitor cell function during these critical processes will help us to develop better interventions to control and regenerate injured or osteoarthritic tissue either directly, or, through the medium of tissue engineering. I will describe our labs' attempts to find answers to these pressing questions, the insights into cartilage biology we have derived from our work and present an overarching hypothesis that attempts to unify various strands of cartilage biology.

Adipose-derived Stroma Cells for Orthopaedic Repair

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INTRODUCTION: The therapeutic potential of mesenchymal stroma cells to stimulate musculoskeletal repair was initially established with cells derived from bone marrow. While the osteochondral differentiation potential of these cells fostered their discovery and characterization, potential therapeutic benefits may also come from their trophic activity.

Adipose tissue is a particularly attractive reservoir for progenitor cells with tissue repair capacity because it is easily accessible, self-replenishing and gives high yields of adherent stroma cells with a demonstrated in vitro multilineage differentiation capacity. Comparative in vitro characterization of human stroma cells derived from adipose tissue (ATSC) with bone-marrow derived mesenchymal stroma cells (BMSC) revealed overlapping as well as diverging characteristics of both cell populations.

ATSC populations grew faster and longer in culture but contained fewer pericytes than BMSC. While BMSC and ATSC showed robust osteogenic in vitro differentiation in culture, the chondrogenic in vitro differentiation capacity of ATSC was inferior.

Molecular characterization revealed ATSC gene signatures with a lower expression of osteochondrogenic inducers like BMP-2, BMP-4, and BMP-6. This questioned their equal suitability for stimulation of bone and cartilage regeneration based on differentiation capacity. In vitro and in vivo studies judged the therapeutic potency of human ATSC versus BMSC for cartilage and bone formation, including comparison of the ectopic bone formation capacity and stimulation of long bone healing. Beyond lower expression of osteogenic inducers, ATSC also seem to display less proangiogenic activity and may have altered immunomodulatory capacity. Implications for biomaterial development and regenerative therapy of musculoskeletal tissue will be discussed.

Human progenitor tenocytes to improve healing in tendinopathies

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INTRODUCTION: Tendinopathies are very common afflictions in developed countries. Healing is time-consuming and doesn't allow to reach the characteristics found prior to injury. Re-injury, loss of mobility or failure in repair are frequent. The use of human progenitor tenocytes could improve the healing process, as other progenitor cells have already been implicated in better regeneration of wounds [1]. In this study, we evaluate such a cell line that has been developed for clinical use.

METHODS: The growth of human progenitor tenocytes in monolayer culture was evaluated with a Cell Titer assay and population doublings were determined. FACS was used to evaluate the HLA class II profile at P3.1, P6.1 and P9.1. The ability of cells to produce matrix was tested in 3D pellet culture and evidenced through staining on histological sections. The capacity of cells to live in a collagen matrix was observed with a Live/Dead assay performed 5 days after seeding. Finally, the stimulating effect of progenitor tenocytes on adult tenocytes was studied through a conditioning media method.

RESULTS: The mean duration for a population doubling is 4.63 days. This growth is steady up to high cellular passages. HLA-DP, DQ, DR epitopes are not expressed at any passages tested.

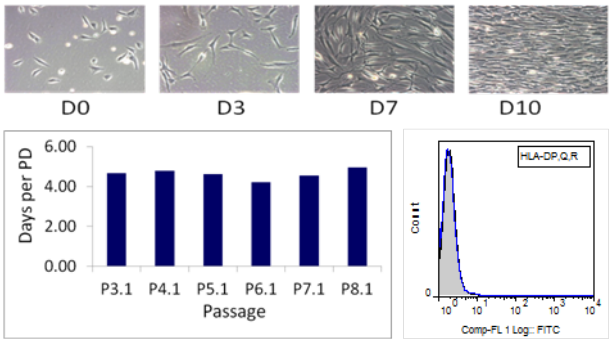

Fig. 1: Results obtained in monolayer culture.

Histological sections highlighted the capacity of progenitor tenocytes to produce glycos-aminoglycans and type-1 collagen when cultured tridimensionnaly.

When seeded on a horse collagen sheet, cells have the ability to adhere and to live as evidenced in the Live/Dead assay by numerous green spots present 5 days after seeding.

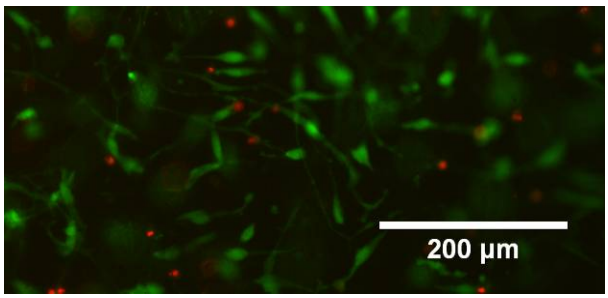

Fig. 2: Live/Dead assay.

Activity of adult tenocytes is slightly improved when they are cultured with media conditioned by progenitor tenocytes.

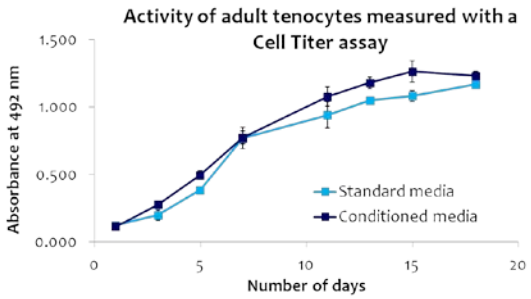

Fig. 3: Stimulation assay with conditioned media.

DISCUSSION & CONCLUSIONS: Human progenitor tenocytes present rapid and steady growth even at high passages, which is important when developing clinical cellular therapies. These cells were also found to have the ability to produce extracellular components and to live in a collagen-based environment. Absence of HLA-DP, DQ, DR is a great attribute if we imagine to transplant those cells. Finally, the results obtained for stimulation with media conditioned by progenitor tenocytes seem encouraging. All those features make human progenitor tenocytes an interesting cell source for the treatment of tendinopathies.

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Reaming material: a vital source for human mesenchymal stem cells with high osteogenic potential

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INTRODUCTION:

The golden standard for treatment of non-union is presently the transplantation of autologous spongy bone from iliac crest. One alternative technique could be stem cell based *Tissue Engineering.* With the Reaming-Irrigator-Aspirator (RIA)-System autologous material could be harvested by femoral reaming. The reaming material is set within the bone defect for treatment of pseudarthrosis. The aim of this study is the *in vitro* characterization and *in vivo* analysis of ectopic bone formation of human mesenchymal stem cells (hMSC) from various tissues like bone marrow from iliac crest, fat and reaming material.

METHODS:

Human mesenchymal stem cells of nine donors were isolated and cultivated from iliac crest aspirate, fat and reaming material. The cells were identified as hMSC by cell surface antigens and differentiated towards osteoblasts, adipocytes and chondrocytes *in vitro*. For *in vivo* study, the ability of new bone formation by colonization of bone substitute β-tricalciumphosphate with hMSC of various tissues was realized. After subcutaneous implantation of these constructs (n=18) into immunodeficiency mice, they were 8 weeks later removed and analyzed by histomorphometry and densitometry.

RESULTS:

The results show that human mesenchymal stem cells can be isolated from the tissues iliac crest, fat and reaming material. The cells could be clearly identified as hMSC by biochemical methods. MSC harvested from reaming material possesses a higher osteogenic potency *in vitro* and *in vivo* compared to hMSC from iliac crest. Ectopic bone formation from reaming material could be detected from every donor, whereas only 75% of all donors exhibited new bone formation from iliac crest. Furthermore, the amount of novel built bone from the hMSC population of reaming material was nearly twice as high compared to hMSC from iliac crest. Human mesenchymal stem cells from fat showed nearly no ectopic bone formation.

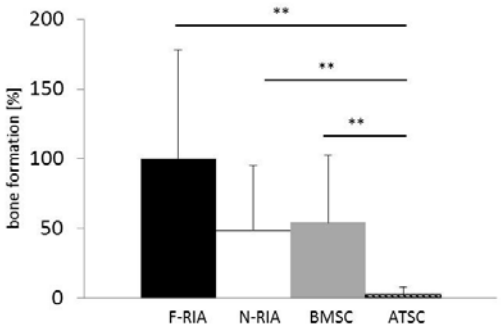

*Figure 1: Osteogenic in vivo differentiation of implants. After HE staining of implant constructs new bone formation was determined by densitometry. n=9. **p≤ 0.001.*

DISCUSSION & CONCLUSIONS:

Reaming material is a promising source of human mesenchymal stem cells. Moreover, it exhibits a higher osteogenic potency than hMSC derived from iliac crest. Their application in clinical treatment is an attractive alternative to the Golden standard.

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ACKNOWLEDGEMENTS:

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Intra-tumoral Heterogeneity in Osteosarcoma and the Cancer Stem Cell Model

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INTRODUCTION: Cells in solid tumors are phenotypically heterogeneous and can vary widely in their rates of proliferation, tumorigenic/metastatic potential, cellular differentiation, synthesis of extracellular matrix, metabolic pathways etc.

Toward developing a clearer understanding of the biology of osteosarcoma (OS) and aiding in the formulation of treatment approaches with improved efficacy, we have been studying the nature of intra-tumoral heterogeneity in this often-fatal form of bone cancer. Based on the cancer stem cell model, we reasoned that cells with the greatest tumorigenic potential within an OS tumor should be the most primitive, and thus should reflect the greatest epigenetic "de-programming."

Consistent with this idea, we found that that populations of tumor-initiating cells in cultures established from primary OS biopsies are selectively capable of activating a fluorescent reporter (GFP) driven by an ES-cell restricted promoter. These cells typically have over 100 fold greater tumorigenic potential following xenograft and generate heterogeneous tumors comprised of tumorigenic (GFP+) and non-tumorigenic (GFP-) cell populations.

Differential analysis of the global expression patterns from the diverse cell populations within individual tumors has provided a detailed roadmap of the specific molecular mechanisms that drive malignancy in the tumorigenic cells in each OS and conversely the pathways that mediate heterogeneity and through which malignancy is naturally silenced.

Counter to our original hypothesis, our data indicate that tumorigenesis and phenotypic heterogeneity in solid tumors are not reflections of "stemness" or hierachical differentiation, but instead are a function of cell cycle dysregulation in rapidly dividing tumor-initiating cells and their innate plasticity as they adapt to the stressful growth conditions of the tumor microenvironment.

Obtaining and Using MSCs during Trauma repair

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INTRODUCTION: Mesenchymal stem cells (MSCs) are key precursor cells that contribute to the osteoblast/osteocyte pool thus supporting bone anabolic responses and are considered to be key cellular players in bone repair and homeostasis in vivo.

MSC therapies commonly require two surgical procedures; harvesting of MSC-containing tissue such as iliac crest bone marrow (IC-BM) and, following culture expansion, their introduction into the defect area. An alternative strategy, termed 'instant stem cell therapy', relies on concentration of large numbers of MSCs and subsequent immediate implantation into the site where bone repair is desirable.

Three different ways of maximising the number of fresh MSC harvest were investigated:

a) Firstly we attempted to isolate MSC's following reaming of human long bones using the Reamer/Irrigator/Aspirator (RIA) (waste bag) and to compare them to 'gold-standard' donor-matched IC-BM MSCs; b) Secondly we investigated the effect of aspirated BM volume on MSC yield; c) Thirdly we assessed the effect of bone marrow aspirate concentration on the number of viable MSC's being available for implantation.

For part a) 12 donors undergoing non-unions treatment were studied. RIA-BM and IC-BM MSCs were transcriptionally similar (76% transcripts, n=12 donors). Similar levels of gene expression were observed for pericyte markers; ANGPT1/angiopoietin, as well as common osteo- (SPP1/osteopontin, OMD/osteomodulin), and adipo-genic (FABP4/fatty acid binding protein 4, adipocyte and PPARG/peroxisome proliferator activated receptor gamma) markers. Total yields of native MSCs from RIA waste bags were established using a colony-forming fibroblast assay; the median value was 314333 CFU-F (range 5x104-1.4x106), equivalent to approximately one litre of IC-BM aspirate.

For part b) of the study (25 patients) aspirating larger marrow volumes gave a significant several-fold reduction in the frequency of CFU-F and CD45 – /low CD271 bright cells per milliliter. Therefore aspirated MSC yields can be maximized through a standardized, low-volume harvesting technique. In order to maximize the number

Improvement of biologically impaired bone fracture healing by potent progenitor cells in the aged

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INTRODUCTION: Delayed healing and pseudarthrosis formation after complex fractures are clinical orthopaedic problems affecting 5-10% of all patients, predominantly occurring within the elderly population. Sufficient angiogenesis is crucial during tissue regeneration and hence essential in fracture repair treatment. Peripheral blood derived CD133+ stem and CD31+ progenitor cells are reported to play a role in revascularisation. Thus, we supposed that a local administration of these cells to a fracture gap is a feasible option to treat biologically impaired fracture healing.

Hence we analyzed availability, angiogenic and osteogenic potential of these cells *in vitro*. As impaired healing situations are particularly often found within elderly women, we focused on differences in regenerative capacities of progenitor cells from different proband cohorts, namely young (18-30 years) and elderly (60-67 years), male and female patients. Besides, we performed first *in vivo* analysis to evaluate the effect of aged cells on fracture healing after local transplantation.

METHODS: CD133+ and CD31+ cells were separated from peripheral blood of the specific patient cohorts via magnetic cell sorting. Flow cytometric (FC) measurements were carried out to check for availability. Angiogenic properties were analyzed in tube formation assays in co-cultures with human endothelial cells. The paracrine effect of the progenitor cells on osteogenesis was investigated in differentiation studies of mesenchymal stromal cells (MSCs) cultivated under conditioned media derived from the respective stem and progenitor cells.

Exemplary, the *in vivo* regenerative potential of CD133+ cells was tested in a 2mm femoral defect rat model with biologically impaired fracture healing. The effect of cell transplantation on bone regeneration was analyzed histological and via µct measurements.

RESULTS: As evident from FC measurements, circulation of CD133+ cells in the human peripheral blood increases with age, independent from the gender, whereas the number of CD31+ cells remains unaltered.

Tube formation assays revealed that CD133+ cells contain higher angiogenic capacities than the respective negative population. The angiogenic properties of these cells were unaltered in aged individuals. In contrast, CD31+ cells and their subpopulation CD31+/CD14- obtained from aged donors show an up to 70% higher angiogenic potential than those isolated from younger ones, independent from the gender.

However, analysis of the osteogenic differentiation proved that young, female CD31+ and CD31+/CD14- cells have an up to 3-fold higher impact on osteogenic differentiation of MSCs then those obtained from male probands. Additionally, we could show that the positive impact of the cells on osteoblast formation is diminished in aged individuals to a level found in untreated controls.

Finally, the regenerative potential of CD133+ cells could be proven *in vivo* by an increased callus formation and higher bone mineral density of callus tissue within the animals treated with the cells, compared to a control group treated identically but without cells. Increased vessel formation and mineralization in the osteotomy gap was visible in histological analysis 42 days after surgery in the cell-treated animals.

DISCUSSION & CONCLUSIONS: We could prove that CD133+ and CD31+ stem and progenitor cells from peripheral blood feature bone regenerative capacities. Thus, an application of these cells to a fracture site is a promising approach for the treatment of impaired healing situations. The present results also indicate that these cells may be promising candidates for a cell therapy even in elderly and hence more often affected patients.

Effects of inflammatory factors and synovial fluid on the expression of adhesion and migration factors in mesenchymal stem cells

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INTRODUCTION: Mesenchymal stem cells (MSCs) are promising candidates for cell therapeutic application in osteoarthritis (OA) because they are the body's natural healers. They can differentiate to generate repair tissue and they have immunomodulatory and trophic capacities. In affected joints of patients with OA there is an inflammatory environment affecting multiple intra-articular tissues like cartilage and synovium. Successful treatment of osteoarthritis will require long-term modulation of inflammation as well as repair processes in the various tissues of the joint simultaneously. To be therapeutic in OA, MSCs need to stay sufficiently long and active in the joint. In an in-vivo pilot study in which labelled cells were administered intra-articular in rat knees, we found a great decrease in the number of cells in the joint already after one week. Factors influencing MSC survival and engraftment in the joint are currently not known. Higher expression of adhesion/migration factors in MSCs will very likely be in favour of their engraftment potential. We studied the effect of inflammation and synovial fluid (SF) on the expression of adhesion/migration factors in MSCs.

METHODS: To study the effect of inflammatory factors and SF on mRNA expression of adhesion/migration factors, MSCs were cultured in monolayer in serum-free medium with 1% ITS with or without 50ng/ml, 20ng/ml, 1 ng/ml IFNγ and TNFα or 20% SF of OA, rheumatoid arthritis (RA) or healthy subjects added to the media. MSCs were cultured with inflammatory factors at 20% O2 or 1% O2 for 24 or 72 hours or with SF at 20% O2 for 48 hours. mRNA expression of adhesion/migration factors CD44, CXCR1, CXCR3, CXCR4, CCR1, CCR4, CCR5, PDGFRa, PDGFRb, CX3CR1, ITGb1 and ITGb2 were analysed and related to the housekeeper: HPRT, which was not affected by either inflammation or oxygen tension.

RESULTS:

Inflammatory factors significantly increased the mRNA expression of CCR1 and CCR4 and significantly decreased the expressions of PDGFRa and PDGFRb. The effects were similar after 24 hours and 72 hours of exposure to inflammatory factors. There was a dose response effect of IFNγ and TNFα on the expression of CCR1 and PDGFRa. Oxygen tension did not influence the expression of the adhesion/migration factors.

Culture in OA or RA SF increased CCR1 and PDGFRa mRNA expression. All SFs decreased the expression of PDGFRb.

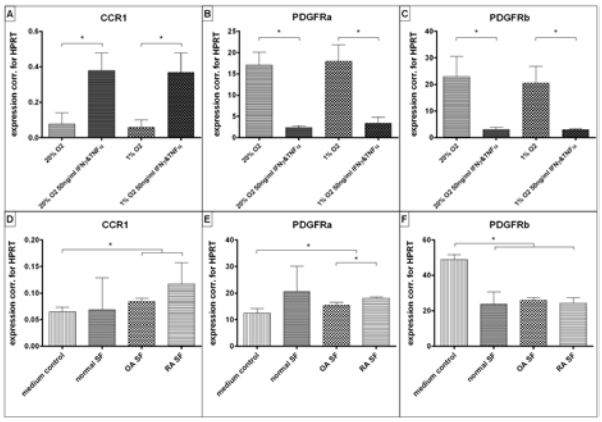

*Fig. 1: mRNA Expression of adhesion/migration factors CCR1, PDGFRa and PDGFRb in MSCs when cultured with inflammatory factors (A-C) and SF (D-F). *P ≤ 0.05*

DISCUSSION & CONCLUSIONS: Up regulation of adhesion proteins on MSCs would hypothetically increase engraftment and a prolonged presence in the joint after injection. Inflammatory factors appeared to increase some of the factors but decrease others. The effect of SF is similar to that of the inflammatory factors, for some but not for all adhesion/migration factors. This can be due to concentrations of the inflammatory factors that are generally low in SF. The up- and down-regulation shows the complexity of the regulation of these migration and adhesion proteins. The role of (regulation of) these factors on the engraftment of MSCs after injection, as well as possible ways to modulate this, is next to be investigated.

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Cell and Protein Delivery Systems to Maximise Efficacy and Safety in Regenerative Medicine Products

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INTRODUCTION : Many regenerative medicine applications require the localisation of potent signals at the site of treatment. These signals may include surface chemistries, exogenous cells and drugs & growth factors.

Delivery of these signals is a key issue in the field because:

1. Many cell therapies have short ranges of action. They need to be located at the site of repair otherwise efficacy is lost ad safety could be compromised.

2. Regenerative drugs need to act over long periods of time to stimulate tissue formation but localised administration may only be possible at the time of surgery/medical intervention.

3. Regenerative drugs cause side effects when delivered outside of the target site/tissue.

4. Cell therapies and to some extent growth factors are fragile and the mode of delivery can compromise their efficacy.

5. The site of administration within the body may be compromised by inflammation, scarring and/or poor vascularisation.

6. Local biomechanics at the site of regeneration may be detrimental to the regenerative process.

There is evidence in the literature on cell (Smith et al., 2012; Zhang, Chen, Wang, Wei, & Hu, 2010.) and protein therapies (Carragee, Hurwitz, & Weiner, 2011) that delivery problems may result in unacceptable side effect incidence or loss of > 97% of the administered cell dose.

PRESENTATION OVERVIEW:

This keynote presentation will begin by presenting the evidence from the literature on successes or problems encouraged in the administration of regenerative medicine products.

A series of delivery systems will then be presented that address the issues described in the INTRODUCTION.

There are many interesting delivery systems being developed by biomaterials groups across the world and musculoskeletal tissues offer important clinical targets for these systems.

We have focussed on developing injectable materials that convert from a fluid to a porous scaffold in response to small temperature changes. A potential advantage of these systems is the ability to control surface chemistry, drug release over periods of days to months, local biomechanics and cell location.

Two thermoresponsive materials will be presented with examples of delivery of small molecule drugs, protein biopharmaceuticals, mesenchymal stem cells and endothelial progenitors.

Applications in minimally invasive delivery of treatments of osteomyelitis, enzyme-free passage of mesenchymal stem cells(Cheikh Al Ghanami, Saunders, Bosquillon, Shakesheff, & Alexander, 2010) and muscle ischaemia(Saif et al., 2010) will be presented. Future applications in 3D printing of cells and polymers to manufacture personalised combination products will be explored.

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Mimicking the stem cell niche with biomaterials

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Proper tissue maintenance and regeneration relies on intricate spatial and temporal control of biochemical and biophysical microenvironmental ('niche') cues, instructing stem cells to acquire particular fates, for example remaining quiescent or undergoing self-renewal divisions. Despite rapid progress in the identification of relevant niche proteins and signalling pathways using powerful in vivo models, to date, many adult stem cell populations cannot be efficiently cultured in vitro without rapidly differentiating. To address this challenge, we and others have been developing biomaterial-based approaches to display and deliver stem cell regulatory signals in a precise and near-physiological fashion, serving as powerful artificial niches to study and manipulate stem cell fate both in culture and in vivo [1]. In this talk I will highlight recent efforts in my laboratory to develop two-dimensional [2] and three-dimensional micro-arrayed artificial niches based on a combination of biomolecular hydrogel engineering and microfabrication. These platforms allow key characteristics of stem cell niches to be mimicked and the physiological complexity deconstructed into an experimentally amenable number of distinct signalling interactions. The systematic deconstruction of a stem cell niche may serve as a broadly applicable paradigm for defining and reconstructing artificial niches to accelerate the transition of stem cell biology to the clinic.

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Injectable polyurethane/alginate composite scaffolds for cell delivery

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INTRODUCTION: Direct injection of cells into the body can result in large-scale death as well as the migration of cells from the injection site. Thus, there is a compelling need for suitable polymeric carriers that can provide a scaffold for adhesion of transplanted cells as well as a template for new tissue formation [1]. Lysine-derived polyurethane scaffolds, which are porous, biodegradable, and biocompatible, have been reported to support cellular infiltration and new tissue formation in subcutaneous, cardiovascular, and bone models [2]. Due to their injectability and settability, two-component lysine-derived polyurethanes were investigated as novel carriers for local delivery of cells using minimally invasive surgical techniques.

METHODS: Injectable polyurethanes (PUR) were synthesized from a polyester triol, an iron acetylacetonate catalyst, and a lysine triisocyanate (LTI)-PEG prepolymer. To protect cells from reacting with the prepolymer, MC3T3-E1 cells or rat BMSCs were encapsulated in 300 - 800 μm oxidized alginate beads [3] prior to embedding in the PUR scaffold. Cell viability was assessed using a Live/Dead viability kit (Invitrogen). The degradation rate of oxidized alginate beads embedded in the scaffolds was evaluated by SEM. In a proof-of-concept experiment, the ability of the carrier to promote matrix deposition *in vivo* was evaluated in 8-mm excisional wounds in rats.

RESULTS: When cells encapsulated in Alg beads were embedded in PUR scaffolds, viability decreased with decreasing bead size, suggesting that the chemical reaction adversely affected the cells. Viability was increased to >70% for 500 μm beads by delaying addition of the beads for 3 min after mixing the reactive liquid PUR components. To improve interconnectivity, scaffolds augmented with 70 wt% oxidized alginate beads (500 μm) were investigated. SEM images revealed interconnected macropores resulting from degradation of the oxidized alginate beads (500 um), as well as smaller pores (50-70 μm) resulting from gas blowing (Fig. 1A). Rat BMSCs injected into 8-mm excisional wounds showed viable cells for up to 14 days. Histological sections at 4 and 7 days revealed that deposition of new extracellular matrix was enhanced for PUR/Alg scaffolds augmented with 10^6 BMSCs/ml (Fig. 1B) compared to the scaffolds alone (Fig 1C), which was quantified by histomorphometry (Fig 1D).

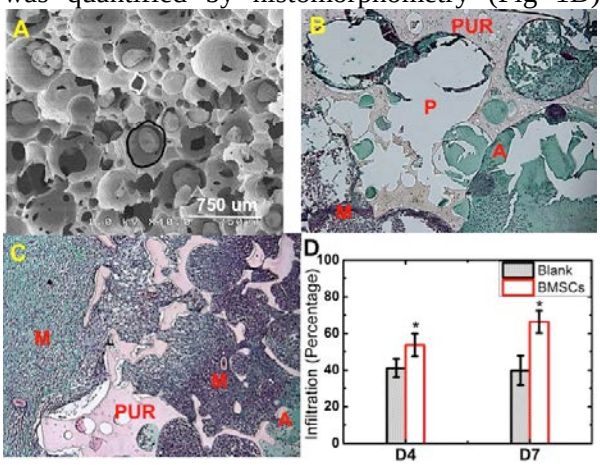

Fig. 1: (A) SEM image of a PUR/Alg scaffold. Images (20X) of PUR/Alg scaffolds injected into 8-mm excisional wounds in rats with (B) 10^6 BMSCs/ml and (C) no cells. (D) Histomorphometry shows significantly more new matrix at 4 and 7 days for scaffolds augmented with BMSCs.

DISCUSSION & CONCLUSIONS: *Previous studies have highlighted the need for macropores in nanostructured hydrogels to facilitate diffusion of nutrients, cellular migration, and matrix deposition [4]. In this study, we have shown that cells are protected from the exothermic and CO_2-releasing urethane reaction by encapsulation in oxidized alginate beads prior to embedding in the injectable PUR. After cure, the beads degrade in 1-2 days to form macropores that support deposition of new matrix* in vivo. *These observations highlight the potential utility of injectable PUR/Alg scaffolds augmented with cells and biologics for healing of musculoskeletal tissue defects.*

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ACKNOWLEDGEMENTS: *This work was supported by the NIH and OETRP.*

HMSC proliferation and differentiation are dependent on chemistry and surface roughness of calcium phosphate bone substitutes

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INTRODUCTION: Ceramic scaffolds have been extensively used as bone substitute materials in order to enhance bone regeneration, thanks to their osteoconductive and biocompatible properties. However, their tendency for granular degradation and incomplete resorption are still a major drawback in orthopaedic surgery.[1] Based on the enhanced sensitivity of HMSCs to surface roughness[2] and to ceramics chemistry, the aim of the present study was to examine the role of these properties on proliferation and phenotypic expression.

METHODS: Three chemically different ceramics were tested: hydroxyapatite (HA), 0.8% silicate-substituted HA (SA), and HA-tricalcium phosphate 60/40 (HA-TCP). Roughness was altered on each material by two different mechanical treatments (using 80 and 1000 Grit SiC pads) and by a chemical treatment (acid-etching, AE, 37% phosphoric acid for 30"). The materials were characterized by SEM, XPS, contact angle and confocal microscopy. HMSCs were seeded on samples and at confluence, cell lysates were used to calculate DNA content and alkaline phosphatase (ALP) specific activity (normalized by total protein). Media were used to determine osteocalcin (OCN), osteoprotegerin (OPG), and vascular endothelial growth factor (VEGF).

RESULTS: Samples showed a statistically significant decreased surface roughness after 1000Grit treatment, higher after acid-etching treatment and highest after 80Grit treatment. Comparing the effect of roughness on cell behaviour, cell proliferation was enhanced on rougher surfaces on HA and HA-TCP, but was not sensitive to the presence of Si. ALP specific activity was sensitive to surface roughness only on HA-TCP, where changes from the original had a negative effect. OPG was enhanced on the smoothest surfaces on HA and SA (fig.1, 2) but not on HA-TCP (fig.2). OCN synthesis was lower on all the surfaces after treatments. VEGF synthesis was enhanced on the smoother and the original surfaces only on HA. Cell proliferation was not dependent on chemistry except on the roughest surfaces (80Grit) on HA-TCP where it was highest. ALP activity was higher on the HA-TCP chemistry on the original and smooth surfaces. OPG was only significantly lower on HA-TCP after all the treatments. OCN was dependent on chemistry only on SA. VEGF was always higher on HA.

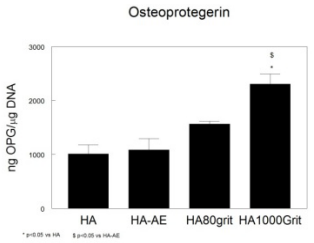

Fig.1: OPG expression on HA

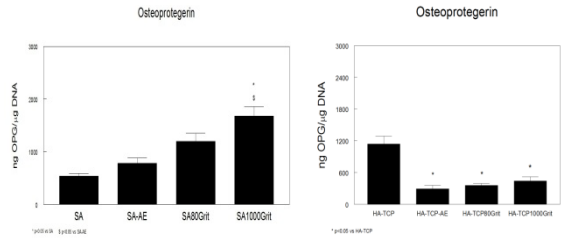

Fig.2: OPG expression on SA (left) and HA-TCP (right)

DISCUSSION & CONCLUSIONS: This study shows that the behaviour of stem cells in contact with bone substitute ceramics is sensitive to properties of the material like chemistry and roughness, which seems to work together to affect the cell response. Cell proliferation was enhanced on the rougher surfaces, but this effect was abolished in the presence of Si; ALP activity was dependent on the roughness, especially in the presence of TCP; OPG was enhanced on smooth surfaces, but in the presence of TCP changes in roughness has a negative effect; OCN wasn't affected by changes in roughness and VEGF was always higher on the HA. The results indicate that modification on HA scaffold chemically and/or structurally can enhance bone regeneration in different conditions.

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Autologous serum in combination with a nanostructured bone graft material improves matrix remodeling and bone formation in the sheep model for tissue engineering of bone grafts in clinically relevant size

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INTRODUCTION:

Therapeutic approaches for large-scale bone defects are currently limited to a few strategies, which are often associated with significant severe side effects for patients. Compared to non-vascularized grafts, the use of vascularized grafts shortens the period of recovery and speeds up the return of function. The sheep AV-loop model was established in the last years to engineer vascularized transplantable tissues in clinically relevant size. Bone substitutes could be vascularized and ectopic bone parts were engineered in this model, but previous studies failed to form a bone block which is stable enough for transplantation in a critical size bone defect. The present study aims at engineering an axially vascularized bone blocks with sufficient primary stability.

METHODS: For that purpose the primary stable bone substitute NanoBone® block was tested subcutaneously with bone marrow, directly re-transplanted or expanded autologous mesenchymal stem cells (MSC) both in combination with and without rhBMP-2. NanoBone® was saturated in fibrin, autologous blood serum or cell culture medium prior to implantation. NanoBone® evaluation in the sheep AV-loop model focusing on the axial vascularization of this bone graft material followed. NanoBone® blocks soaked with blood were implanted in a completely isolating Teflon or a perforated titanium isolation chamber. Vascularization was assessed by different imaging procedures and bone formation was analyzed using immunohistochemistry and molecular biology methods.

RESULTS: Autologous serum led to increased remodeling and earlier bone formation compared to fibrin within NanoBone® scaffolds. Real-time PCR analyses proved the upregulation of osteogenic genes. Best results were achieved using $6*10^6$ expanded MSC in combination with 60 µg/ml rhBMP-2 in autologous serum (Fig. 1). The NanoBone® block in the AV-loop model showed a good vascularization pattern with beginning bone formation and remodeling of the nanostructured bone graft material within this time period. Using the perforated titanium chamber in the AV-loop model an accelerated vascularization of the constructs inside the chamber could be shown over time (Fig. 2). This earlier vascularization led to increased remodeling and earlier bone formation compared to the closed Teflon chamber.

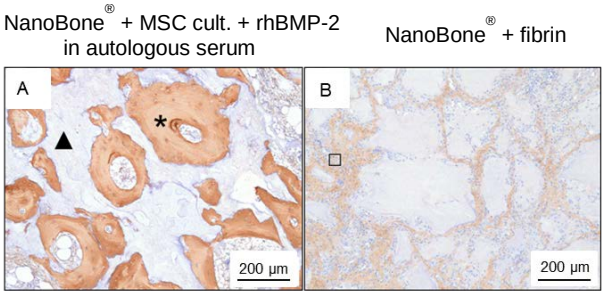

Fig.1: (A) Immunohistochemical staining of collagen type I (= bone, ▲ = NanoBone®, □ = ingrowth of connective tissue). A: NanoBone® s.c. in combination with 6 x 10^6 cultivated MSC, 60 µg/ml rhBMP-2 and autologous serum B: NanoBone® in combination with fibrin*

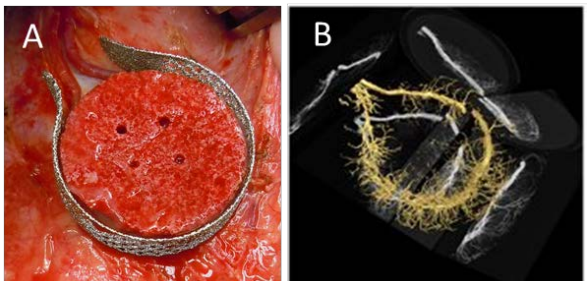

Fig. 2: (A) Titanium implantation chamber with a NanoBone® block and the AV-loop in the sheep model. (B) µCT of an AV-loop and a three-dimensional reconstruction of the vessels filled with the contrast agent shown as an isosurface extraction.

DISCUSSION & CONCLUSIONS: The next step will be to test further formulation of the nanostructured bone graft material and subsequently the transplantation of an engineered bone block in the sheep AV-loop model into a critical size tibial defect. In the future this concept could possibly replace current therapeutic concepts for treatment of bone defects.

Immunomodulation by adult stem cells

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INTRODUCTION: Over the past 10 years, adult stem cells have received much attention, not only for their regenerative potential but also for their immunomodultory capacity. We have focused our attention on two different types of stem/stromal cells and investigated their immune suppressive characteristics. The first is mesenchymal stromal cells (MSC), a population of heterogenous adult stromal cells first identified in the bone marrow. The second is a vessel associated stem cell derived from muscle tissue named mesoangioblasts[1]. These vessel associated stem cells have been demonstrated to ameliorate signs of muscular dystrophy in both mouse and dog models of muscular dystrophy. Currently mesoangioblasts are in phase I/II clinical trial for treatment of patients with Duchenne muscular dystrophy. MSC and mesoangioblasts share a number of surface markers and have similar differentiation capacity, however unlike mesoangioblasts MSC do not differenciate into skeletal myocytes. MSC have the capacity to modulate the immune response both in vitro and in vivo[2-3]. However, until recently the immunosuppressive capacity of mesoangioblasts remained to be elucidated. Herein, we characterized the in vitro immunomodulatory characteristics of human MSC, mesoangioblasts and induced pluripotent stem cell derived mesoangioblasts (HIDEM) and examined their mechanisms of action.

METHODS: Human mesoangioblasts were isolated from adult skeletal muscle, and human MSC were isolated from iliac crest of healthy adult donors. Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats. Proliferation of PBMCs was measured using the 5,6 carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay or [3H]-thymidine incorporation assay. Standard flow cytometry, ELISA and RT-PCR were used to analyse surface markers, intracellular cytokine production, cytokine protein production and gene expression. Neutralising and blocking studies were carried out using neutralising antibodies to IFN-γ and TNF-α, recombinant IL-1 receptor antagonist and inhibitors of IDO (1-Methyl-L-tryptophan) and Cox-2 (Indomethacin and NS-398).

RESULTS: MSC, mesoangioblasts or HIDEM did not evoke but suppressed T cell proliferation and effector function in vitro in a dose and time dependent manner. Furthermore, these cells modulated both $CD4^+$ and $CD8^+$ T cells in a reversible manner without inducing a state of anergy. Neutralising studies demonstrated the important role played by IFN-γ and TNF-α in the initial activation of MSC and mesoangioblasts and identified IDO and PGE-2 as the mechanisms of action involved in mesoangioblast suppression of T cell proliferation.

DISCUSSION & CONCLUSIONS: Together, these data demonstrate a previously unrecognised capacity of mesoangioblasts and HIDEM to modulate immune responses[1-2] and highlight the many similarities with MSC in this context. These finding support the idea that mesoangioblasts may function in muscle repair on two fronts through direct regeneration and paracrine effects on the inflammatory environment.

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M1 polarised macrophages are potential mediators of the anti-chondrogenic effects of osteoarthritic synovium

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INTRODUCTION: Osteoarthritis (OA) is a disabling degenerative joint disease affecting synovial joints, characterised by the loss or damage of articular cartilage and inflammation of the synovial membrane. The release of pro-inflammatory cytokines and other soluble mediators by inflamed synovium may accelerate cartilage matrix degradation and inhibit cartilage repair. Synovial macrophages are considered to play a prominent role in the production of such pro-inflammatory mediators. Both osteoarthritic synovial fluid and synovium conditioned medium (SCM) have been reported to inhibit the chondrogenic differentiation of Mesenchymal Stem Cells (MSCs) (1-2). Therefore the presence of a catabolic environment may limit the use of MSCs in cell-based cartilage regeneration strategies. In this study, we sought to investigate:

1) Whether the inhibition of chondrogenesis of MSCs by osteoarthritic synovium is due to macrophages

2) The role of macrophage subtype in this inhibition

METHODS:

Osteoarthritic synovial tissue was cut in to pieces between 1-3 mm^2 and 200 mg of tissue was cultured in 1 ml of serum free medium. Following 3 days of culture, Synovium Conditioned Medium (SCM) was harvested.

Peripheral blood monocytes were stimulated with IFN-γ (10 ng/ml) & LPS (100 ng/ml) or IL-4 (10 ng/ml) for 72 hr, for differentiation towards an M1 or M2 phenotype respectively. Cells were further cultured for 24 hr in serum-free medium without additional cytokines prior to harvesting of CM.

Human MSCs (hMSCs) were encapsulated in 1.2% alginate and cultured in chondrogenic differentiation medium. After 14 days 5% SCM, or 20% M1 or M2 CM was added for a further 3 days. Samples were harvested for mRNA analyses of collagen II and aggrecan.

Immunohistochemical staining for CD11c (M1 marker) and CD206 (M2 marker) was performed on osteoarthritic synovium sections.

RESULTS:

Chondrogenic gene expression in hMSCs was downregulated (74% ± 22.2% inhibition compared to positive control values for COL2, 23% ± 28.4% for AGCN), following 3 days of 5% SCM treatment. Next to this variation in inhibition of chondrogenesis, different synovium donors secreted variable amounts of pro- and anti-inflammatory cytokines. Histology demonstrated both M1 and M2 macrophages in the synovium. Furthermore, treatment of hMSCs during chondrogenesis with 20% M1 CM, reduced gene expression of COL2 and AGCN (Fig.1).

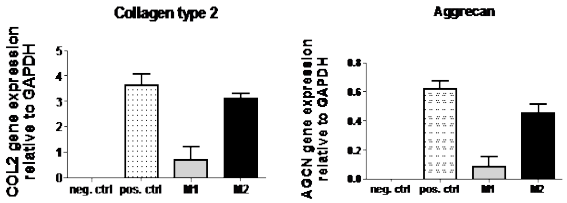

Fig.1: M1 CM negatively impacts collagen II (COL2) and aggrecan (AGCN) gene expression by hMSCs (M1 & M2 CM donor n=3)

DISCUSSION & CONCLUSIONS: Chondrogenic gene expression was downregulated in hMSCs treated with M1 CM, however this effect was not observed with M2 CM. These findings highlight a potential role of M1 macrophages in the anti-chondrogenic effect of osteoarthritic synovium. Modulation of M1 macrophages may act as a beneficial strategy to alleviate the negative impact of osteoarthritic synovium on hMSCs in cell replacement strategies.

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ACKNOWLEDGEMENTS: The research has received funding from the European Union's 7th Framework Programme under grant agreement no NMP3-SL-2010-245993.

Mesenchymal Stroma Cells implanted in fibrin hydrogel trigger attraction of M1 macrophages, endothelial cells and early immune modulation stimulating long bone healing without long-term engraftment.

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INTRODUCTION: Non-healing or delayed healing bone defects remains a major problem in orthopedic surgery. Implantation of Mesenchymal Stroma Cells (MSC) on suitable carriers is a promising approach to stimulate closure of such bone defects. According to a changing paradigm of MSC function [1, 2], trophic and/or immunomodulatory features of MSC may be more important than a high osteogenic activity. Aim of this study was to unravel relevant actions of MSC on early bone healing with regard to host cell recruitment, immune modulation and long-term engraftment.

METHODS: A plate-stabilized 2 mm gap was created in the right femur of female rats (n=42) and MSC expanded from male rats were embedded in a fibrin hydrogel and implanted into the bone defect. Control animals received an empty fibrin clot. After 3, 6, 14 or 28 days callus was removed and analysed for host cell invasion (histology), local cytokine expression (quantitative PCR) and persistence of male MSC (SRY-gene PCR).

RESULTS: While only a few cells invaded the cell-free fibrin clot, the implanted MSC attracted many host cells into the fibrin carrier. A migration front dominated by M1 macrophages and endothelial progenitors was formed and tissue maturation and primitive vessel formation were apparent in the MSC-group at day 6. No changes in expression of relevant immune mediators could be detected on single immune cell level at day 3 and 6. Male MSC persisted at day 14, but were not detectable at day 28 after implantation.

DISCUSSION & CONCLUSIONS: Rather than directly forming permanent bone, MSC expressed bioactive factors like IL-6, VEGF and MIP-2 deemed to promote tissue repair, which complemented inflammatory cytokines secreted by recruited immune cells. In conclusion our results support a mainly trophic rather than osteogenic role for MSC in long bone repair.

ACKNOWLEDGEMENTS: This study was funded by the priority program SPP1468 “Immunobone” of the German Research Foundation (DFG).

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Some Practical Aspects of Cell Therapy

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INTRODUCTION: Stem cell and progenitor cells have long been the central element in medicine. They have determined our development as individuals, our growth and maturations and our response to injury, disease and therapies. We have long known that no new tissue forms in the absence of an underlying population of progenitor cells that are capable of responding to an activating event by dividing and giving rise to a wave of progeny that are capable of migrating, surviving and differentiating to take on the phenotype of one or more of the innumerable cells comprising mature adult tissues. Neither scaffolds, or growth factors, or biophysical stimuli nor pharmacologic agents can ever achieve an optimal effect in a setting where the underling population of target cells is deficient or suboptimal.

While this has been long evident, it is only in the past half century and particularly in the last decade that research has enabled clinicians and patients to consider the tangible possibility of purposefully targeting stem and progenitor cells therapeutically. It is even more recently that practical methods have can be considered for harvesting, isolating, processing, characterizing or transplantation of progenitors as a means of repair, augmentation, replacement or regeneration of diseased or missing tissues.

As the promise of stem and progenitor cell therapies becomes a clinical reality the practical scientist is inevitably drawn back to a series of nagging questions. What are the cells I want? Where can I find them? How many are there? What are their characteristics? How can or should we define them? What should we call them? Which cell don't I want? How are they different from other cells? How are they the same? What factors most influence their behavior (activation, migration, proliferation, survival differentiation, death) to accomplish my goals? It is possible to efficiently separate cells that I want from those that I don't? Which of the available methods work best? Would it make a clinical difference if certain cell populations were concentrated, selected, purified? Would it make a clinical difference if some cell populations were depleted or removed?

These practical questions in turn lead to the confrontation with practical issues. How can stem cells and progenitor cells be assayed reliably? How reliable are current methods? Can these assays be made better? What standards can and should be created for assay of cells, their number, characteristics, and particularly their biological potential or potency? Is our existing nomenclature and our conventional use of names for cell types and cell populations helping us communicate? Does our nomenclature differentiate between cells and populations with different functional characteristics and/or biological potential? If so, where? If not, where not?

DISCUSSION & CONCLUSIONS: This presentation will explore some paths through this maze of questions, opportunities and options, and open a discussion for how these questions can and may be answerable.

Processing MSCs for clinical uses

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INTRODUCTION: Due to their multipotency and immunosuppressive properties Mesenchymal Stem/Stromal Cells (MSCs) are important tools for treatment of immune disorders and tissue repair. The increasing use of MSCs, their definition as advanced-therapy medicinal products (ATMP) in European regulations, and the US Food and Drug requirements for their production and use imply the use of production processes that should be in accordance with Good Manufacturing Practices (GMPs).

Recently, important concerns have appeared regarding the risk of transformation after long-term cultures, corresponding to some cells escaping the senescence program. Using hTERT transduced immortalized MSCs, it was demonstrated that transformation was a long (over 200 population doublings) and multistep process involving the deletion at INK4a locus. MSCs cultured during reasonable term reaching less than 25 population doublings do not undergo transformatio. Moreover, since the first clinical trial in human (1995) emerging tumours are never reported following injections of MSCs. For avoiding different side effects linked to senescence and transformation, it is important to limit the number of population doublings (PL) experienced by MSCs during culture processes.

Complying with GMPs requires precisely defining the production process(es) as well as the multiple criteria needed for a quality final product. Such variables include the environment, staff training and qualification, and controls. Developing processes based on well- or completely defined media and operating in closed systems or bioreactors is important and will increase safety and reproducibility. One of the most challenging issues remains implementation of relevant and reproducible controls for safety and efficacy. A linking of researchers, research and development teams, producers and clinicians is mandatory to achieve GMP-compliant processes with relevant controls for producing well-defined, safe and efficient MSCs

Characterisation of a novel, clinically compliant, serum-free culture system for bone marrow derived mesenchymal stem cells

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INTRODUCTION: Mesenchymal stem cells isolated from human bone marrow (hMSCs) represent a clinically proven cell source for the treatment of a number of disease states. Currently, regulatory acceptance of either autologous or allogeneic human MSCs for clinical tissue repair or regeneration is hampered by the requirement of fetal bovine serum (FBS) which is necessary to generate sufficient cell numbers [1]. The aim of this project was to evaluate a novel animal and serum-free culture system for generation of hMSC for clinical use.

METHODS: Bone marrow aspirates were obtained, after informed consent and with ethical approval from the research ethics committee of University College Hospital Galway or from Lonza (Maryland, USA), from the iliac crest of healthy donors between 18 and 30 years old. Mononuclear cells were plated at 2.4-2.9 x 10^5 cells/cm^2 in normoxia and hypoxia. Serum-free MSCs (MSC-SF) were plated onto fibronectin-coated plates to facilitate attachment while MSCs cultured with serum (MSC-SC) were plated directly onto tissue culture plastic. Media changes were performed every 3-4 days and cells were passaged at 80-90% confluency. Analysis of growth kinetics was performed and cells induced to undergo osteogenic, chondrogenic and adipogenic differentiation at passage 3 (P3). In vitro evaluation of angiogenic potential was assessed by the addition of hMSC conditioned medium to human umbilical vein endothelial cells (HUVEC) cells on Matrigel®, to assess tubule formation.

RESULTS: Cells cultured in serum containing and serum-free conditions displayed equivalent growth when cultured in normoxia. Serum-free cultured cells displayed greater growth when cultured in hypoxia (*Fig 1*). MSC-SF were shown to have a distinct morphology and were more compact than MSC-SC (*Fig 2*). Flow cytometry data indicated an MSC phenotype for both cell types. MSC-SF showed increased osteogenic differentiation and equivalent chondrogenic and adipogenic differentiation compared to MSC-SC. Pro-angiogenic potential of MSC-SF was superior to MSC-SC (data not shown).

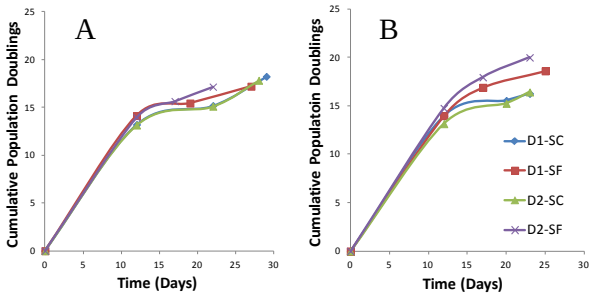

Fig.1: Growth of MSC-SF and MSC-SC in normoxia (A) displaying equivalent growth. MSC-SF grown in hypoxia (B) display greater growth rates compared to MSC-SC (n=2).

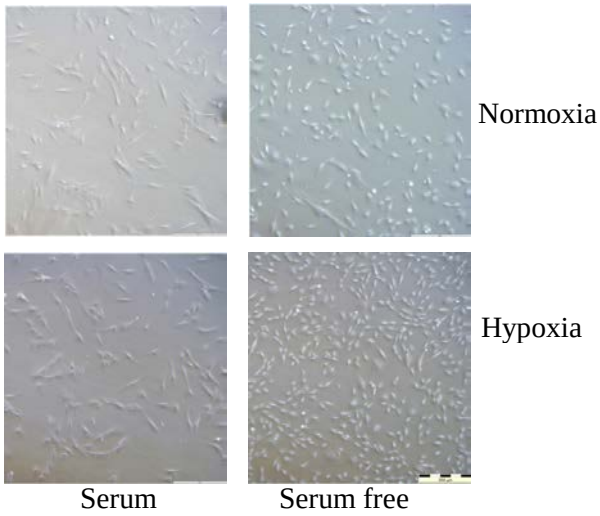

Fig 2: Morphology of MSC-SC and MSC-SF in normoxia and hypoxia. (Scale bar: 200µM).

DISCUSSION & CONCLUSIONS: Serum-free culture of bone marrow derived hMSCs using this novel system represents a viable alternative to the current practice of using animal serum. Promising data to date indicate that this culture system will address the current unmet need of production of a GMP compliant, serum- and zeno-free hMSC product for clinical use.

ACKNOWLEDGEMENTS: Funding was from Science Foundation Ireland/Grant 09/SRC/B1794 and the Irish PRTLI-5 programme co-funded by European Regional Development Fund.

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Safety of mesenchymal stem cell application for regenerative therapy: Expression and functional role of c-Myc for expansion and cell differentiation

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INTRODUCTION: Mesenchymal stem cells (MSCs) are an attractive cell source for regenerative therapy owing to their high proliferation capacity, multipotent differentiation ability and trophic properties. In the context of extensive *in vitro* cell propagation before clinical application, a thorough investigation of safety issues of MSC populations is desired. Beside immune activation after exposure to xenogeneic compounds during culture, the risk of tumorigenic degeneration is one major concern for therapeutic application of MSCs. Expansion protocols optimized for highly proliferative cells could preselect MSCs with elevated levels of growth promoting transcription factors with oncogenic potential like c-Myc. C-Myc has a dual role for tumour pathogenesis and cell proliferation and is among the four classical factors applied to generate induced pluripotent stem cells from differentiated cells. Expression and function of c-Myc during expansion and *in vitro* differentiation of MSC was so far unknown, although such information is of major interest regarding safety concerns. Aim of this study was to assess c-Myc expression during extensive *in vitro* expansion of MSC, modulate its expression level and follow effects on cell growth, differentiation ability and ectopic bone formation capacity of the cells.

METHODS: Bone-marrow derived MSCs were transduced with a retrovirus in passage 1 to enhance c-Myc expression or GFP expression or were left untreated. Cells were expanded over 15 passages and subjected to differentiation into chondrocytes, osteoblasts and adipocytes at regular intervals during expansion. C-Myc overexpression was investigated by Western-blotting and DNA-binding activity was recorded. Gene expression levels were quantified by qPCR and ectopic bone formation capacity was tested on ß-TCP in immunocompromised mice.

RESULTS: C-Myc levels were lower in primary human MSC compared to culture expanded cells. Elevated c-Myc levels stimulated proliferation of MSC allowing production of more cells and an expansion to higher passages compared to control cells. Accelerated proliferation of MSCs by overexpression of c-Myc conferred a selection advantage for the cells during expansion culture, since GFP-labelled control cells were lost within a few passages in co-cultures between c-Myc overexpressing and GFP-overexpressing MSC. Elevated c-Myc (passage 4 – 10) had no apparent effect on adipogenesis according to Oil Red O staining per protein. At passage 4 – 10 c-Myc overexpression showed similar mineral deposition per well according to Alizarin Red quantification but reduced activity per cell arguing for a reduced osteogenic activity on a per cell basis. Expression of SOX9, the master transcription factor of chondrogenesis was significantly reduced in c-Myc-overexpressing MSCs versus GFP cells while COL2A1 was only little affected. c-Myc- and GFP-MSCs of passage 2 were both capable to form ectopic bone in immunocompromised mice without evidence of tumour formation.

DISCUSSION & CONCLUSIONS: Higher c-Myc expression appears to be a positive selection mechanism active in MSC populations during expansion culture which is mediated by the c-Myc driven stimulation of cell proliferation. Higher c-Myc levels did not eliminate the *in vitro* differentiation capacity or the ectopic bone formation ability of the cells at low passages. Safety concerns and assessment of tumour formation should thus be further dissected at high passages for therapeutic applications for which a long term expansion of MSC is required.

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

Modelling the mesenchymal stem cell niche in bone marrow

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INTRODUCTION: Standard isolation and culture techniques for bone marrow-derived mesenchymal stem cells (MSCs) do not mimic the native MSC environment and lead to pronounced phenotypic changes *in vitro*[1,2]. Current cell therapies rely on an *in vitro* expansion phase which may not only compromise the therapeutic potential of these cells but is a lengthy, costly process. Manipulation of MSCs in the mononuclear cell fraction of bone marrow, using growth factors or gene transfer could negate these cellular and clinical challenges. In order to develop such therapies, we must better understand the regulation of MSC behaviour *in vivo* where interactions with haematopoietic cells would be present. Here, we describe the development of an *in vitro* culture system to model the MSC niche in bone marrow. The maintenance and proliferation of haematopoietic (CD45+) and mesenchymal (CD45-) cells were investigated.

METHODS: Bone marrow from consenting patients undergoing spinal fusion or joint replacement was processed by Ficol density centrifugation. Mononuclear cells were either seeded directly into polyurethane-fibrin scaffolds or cultured in monolayer in order to expand an adherent MSC population. *In vitro* expanded MSCs and the haematopoietic cell line KG1a were co-cultured in scaffolds under static or perfusion conditions and supplemented with FGF2. Cell proliferation and CD45 expression were assessed by BrdU incorporation and flow cytometry.

RESULTS: Our polyurethane-fibrin culture system supported viable populations of MSCs and KG1a over 7 days under both static and perfusion conditions. MSCs cultured in scaffolds were less proliferative than cells cultured in monolayer as shown by reduced BrdU incorporation (Fig.1). This reduction in proliferation was more evident in perfusion compared to static culture conditions. Co-culture of MSCs with KG1a resulted in enhanced proliferation of the CD45- population.

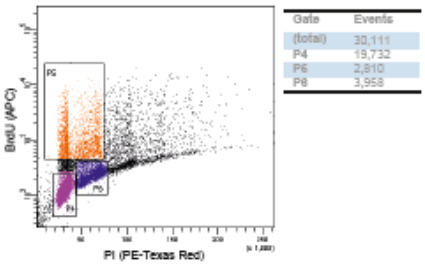

Fig. 1: Cell cycle analysis of MSCs cultured in polyurethane-fibrin scaffolds under static conditions.

DISCUSSION & CONCLUSIONS: Here, we describe a 3D culture system which maintains MSCs in a quiescent state, as they are in bone marrow prior to activation. MSC-haematopoietic crosstalk appeared to stimulate MSC proliferation in the presence of FGF2. Future work using this system to culture mononuclear cells will allow us to investigate how MSCs are activated in their niche environment to promote tissue repair *in vivo*.

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Testosterone enhances extracellular matrix synthesis by male intervertebral disc cells *in vitro*

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INTRODUCTION: Testosterone (T) is a hormone and regulator involved in the processes of development of the organism, *i.e.* promoting development of bone and muscle mass. Although T effects on the mesenchym derived muscle, bone and adipose tissues are well studied, T effects on intervertebral disc (IVD) have not been reported.

The aim was to test the hypothesis if physiological concentration of 10 ng/mL T (~30 nM) can improve *in vitro* chondrogenesis of human IVD cells and mesenchymal stem cells (MSC).

METHODS: Human IVD cells and MSCs were differentiated to chondrogenic lineage on gelatine scaffolds for four weeks, with or without T.

Chondrogenesis was assessed by cell viability, by measuring gene expression with quantitative PCR and extracellular matrix (ECM) accumulation with immunoblotting, immunohistochemical and biochemical methods.

RESULTS: Supplementation of T to chondrogenic culture promoted nuclear translocation of the cytoplasmic androgen receptor in both IVD cells (Fig. 1) and MSCs. Cell viability was not affected by T. In male IVD cells, T had a beneficial impact on chondrogenesis, especially in nucleus pulposus cells, demonstrated by increased expression of aggrecan, collagen type I and especially collagen type II (Fig. 2). Conversely, T had no effects on chondrogenesis of female IVD cells or MSC from both genders.

A gene expression array of TGFβ/BMP signalling cascade showed that in male IVD cells, T promoted a stable general but non-significant increase in gene expression. Furthermore, aromatase inhibitor anastrazole repressed the effect of T on ECM expression by IVD cells.

The results suggest that T increased extracellular matrix accumulation in male IVD cells in combination with its conversion to estradiol by the enzyme aromatase.

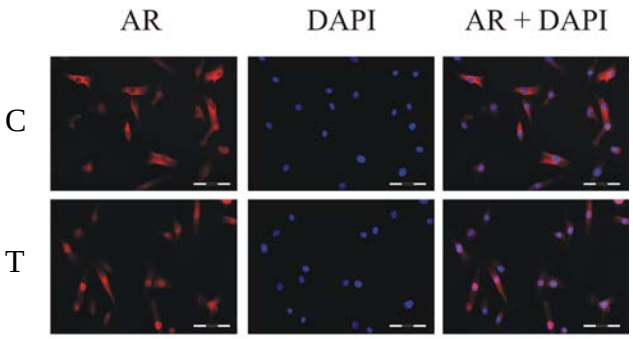

Fig. 1: Androgen receptor (AR) presence (red fluorescence) in IVD cells cultured without (top row) or with 10 ng/mL testosterone (bottom row) was determined by immunocytochemistry. Nuclear translocation of the cytoplasmic AR occurs in presence of testosterone in IVD cells cultured for 24 hours in monolayer. (Entire scale bar is 120 µm).

DISCUSSION & CONCLUSIONS: We demonstrated that T is effectively enhancing *in vitro* chondrogenesis in male IVD cells, rising the interest in the possible role of sex hormones in IVD degeneration. Nevertheless, T does not affect chondrogenic differentiation of female IVD cells and MSCs from both genders.

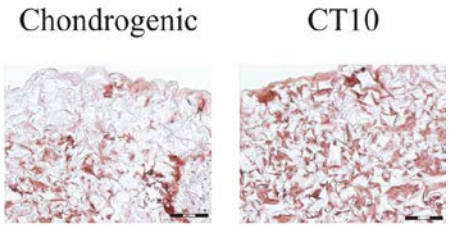

Fig. 2:Immunohistochemistry microphotographs of sections of male IVD cell-constructs cultured in chondrogenic medium without and with T (CT10) for 28 days. Collagen type II accumulation was depicted by positive red-coloured signal (Entire scale bar is 160 µm).

ACKNOWLEDGEMENTS: This work was supported by the Swiss Paraplegic Foundation and Swiss National Foundation Grant CR3I3_140717/1.

Determining the viscoelastic properties of trachea cartilage for scaffold design in tissue engineering

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INTRODUCTION: The trachea can be damaged as a result of compression, like blunt trauma, or as a result of tension, such as the increased internal pressure that presents in patients with a mechanical ventilation aid. In cases of severe tracheal trauma, it is desired to generate artificial trachea replacements by means of tissue engineering. Such replacements typically incorporate a tubular scaffold seeded with stem cells. By mimicking the appropriate physiological environment of the trachea, these stem cells will hopefully differentiate into cartilage cells. Early tissue engineering efforts included linearly elastic scaffold materials; however, the trachea is actually viscoelastic. Because stem cells are highly sensitive to the surrounding mechanical environment, it is fair to reason that if the viscous component of native trachea is large, then this property should be taken into account when designing a scaffold. In this study, trachea cartilage was mechanically tested to determine the importance of viscoelasticity when designing artificial trachea constructs to direct stem cell fate.

METHODS: Six-month-old porcine trachea was cross-sectioned between cartilage rings to obtain 9 ring samples for 3 different loading protocols (n=3 per test). The average width, inner diameter, and outer diameter were measured to be 13 mm, 14 mm and 22 mm, respectively. An ElectroForce 5500 Test Instrument (Bose, Eden Prairie, MN, USA) was used to characterize the viscoelastic properties of the cartilage rings under several loading configurations. Samples were mounted to the test instrument in three ways. For compression tests, samples were placed between platens in an axial or longitudinal position. For tensile testing, the samples were clamped between two grips in an axial position.

RESULTS: The properties of a viscoelastic material, including creep, stress relaxation and hysteresis, were demonstrated by compressive or tensile loading of the trachea cartilage rings. To investigate the creep response, rings were compressed axially or longitudinally with 120 g of force for four minutes. The nonlinear deformation responses indicated that the trachea rings have greater compressive strength in the longitudinal direction than in the axial direction. Stress relaxation tests were then performed with the cartilage rings under axial tension or compression. Samples were either pulled or compressed to 25 % axial strain for four minutes while the nonlinear load response was allowed to equalize. The rings were shown to have greater tensile strength than compressive strength in the axial direction.

Finally, the hysteresis response of the cartilage rings was investigated. Rings were cyclically preconditioned with sinusoidal application of a 25 g compression at 1.0 Hz for 10 cycles. The sample was then immediately compressed with 50 g of force and the load was subsequently removed. The area between the loading and unloading portions of the curve (Fig. 1) signifies the energy that is dissipated as a result of the material's viscoelastic nature. This area was calculated to be 1.5 mJ, a 74% dissipation of energy.

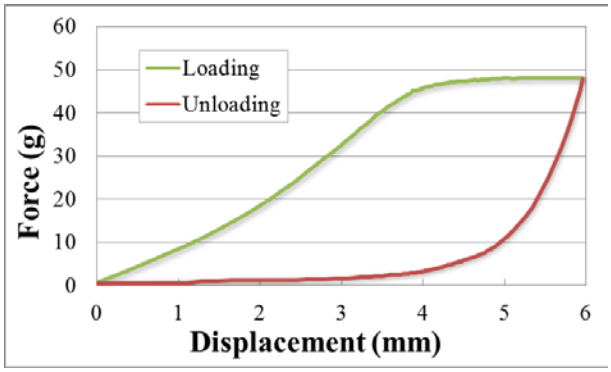

Fig. 1: A preconditioned ring of trachea cartilage was compressed and unloaded to demonstrate tissue hysteresis.

DISCUSSION & CONCLUSIONS: The large dissipation of energy that was observed in hysteresis tests indicates that the viscous behavior of trachea cartilage is just as important as the elastic behavior. Viscoelastic properties of trachea were also illustrated with nonlinear creep and stress relaxation tests. Taken together, these results indicate that a viscoelastic scaffold would provide a more physiological environment to direct stem cell fate in regenerative medicine efforts at engineering an artificial replacement trachea.

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Inferior ectopic bone formation of Mesenchymal Stroma Cells from adipose tissue compared to bone-marrow: rescue by chondrogenic pre-induction.

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INTRODUCTION: Human mesenchymal stroma cells derived from bone marrow (BMSC) and adipose tissue (ATSC) represent a useful cell source for bone tissue engineering. While ectopic bone formation is a standard activity for BMSC, this capacity for ATSC was so far unclear. Aim of this study was to judge the bone formation potency of ATSC in an ectopic mouse model and search for molecular differences between both cell sources.

METHODS: BMSC and ATSC (n=7) were cultured over 2 passages, seeded on β-TCP granules and implanted either directly or after 6 weeks of chondrogenic pre-induction into SCID mice. Bone formation of explants was quantified by histomorphometry. Molecular differences between BMSC and ATSC were assessed by RT-PCR.

RESULTS: Although ATSC showed better proliferation than BMSC and displayed a similar osteogenic *in vitro* differentiation, they did not form bone within 8 weeks *in vivo*. Chondrogenic pre-induction of ATSC/β-TCP constructs installed bone formation in >75% of samples at comparable levels to BMSC. ATSC expressed less BMP-2, BMP-4, VEGF, angiopoietin and IL-6, and more adiponectin mRNA compared to BMSC.

DISCUSSION & CONCLUSIONS: In conclusion, chondrogenic pre-induction was necessary to enable efficient ectopic bone formation by ATSC and to overcome their lower osteochondral commitment, reduced pro-angiogenic activity and altered immune modulation. This points towards a need for enhanced inductive conditions to make this more easily accessible cell source attractive for future applications in bone regeneration.

Enhancing therapeutic potential of MSC cells for bone regeneration

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INTRODUCTION: Stem cell therapies have been successfully used for treating various pathologies and disorders due to their potential for multilineage differentiation. Accelerating controlled differentiation of MSC in treating bone and cartilage defects requires the pre-culture of cells before inducing them towards specific lineage. This, however, has many drawbacks including the need of a secondary surgical procedure and maintenance of cells for long time out of their natural milieu. It is known that following injury different cells secrete chemotactic and proinflammatory factors that are crucial for triggering tissue regeneration. Hence, we aimed to evaluate the effects of one of these factors, IL1β, on bone mesenchymal stem cells (MSC) differentiation.

METHODS: Human bone marrow was obtained from vertebral bodies or iliac crests of donors with full ethical approval. MSCs were isolated through Ficoll gradient followed by adhesion to the plastic. Expanded cells were stimulated for 2 hours with 10 ng/ml IL1β either in suspension or after the adhesion to the tissue culture plastic. The effects of IL1β stimulation on MSC cytokine mRNA expression and protein secretion were analysed 48 hours after the treatment. The influence of IL1β stimulation on MSC differentiation towards osteogenic lineage was assessed on gene expression (Runx2, Sox9, Sp7) at day 1, 3, 7, 14 and 21. Matrix mineralisation was analysed with Alizarin Red S (ARS) staining and calcium incorporation assay. For osteogenesis cells were cultured in differentiation medium (DM) containing 10 nM dexamethasone, 5 mM β-glycerol phosphate and 50 µg/ml ascorbic acid or in medium without supplements (CM, control medium) for 28 days at 37°C, in 5% CO_2 in air. Medium was changed every 2-3 days.

RESULTS: The results showed that the stimulation with IL1β regulates the expression of cytokines implicated in various processes during fracture healing. Specifically, IL1β induced the up-regulation of genes involved in angiogenesis (IL6, IL8, IL11), bone formation (BMP2, BMP6, IL11) and immunomodulation (IL17B, IL12A, GMCSF). This was coupled with downregulation of mRNA expression of proteins involved in chondrogenesis, myogenesis and bone remodelling (GDF5 and GDF8, $TGF\beta_3$). Moreover, we report a positive effect of IL1β stimulation on osteogenic differentiation of MSCs. Sox9 gene expression was down-regulated in cells stimulated with IL1β already at day 1, whereas mRNA expression of two main transcription factors, Runx2 and Sp7 involved in the development of an osteoblastic phenotype, were upregulated. At day 21, we report a higher level of mineralisation (Fig.1) and calcium incorporation (Fig.2) in pre-stimulated MSC cultured in osteogenic medium.

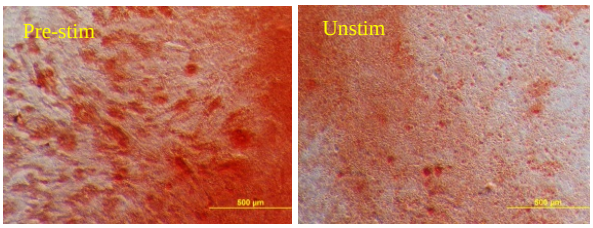

Fig. 1: ARS staining of MSC cells at day 21.

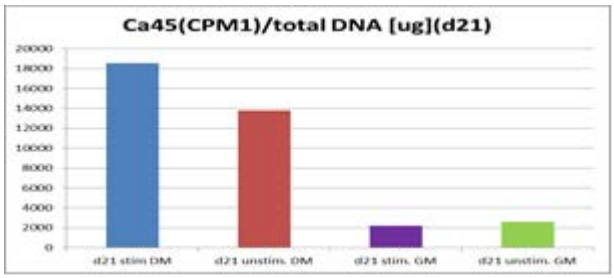

Fig. 2: Ca incorporation in MSC cultures.

DISCUSSION & CONCLUSIONS: The presented results following 2 hours of stimulation of MSCs with IL1β demonstrate the influence of this factor on the expression of multiple genes involved in osteoblastic lineage differentiation, as well as various stages of bone regeneration. Integrating inflammatory modulation in bone tissue engineering coupled with stem cell therapy would provide more powerful strategy to enhance bone regeneration processes.

Study of the cell/polyelectrolytes interaction: the role of the hyaluronan-based pericellular matrix

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INTRODUCTION: Polyelectrolyte multilayer (PEM) films are promising bioactive materials for the development of biomimetic films to be used in vitro or in vivo to control cellular behaviours. The formation of focal adhesion complexes, and the consecutive cytoskeletal reorganisation, influence important functions such as proliferation, differentiation, and migration. The initial cell-ECM/surface interaction is mediated by a pericellular matrix (PCM). PCM is a thick coat, constituted mainly by hyaluronan, lying between the cell membrane and the extracellular matrix, which recognizes the substrate and holds the cells close to the surface until stable contacts are established. In this study we explore how PCM mediates the cell interaction with positive (PEI) and negative (PSS) charged polyelectrolytes.

METHODS: Poly(ethylene imine) (PEI) and poly(sodium 4-styrene sulfonate) (PSS) were assembled on glass as layers by using the layer-by-layer method. The resulting films were 6±1.2 nanometer thick characterized by AFM and XPS analyses. GFP-expressing MG63 were seeded on such multilayer films and the very early events (30 minutes to 6 hours) of cell/surface interaction were highlighted. The presence of PCM was assessed by using specific hyaluronan binding proteins (HABP). The organization of cytoskeleton and focal contacts was then analysed via immunostaining of integrin b1, paxillin vinculin and actin. Adhesion and spreading of MG63 cells were evaluated by image analysis using LUCIA software. To evaluate the role of the PCM, the matrix was removed by hyaluronidase-treatment.

RESULTS: MG63 cells produce an extensive PCM, removable by hyaluronidase treatment. On PEI and Permanox® the cells mantained a round shape after 3 hours, and organized a thick homogeneous PCM. Instead, MG63 on PSS and fibronectin were spread and the PCM was present only on a few cells and mainly distributed along the flanks of the cells (fig 1).

Many cells adhered on PSS and no differences were evident after hyaluronidase treatment. On PSS cells were elongated, and cell area increased over time. Cells showed focal adhesion contacts organized, and evident actin stress fibers. The treatment of cells with hyaluronidase induced a reduction of focal contacts formation. On PEI the cells tend to detach and the number of adherent cells was significantly reduced over time. Moreover, hyaluronidase treatment induced on cells a significant lower ability to adhere. MG63 maintained a round shape over time, the actin disorganized and present only at the cell edge as a cortical layer. The adhesion molecules were present only as a cytoplasmic pool, and did not organize into focal contacts.

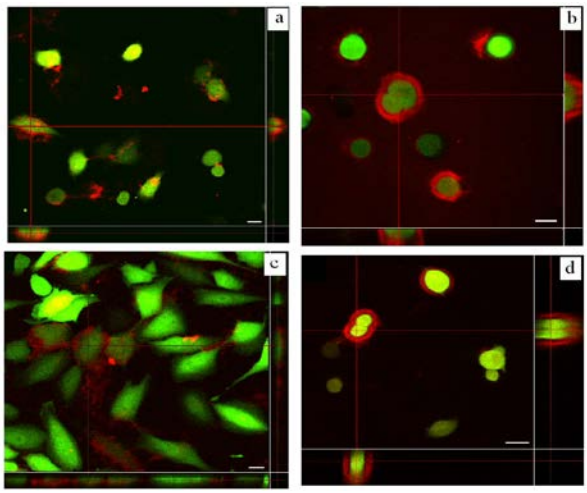

Fig. 1: Distribution of the pericellular matrix (red) of GFP cells (green), seeded on the substrates. a) PSS; b) PEI; c) fibronectin; d) Permanox®.

DISCUSSION & CONCLUSIONS: PCM is fundamental in cell adhesion to material surface, in particular for surfaces that do not favour cell adhesion, such as on PEI. Cell/material interaction not simply based on electrostatic forces, but on a more complicated system, not yet fully understood. Further studies will be necessary to increase our knowledge on the nature and function of this matrix and to evaluate its contribution to cell-matrix interactions.

Transfection of primary human mesenchymal stem Cells with growth and differentiation factor 5 (GDF-5) – A non-viral gene transfer therapy for the disc?

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INTRODUCTION: Mesenchymal stem cells (MSC) harvested from human bone marrow show great promise for therapeutic interventions. The relatively easy isolation protocol, the high proliferation capacity and their ability to differentiate into different cell types of the mesenchyme puts them into primary focus of regenerative medicine.[1] Insertion of targeted DNA into MSCs gives raise to new therapeutic strategies. The phenotype of intervertebral disc (IVD) cells is still obscured. Recent evidence demonstrated that growth and differentiation factor 5 (GDF-5) is a promising cytokine pushing stromal progenitor cells possibly towards a disc-like phenotype.[2,3] The aim of this study was to transfect MSCs with a plasmid containing the full open reading frame (ORF) of GDF-5. The procedure included a combination of electroporation (nucleofection) and sub-sequent lipofection with the aim to differentiate MSCs to IVD cells with the over-expression of GDF-5. Nucleofection is a straightforward method to insert genes of interest into target cells without the need of silenced viruses, which are not well accepted as therapeutic vectors.[4].

METHODS: MSCs were harvested from bone marrow aspirations of 4 patients undergoing spine surgery and isolated through histopaque density centrifugation and plastic adhesion (Ethical permit #187/10 of ethical authorities of the canton of Bern). These isolated cells were cultured and expanded for 2 weeks and then transfected with a GFP-tagged ORF clone of the GDF-5 gene by nucleofection using the nucleofector from Amaxa (Lonza, Basel, Switzerland). In addition, lipofection (Invitrogen, Basel, Switzerland) was applied and the cells were analyzed for RT-PCR, immunohistochemistry over time to see the effect of the GDF-5 transfection.

RESULTS: Increased levels of GDF-5 gene expression by nucleofected-MSC were confirmed using RT-PCR (Figure 1). We found ACAN upregulated but not collagen type 2. GDF-5 specific antibody confirmed presence of intracellular GDF-5 compared to untransfected control cells by immunohistochemistry.

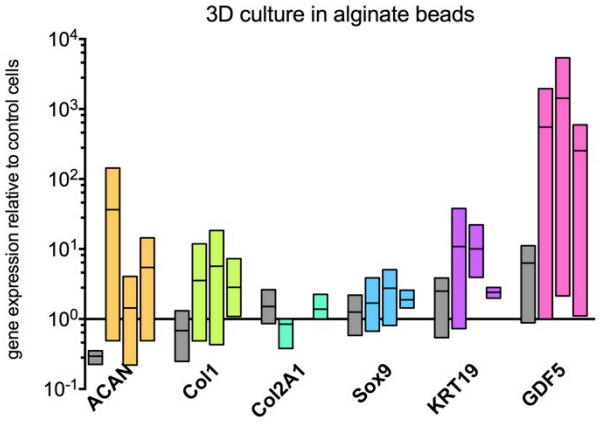

Fig. 1 *Gene expression of MSC nucleofected with plasmid GDF-5 and encapsulated in alginate. Expression levels were adjusted to control cells. Per gene 3 time-points (colorized bars) were monitored: from left to right day 7, day 14, day 21. Gray bars correspond to day 0 (day 7 in monolayer culture). Bars represent min to max with a line at the mean. N=4).*

DISCUSSION & CONCLUSIONS: These results suggest that gene delivery by nucleofection of GDF-5 is an attractive approach for the release of GDF-5, which was produced by the cells themselves. Previous studies point towards an IVD-relevant role of GDF-5 to rescue IVD degeneration. Future studies will have to demonstrate longevity of the over-expression of GDF-5 *in vitro* and *in vivo*. Injection of transfected MSCs into 3D organ culture will show performance of cells in the IVD environment. Non-viral gene therapy seems a more promising approach from a translational medicine perspective than viral methods.[4]

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M. Bilici, J. Pierog, E. L. Ayuni, et al (2006) *J Gene Med* **8**: 910-8.

Mesenchymal stromal cell (MSC)-seeded dense collagen scaffold and VEGF promote healing of large bone defect

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INTRODUCTION: Traumatic injury and excision of infected or neoplastic tissue all result in large bone defects that will not heal spontaneously. Interventions to promote bone healing under these circumstances using allograft bone are largely ineffective due to its poor osteo-inductive capability[1]. The current study investigates the potential of bone marrow derived MSC embedded in a dense collagen scaffold, in combination with vascular endothelial growth factor, to repair a large defect in the femoral diaphysis of inbred mice.

METHODS: ***Ex vivo*** MSCs isolated by adhesion to tissue culture plastic from whole bone marrow of health young adult donor mice were expanded in 2D culture before seeding at 3 x 10^5 /ml in hydrated rat tail collagen to form 3D gels. The collagen gels were then subjected to unconfined compression to expel 98% of the fluid thus generating cell-seeded dense collagen scaffolds[2]. Metabolic activity of the MSC in osteogenic medium was monitored for the first 15 days using Alamar Blue® and differentiation was evaluated using qPCR and histology. ***In vivo*** A 3mm x 1mm full thickness defect was drilled in the lateral aspect of the femur of 10 month old recipient mice which were randomized into one of the following treatment groups: 1) dense collagen scaffold implant on Day 0; 2) cell seeded scaffold on Day 0; 3) VEGF injection on post-operative Day 4; 4) cell seeded scaffold on Day 0 + 10ng VEGF on Day 4. Bone repair was evaluated after 4 weeks using quantitative micro computed tomographic imaging and histological analyses.

RESULTS: ***Ex vivo*** The dense collagen scaffold supported survival of the MSC up to 15 days when cultured *ex vivo.* Progressive up-regulation of osteoblast markers and increase in mineralization of the dense collagen scaffold were observed over the 15 day period. ***In Vivo*** Little healing of defects with no treatment (Fig 1A) or those treated with a dense collagen gel without (Fig 1B) or with MSC (Fig 1C). Some healing occurred in the presence of VEGF (Fig 1D) with significantly more in defects treated with the combination of cell-seeded scaffold and VEGF (Fig 1E). Histological analysis of the mineral content of the defects treated with cell-seeded scaffolds and a bolus dose of VEGF revealed active osteoblasts (ALP) and osteoclasts (TRAP) around and within the transplanted scaffold. Their presence suggested robust "turnover" similar to that seen in the growth plates during endochondral bone development.

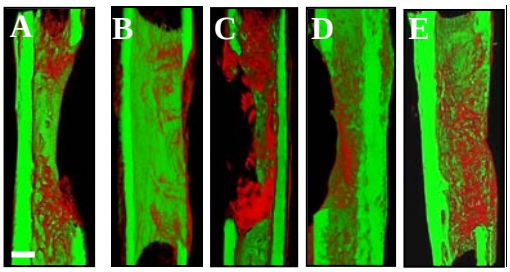

Fig. 1 Volume rendering MicroCT images separates trabeculae in bone callus (red) from cortical bone (green) in femurs with A) No intervention; B) Dense collagen scaffold without MSCs; C) MSCs seeded-Dense collagen scaffold; D) VEGF alone; E) Combined MSCs-seeded dense collagen gel and VEGF treatment. Scale bar represents 500µm.

DISCUSSION & CONCLUSIONS: A dense collagen scaffold induced the osteoblastic differentiation of MSC when folded into a large defect in the mouse femur. Deposition of mineral within the scaffold effectively filled the defect with "tissue" that resembled trabecular bone. A single post-operative dose of recombinant VEGF promoted integration of the mineralized scaffold with endogenous bone around the implant.

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ACKNOWLEDGEMENTS: CHIR, FRQ-S RI-MUHC, CIHR-MENTOR, FRQ-S-RSBO, NCE-MITACS

Low intensity pulsed ultrasound (LIPU) enhances ectopic bone formation by intrinsic mesenchymal stem cells in rabbit spine

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INTRODUCTION: Decortication was considered essential for posterolateral spinal arthrodesis, as this procedure provides not only a rich source of vascular supply from the underlying cancellous bone, but also access to pluripotent stem cells within the marrow. However, it has been demonstrated by our previous studies that Recombinant human bone morphogenetic protein-4 (RhBMB-4) has the ability to induce heterotopic bone formation that results in posterolateral spinal fusion (PLSF) without decortication in rabbits[1]. Aim of the present study was to investigate whether LIPU can enhance RhBMP-4 induced ectopic bone formation in a rabbit model of PLSF without decortications.

METHODS: Sixteen adult New Zealand white rabbits were used. Bilateral transverse processes at L5–L6 were surgically exposed and a PDLLA scaffold loaded with rhBMP-4 was implanted onto the cortical surface of transverse processes of each side to establish an ectopic ossification model. Each rabbit received LIPU treatment at the surgical site unilaterally, and the other side served as control. Animals were sacrificed at 3 days, 1week and 3 weeks following surgery for histomorphological assessment of ectopic bone formation and immunohistochemical assessments of ingrowth of calcitonin gene related peptide positive (CGRP+) nerve in ectopic bone.

RESULTS: Histomorphology showed that 1 week after surgery, there were more cartilage tissues on LIPU side than control side. Chondrocyte proliferation rate, cell nuclear size, and the ratio of cell nuclear to whole cell were significantly higher on LIPU side than control side (Figure 1). Size of ectopic bone mass was significantly larger in LIPU side 3 weeks after surgery.

Immunohistochemistry showed that the density of CGRP+ nerve fibers was significantly higher in ectopic cartilage and bone at the LIPU side than in those at the control side at 1 week and 3 week postoperation (Figure 1).

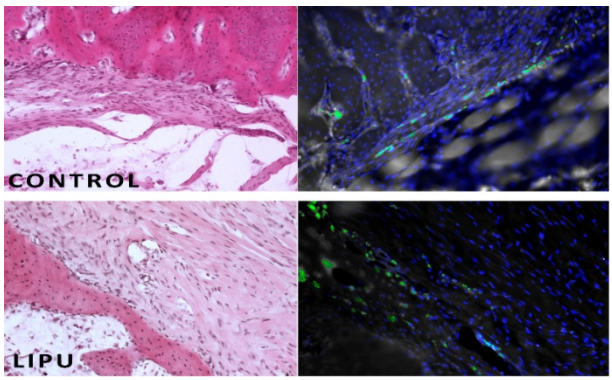

Fig. 1: Ectopic bone formation 3 weeks postoperation. Left: HE staining. The bony tissue in LIPU treated side is lamellen bone while it is still vowen bone in control side. Right: Iinnervations of ectopic bone formed in PLSF with CGRP+ nerves.

DISCUSSION & CONCLUSIONS: *LIPU promotes CGRP+ nerve ingrowth into ectopic cartilage and bone, which in turn may contribute to the promoting effects of LIPU on ectopic bone formation by intrinsic mesenchymal stem cells.*

REFERENCES: [1] X. Guo, K.M. Lee, L.P. Law et al. (2002) *J Orthop Res* **20**: 740-6.

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Innervation of ectopic bone formed by intrinsic mesenchymal stem cells in rabbit spine

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INTRODUCTION: Decortication was considered essential for posterolateral spinal arthrodesis, as this procedure provides not only a rich source of vascular supply from the underlying cancellous bone, but also access to pluripotent stem cells within the marrow. However, it has been demonstrated by our previous studies that Recombinant human bone morphogenetic protein-4 (RhBMB-4) has the ability to induce heterotopic bone formation that results in posterolateral spinal fusion (PLSF) without decortication in rabbits[1]. It is known that fracture callus is innervated withsensory nerves expressing calcitonin gene related peptide (CGRP+)[2]. Aim of the present study was to investigate whether CGRP+ nerve fibers present also in RhBMP-4 induced ectopic bone formation in a rabbit model of PLSF without decortications.

METHODS: Sixteen adult New Zealand white rabbits were used. The left transverse processes at L5–L6 were surgically exposed and a PDLLA scaffold loaded with rhBMP-4 was implanted onto the cortical surface of transverse processes of each side to establish an ectopic ossification model. Animals were sacrificed at 3 days, 1week and 3 weeks following surgery for histomorphological assessment of ectopic bone formation and immunohistochemical assessments of ingrowth of calcitonin gene related peptide positive (CGRP+) nerve in ectopic bone.

RESULTS: Histomorphology showed that cartilage presented 1 week after surgery. The ectopic bone was a mixture of cartilage and woven bone 3 weeks after surgery.

Immunohistochemistry showed that CGRP+ nerve fibers presented as early as 3 days postoperation in the fibrous tissue. Early cartilage formation was in close contact to the CGRP+ nerve fibers. The density of CGRP+ nerve fibers was increasing from day 3 to week 3 postoperation (Figure 1).

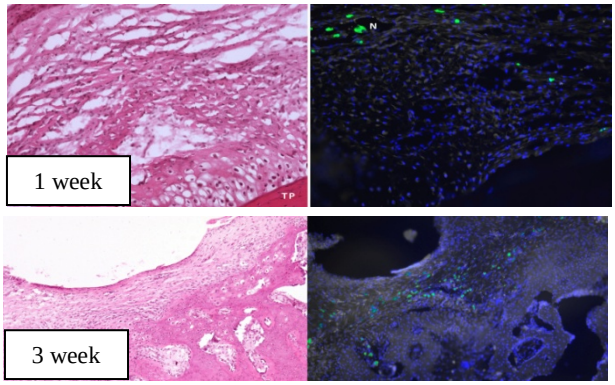

Fig. 1: Iinnervations of ectopic bone formed in PLSF with CGRP+ nerves 1 week and 3 weeks postoperation. Left: HE staining, Right: Immunohistochemical staining.

DISCUSSION & CONCLUSIONS: It has been demonstrated that ectopic bone is also innervated with with CGRP+ nerve fibers. The nerve ingrowth presented prior to cartilage formation and the density of CGRP+ nerve fibers increases during ectopic bone formation. It is suggested that CGRP+ nerve plays a role in ectopic bone formation by intrinsic mesenchymal stem cells.

REFERENCES: [1] X. Guo, K.M. Lee, L.P. Law et al. (2002) *J Orthop Res* **20**: 740-6. [2] A.K. Huebner, J. Keller, P. Catala-Lehnen et al. (2008) *Arch Chem Biophys* **473**:210-7.

ACKNOWLEDGEMENTS: This research project was supported by Hong Kong RGC Grant PolyU5406/03M and Hong Kong Polytechnic University Research Grant GU-671.

Towards an autologous culture of human endothelial progenitor cells

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INTRODUCTION: The sufficient supply of oxygen, nutrients and growth factors is of critical value for tissue engineered implants of large size. This can be achieved by promotion of neovascularization within scaffolds. Human bone marrow derived endothelial progenitor cells (EPC) can serve as an autologous source of cells promoting neovascularization. Here, we aim to optimise the isolation and culturing procedure of EPCs with regard to their proliferation capacity as well as their endothelial differentiation capability. To meet the needs of clinical application we used autologous platelet-derived growth factors (PL) as a source of pro-angiogenic cytokines [1].

METHODS: EPC were isolated from human bone marrow using Ficoll. EPC (CD34+/CD133+) were enriched using MACS® Technology. Cells were cultured in IMDM supplemented with varying concentrations of PL and FCS. PL was prepared out of platelets as described before [1]. The cytokine content of the PL preparation was analysed by ELISA. Commercial endothelial growth medium (EBM-2) and IMDM containing 5% FCS supplemented with or without recombinant VEGF (50ng/mL) were used as control. EPCs from passage 1 and 2 were characterised by flow cytometry using endothelial-cells specific antibodies. In addition, we evaluated binding of lectins (*Ulex europaeus* agglutinin-1) and uptake of Dil-labeled acLDL by EPCs. Tube-formation capability was analysed on growth-factor reduced MATRIGEL™.

RESULTS: We show that human bone marrow derived EPCs cultured in commercial endothelial growth medium are highly efficient in promoting tube formation in MATRIGEL™. The cells also showed typical characteristics of endothelial cells including lectin binding and uptake of acLDL. Interestingly, we demonstrate that IMDM supplemented with PL did likewise promote the endothelial properties of EPCs in a dose-dependent manner, while supplementation with VEGF alone was not sufficient. In addition, supplementation with PL allowed reduction of the FCS content without affecting the proliferation capacity of cells.

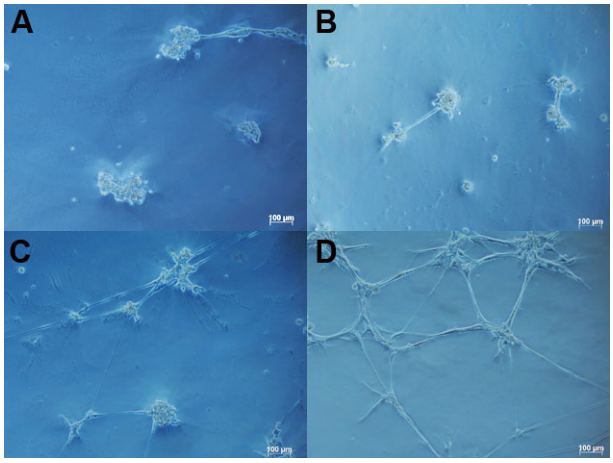

Fig. 1: MATRIGEL™ seeded with EPCs cultured in (A) IMDM 5% FCS, 50 ng/ml VEGF; (B) IMDM 5% FCS-5% PL; (C) IMDM 2 % FCS-8% PL or (D) EBM-2. After 24 h sprouting and tube-formation was observed in the presence of growth factors and cytokines contained in commercial endothelial medium (D). PL did mediate tube formation in a dose-dependent manner (5% in B vs. 8% in D), whereas inhibitory clustering of cells was observed for EPC cultures supplemented with VEGF only (A). Scale bars depict 100 µm.

DISCUSSION & CONCLUSIONS: Our results demonstrate that human bone marrow derived EPC preserve their potential to differentiate towards mature endothelial cells upon in vitro expansion. Thus, EPC may be used to trigger neovascularization in tissue engineered implants. Most importantly, we present an autologous culture method of EPCs, enabling a potential translation into clinics.

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Serum albumin markedly increases bone formation in vivo

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INTRODUCTION: Blood serum fractions such as platelet rich plasma or activated serum are possible adjuvants in bone replacement therapies. In previous experiments we have shown that serum albumin coating on bone allografts can significantly increase stem cell adherence and proliferation in vitro, raising the possibility that serum albumin alone may be an effective autologous agent in bone remodeling. We investigated the bone formation by filling critical size defects with serum albumin coated allografts in a rat cranial defect model.

METHODS: Under ketamine/xylazine anesthesia 3.5 mm bone defects were created on the cranium of Wistar rats with a trephine bur. Mineralized bone allografts coated with serum albumin were used to fill the defect. Uncoated bone grafts served as positive controls and defects left empty served as negative controls. After 1,3,5,7,9,11 weeks postoperatively computed tomography (CT) was performed in order to follow bone formation. Bone density and the area of the remaining defect were calculated. At the 11th week parietal bones were harvested and engraftment was tested with an Instron 5566 mechanical testing machine. All methods, including surgery and evaluations were performed according to Nature Protocols [1].

RESULTS: CT scan showed bone formation already after three weeks in every experimental group. At five weeks significant difference can be observed between the control groups and the albumin-coated group. The remaining bone defect in the albumin-coated group was 15.21% ± 6,57, while in the control groups it was significantly larger (uncoated: 63,4% ± 12.8 and sham 62,07% ± 7.8). Furthermore, in the albumin-coated group every bone defect healed completely by the 9th week while a significant defect was still present in the control groups (uncoated: 29,6% ± 8,4; sham: 43,6% ± 8,8). Bone formation was also measured by densitometry: from the 5th week the albumin-coated group showed markedly higher values compared to the controls. By the 11th week the defects treated with the albumin coated graft reached over 1000 Hounsfield unit (1061.25HU ± 79,1), while density in the control groups were 699.9 ± 104,6 HU (uncoated) and 655.70HU ± 84,04 HU (sham). In addition, albumin coated grafts showed significantly higher breaking force values after mechanical testing compared to the uncoated grafts (albumin 46.1 ± 10 N, uncoated 15.7N ± 5 N) .

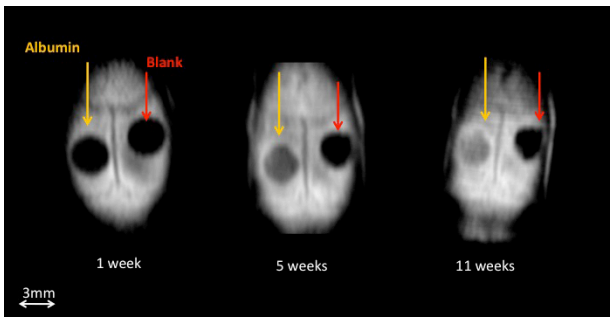

Fig. 1: Horizontal reconstructional CT images of bone formation after 1, 5 and 11 weeks. Yellow arrows indicates ossification of albumin coated grafts, red arrows show defects left empty (sham).

DISCUSSION & CONCLUSIONS: In the present investigation we showed that implanting serum albumin coated allografts significantly reduce healing time in critical size defects. These results also support the idea that albumin coating provides a convenient milieu for stem cell function, therefore cell therapy can be achieved without the use of exogenous stem cells.

REFERENCES: [1] Patrick P Spicer, James D Kretlow, Simon Young, John A Jansen, F Kurtis Kasper, Antonios G Mikos (2012) *Nature Protocols* Nature America, Inc. Oct;7(10):1918-29.

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Cell coated suture: a novel cell transplantation technique

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INTRODUCTION: Cell therapy holds the promise for a novel modality in the surgical toolkit, however, delivery of cells into damaged soft tissues constitutes a challenge. We hypothesized that first growing stem cells on the surface of absorbable sutures in vitro and then implanting them via stitching into a dissected soft tissue would be a suitable delivery route for cell therapy.

METHODS: Poly-filament absorbable sutures were used for the in vitro and in vivo experiments. Fibronectin, poly-l-lysine and albumin coating were used to increase attachment of human or rat bone marrow derived stem cells (BMSCs). After 24 or 48 hours of incubation fluorescence microscopy was performed to visualize the cells on the suture. In the in vivo experiments, under halothane anesthesia a 10 mm incision was made on the triceps surae muscle of male Wistar rats. Rat BMSC coated sutures were placed into the muscle. Fluorescence microscopy was performed at 48h, while histology was performed at 5 weeks.

RESULTS: After 48 hours of incubation the albumin-coated sutures had the highest cell number compared to the other coatings or native sutures with both cell types. After 168 hours cell number reached confluency. In the in vivo experiments 48 hours after the implantation cells were seen on the surface of the sutures as well as in the surrounding muscle tissue. Long-term results at 5 weeks showed that the surviving cells integrated into the tissue and the sutures were partly absorbed.

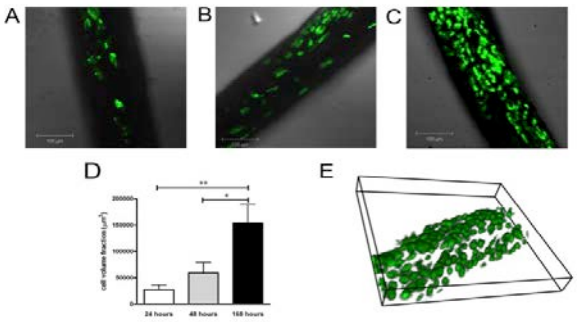

Fig. 1: Rat BMSCs on albumin-coated suture after 24, 48 and 168 hours. Rat BMSCs successfully attached to the albumin coated suture already after 24 hours. Green color represents the nuclei of attached cells. The nucleus volume fraction of adhered cells increased as the incubation time increased (Panels A, B, C). The image was taken at 20x magnification. Scale bar represents 100 µm. Panel D shows the significant difference between the 24 hours and 48 hours incubation compared to 168 hours incubation. Panel E shows 3D reconstruction of the suture after 168hours incubation.

DISCUSSION & CONCLUSIONS: We showed in vitro that coating absorbable sutures with proteins, especially serum albumin improves attachment and proliferation of cells so only 48 hours in culture is enough to cover the sutures sufficiently. Using these stitches in vivo resulted in short and long term survival of cells. As a result, albumin coated suture can be a vehicle for stem cell therapy in soft tissues.

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Substrate-dependent properties of biomaterials-derived MSC spheroids

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INTRODUCTION: The cellular function and properties of three-dimensional (3D) spheroids more faithfully replicate those of cells in vivo, compared to the traditional 2D monolayer culture. Mesenchymal stem cell (MSC) spheroids own higher differentiation capacities, but the associated mechanisms remain unclear. In the current study, we used a series of biomaterials membranes including chitosan and that grafted with different densities of HA (chitosan-HA) to generate dynamic MSC spheroids and to investigate the different cell fate of these substrate-derived 3D spheroids.

METHODS: MSCs were obtained from the adipose tissue of Sprague-Dawley rats. The adipose tissue was treated with type I collagenase. The cellular pellet was washed, centrifuged, and resuspended. The homogenate was cultured in basal medium, which consisted of Dulbecco's modified Eagle medium-low glucose/F12 (1:1) supplemented by 10% fetal bovine serum, 20 mM HEPES, 50 mg/mL bovine serum albumin fraction V, 10 mg/L L-glutamine, and 1% penicillin-streptomycin (antibiotics). Cells of the third to the fifth passages were used in this study.

Chitosan was purchased from Sigma (USA, Mw 510 kDa). HA (sodium salt) was obtained from SciVision Biotech (Taiwan, Mw 2,500 kDa). Chitosan solution (1% in acetic acid) was coated on coverslip glass placed in petri dish, where membranes formed after solvent evaporation. HA solution containing different amounts of HA was added on each chitosan-coated coverslip glass so the initial amount of HA was 0.1, 0.5, or 2.5 mg per cm^2 of the membranes. The membranes were then further crosslinked with ethyl(dimethylamino propyl) carbodiimide/N-hydroxysuccinimide (EDC /NHS). The amount of surface bound HA was determined by the glucuronic acid assay.

MSCs (5×10^4 cells) were seeded on each membrane in 24-well tissue culture plates. Cells seeded in the culture well (tissue culture polystyrene, TCPS) served as the control. The expressions of OB-cadherin and N-cadherin were analyzed at 3 and 10 days by Western blot (WB).

The protein expressions of Wnt3a, Wnt5a, and Wnt5b were analyzed at 17 days by WB. The expression of cytoplasmic and nuclear β-catenin was analyzed at 3 and 17 days by WB. For the induction groups, chondrogenic or osteogenic induction medium was given after 3 days.

RESULTS: MSCs vigorously migrated and were self-assembled into highly mobile 3D spheroids with substrate-dependent upregulation of adhesion molecule N-cadherin. MSC spheroids showed increased expression of Wnt genes/proteins and substrate-dependent cell fate. The correlation of differentiation with Wnt signaling and crosstalk with other pathways (ERK1/2 and Smad2/3) were observed for MSC spheroids but not for the 2D cultured cells on TCPS. Wnt3a-mediated canonical Wnt signaling was more active for MSC spheroids derived on chitosan, which were prone to osteogenesis. Wnt5a-mediated non-canonical Wnt signaling was more active for MSC spheroids derived on HA-grafted chitosan, which were prone to chondrogenesis (*Figure 1*). The relative importance of Wnt5a- vs. Wnt3a-mediated Wnt signals in determining the cell fate was controlled by the grafting density of HA on chitosan.

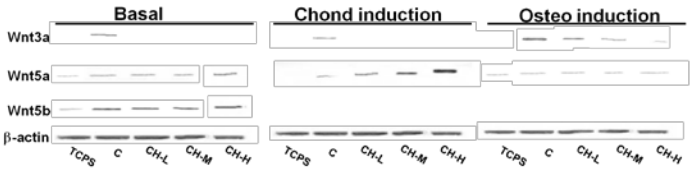

Fig. 1. Wnt expressions for MSCs on chitosan (C) and on chitosan grafted with HA in the low (CH-L), medium (CH-M), or high (CH-H) density.

DISCUSSION & CONCLUSIONS: This study demonstrates that Wnt signaling of MSCs is distinct in a 3D environment and is substrate-dependent. The convenient platform of 3D spheroids may be used to examine the role of Wnt signaling in controlling MSC fate under different extracellular environments, and potentially applied to study stem cell behavior in regenerative medicine, normal development, and cancer.

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A novel magnesium composed PLGA/TCP porous scaffold for bone regeneration

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INTRODUCTION: Bone regeneration is a crucial event in bone tissue engineering, and bioactive scaffold has become a focused strategy in bone regeneration. Magnesium is a biodegradable and bioactive metal with needed mechanical strength for bone healing [1]. We designed and fabricated a biodegradable bioactive porous scaffold composed of poly (lactide-co-glycolide, PLGA), β-tricalcium phosphate (TCP) and magnesium (Mg) using a low-temperature rapid-prototype biospinning technology. This study presented the enhancement of magnesium in mechanical properties and biocompatibility of the composite scaffold. The structure characterization, mechanical strength and *in vitro* biocompatibility of this innovative scaffold were investigated.

METHODS: The PLGA/TCP/Mg porous scaffolds were fabricated according to an established protocol by low-temperature rapid-prototyping machine with homogeneous mixture solution of Mg powder, PLGA (LA: GA = 75:25) and TCP in 1, 4-dioxane [2]. Four group scaffolds with different ratio of Mg to TCP (M: T = 1:2,1:1, 2:1, and TCP-free) were designed to estimate the effect of Mg in mechanical properties and biocompatibility. PLGA/TCP scaffold and PLGA scaffold as control group. All the porous scaffolds were fabricated in size of 30×30×30 mm^3 at -30 ^oC and lyophilized after. The surface morphology of scaffold was analyzed by scanning electron microscope (SEM) and high-resolution micro-computed tomography (micro-CT). The porosity was determined by ethanol replacement method and micro-CT, and the mechanical properties were tested by mechanical testing machine. The MC3T3-E1 osteoblast were used to study the *in vitro* biocompatibility of the scaffold and analyzed by cell counting kit-8 (CCK-8) assay.

RESULTS: The composite PLGA/TCP/Mg porous scaffold fabricated by low-temperature rapid-prototype manufacture had high porosity with regular macropores and numerous micropores. The micro-CT and SEM results showed that the diameter of the macropores was around 450 μm and the porosity of the scaffolds was above 85%. The connectivity of the scaffold was almost 100%. By using phase-separation technology, numerous micro pores were found distributed on the pore wall of the scaffold ranged from 2.5 μm to 90 μm. The mechanical strength of the PLGA/TCP/Mg scaffolds was enhanced with increasing Mg content. The Young's modulus of PLGA/TCP/Mg scaffolds (M:T=2:1) was around 104 Mpa, statistically significantly stronger than that of M:T=1:1 (83Mpa) and 1:2 group (82 Mpa), as well as PLGA/Mg group, 66Mpa. All the Mg contented scaffolds were statistically significantly stronger than PLGA/TCP and PLGA group, 45 Mpa and 30 Mpa, respectively. The results of cell counting kit-8 (CCK-8) assay demonstrated the MC3T3-E1 osteoblasts grew very well and proliferated rapidly on PLGA/TCP/Mg scaffolds compared to PLGA/TCP scaffold after 7 days of culture.

DISCUSSION & CONCLUSIONS: The novel porous PLGA/TCP/Mg scaffold fabricated by low-temperature rapid-prototype technology has well-defined interconnected porous structure, suitable mechanical strength for bone repair. The pre-designed regular macropores and micrometer-sized pores by solid–liquid phase separation gave an unique structure to PLGA/TCP/Mg scaffold with high osteoconductive which allow nutrition to penetrate and enhance cell attachment, proliferation and migration within the pore walls of the scaffold, and form tissue. The metal magnesium greatly strengthens the PLGA/TCP/Mg scaffold to a proper initial Young's modulus with 104 Mpa compared to the 45Mpa Young's modulus of PLGA/TCP scaffold. The in vitro study also demonstrated a good biocompatibility and bioactivity of the PLGA/TCP/Mg scaffold that was in favor of accelerating and inducing the proliferation and differentiation of osteoblasts.

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The properties of equine chondroprogenitor cells from articular cartilage after extended i*n vitro* expansion

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INTRODUCTION: One sign of '*in vitro* aging' is the diminishing capacity for cell division. In contrast to embryonic stem cells that show no loss of proliferative potency, the maximal population doublings (PD) for mesenchymal stem cells (MSCs) *in vitro* is reported to be between 30 and 40 replications[1,2,3,]. We have isolated a population of chondroprogenitor cells from articular cartilage of several species, including equine[4]. These cells have demonstrated functional equivalence in their differentiation capacity when compared with MSCs but have the advantage of retaining the highly desirable stable (permanent) chondrocyte phenotype. In this study, we examined the age-related capacity of these cells for extended division and retention of potency.

METHODS: Chondroprogenitors were isolated from the surface zone of equine articular cartilage from the metacarpophalangeal joint by adhesion onto fibronectin[5]. Cells were isolated from both skeletally immature (1 year-old) and a mature animals (8 year-old). Clonal and polyclonal cell lines (at least 5 of each for each age) were cultured in the presence of 10% FCS, 1ng/ml TGFβ-1 & 2.5 ng/ml FGF-2. Cells were seeded at low density and passaged weekly.

RESULTS: Chondroprogenitors from both animals reached over 40 (mean) PD in 50 days with growth remaining linear. Little difference in growth rates was observed between clonal and polyclonal cell lines. For the mature animal, 96% of cells were BrDU positive at 22 PD whilst none of cells were (senescence associated) β-gal positive. At 44 PD, 88% of cells were BrDU positive and just 15% of cells were β-gal positive. Three clonal and three polyclonal cell lines from the mature animal were cultured beyond the 50-day time point. At 120 days, cells reached up to 90 PD with the same pattern of linear growth observed (figure 1). When tested at 70 PD, 79% of these cells were still BrDU positive (range 55-97%) and just 11% of cells were β-gal positive (range 2-22%). Furthermore, little difference in cell morphology was observed throughout this extended expansion. At 70 PD, we found that both clonal and polyclonal cell lines in monolayer culture were still expressing the chondrogenic transcription factor; *Sox-9* (figure 2). Expression of genes for aggrecan and collagen type II was also detected in cells that were chondrogenically induced for 72 hours.

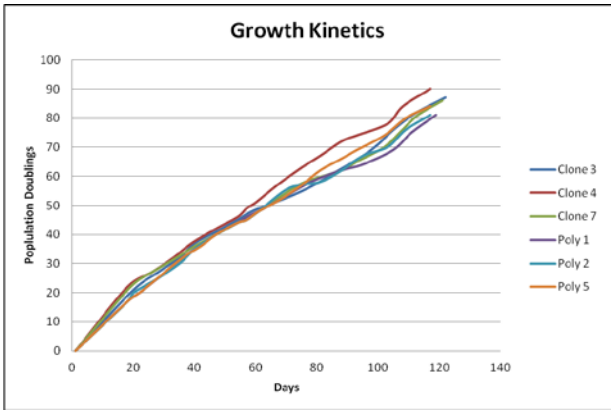

Fig.1: In 120 days, cells reached up to 90 PD

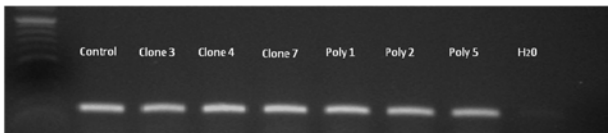

Fig 2: Sox-9 expression of clonal and polyclonal cell lines at 70 PD.

DISCUSSION & CONCLUSIONS: We have demonstrated for the first time the extended expansion of cells derived from articular cartilage that retain chondrogenic potency. These equine cells have since been cultured to over 100 PD without evidence of senescence. One hundred PD is equivalent to 1 x10^{30} cells originating from a single cell. We have previously reported that the human equivalents of these cells surpass MSCs in doubling capacity but senesce at approximately 60 PD[6]. The properties of these equine chondroprogenitor cells make them ideal candidates for allogeneic cell therapy for articular cartilage repair. In addition, the data suggest the reclassification of these cells from progenitor cells to stem cells.

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STEM CELLS DERIVED FROM HUMAN OSTEOARTHRITIC CARTILAGE ELICIT *IN VITRO* REGENERATIVE PROPERTIES.

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INTRODUCTION: Osteoarthritis (OA) is the most common form of joint disease leading to disability and dependence. In severe cases of knee OA, the joint is deemed irrecoverable and total knee replacements are indicated. Tissue engineering is a possible solution for this pathology and previous work from the Archer laboratory has demonstrated that it is possible to isolate and expand articular cartilage derived progenitor/stem cells from several species, including human[1,2,3]. Work presented here describes the detection and isolation of progenitor/stem cells derived from human osteoarthritic cartilage following total knee replacement in patients with severe OA.

METHODS: Human articular cartilage was excised from tibial plateaux (TP's) obtained from total knee replacements following the diagnoses of severe OA. Cells were isolated by a sequential pronase and collagenase digestion and subject to a fibronectin adhesion assay[1]. Cells were expanded in monolayer culture in supplemented growth medium. Clonal 3D pellet cultures were established in chondrogenic and osteogenic differentiation media. Adipogenic cultures were also established in monolayer cultures. Immunohistochemistry and gene expression analysis were undertaken in order to determine the extent of differentiation. In addition, osteochondral plugs were excised from the TP's and wax embedded for further histological and immunohistochemical analysis.

RESULTS: Clonal cell lines obtained from osteoarthritic knee-joint cartilage using the fibronectin adhesion assay were isolated and successfully cultured to a maximum of 60 population doublings (PD) whilst still demonstrating a chondrogenic capacity. This finding was not consistent however as a small number of cell lines failed to reach 30 PD. Three-D pellet cultures after 21 days of chondrogenic induction produced smooth and iridescent pellets which stained positively for toluidine blue and safranin O. The pellets varied in size and staining intensity between clonal cell lines s well as between patients. Positive labelling for collagen type II and aggrecan was observed in addition to the expression of the chondrogenic transcription factor; *Sox-9*. Following osteogenic induction, evidence of mineralisation was indicated by the von Kossa stain. Adipogenic induction revealed a positive oil-red O stain along with the expression of the lipoprotein lipase gene. Histological analysis of osteochondral plugs from the same OA patients demonstrated positive labelling in the surface region for the putative stem cell markers Stro-1 and Notch-1 as well as PCNA.

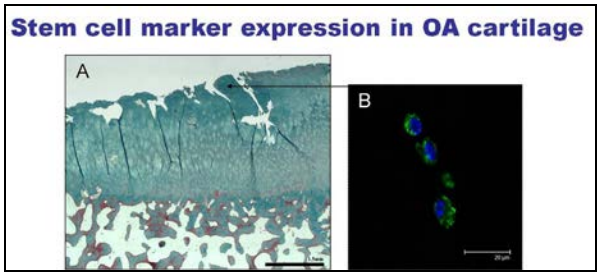

Fig. 1: Stro-1 labelling in human OA cartilage.

DISCUSSION & CONCLUSIONS: Previous studies have demonstrated the presence of a sub-population of progenitor/stem cells present in normal hyaline cartilage. We have demonstrated in this study that a similar group of cells reside within osteoarthritic articular cartilage. These cells have the capacity to proliferate to over 50 PD whilst maintaining their chondrogenic phenotype. As well as having the capacity to proliferate beyond the potential of chondrocytes, phenotypic plasticity was also demonstrated. Importantly, a significant amount of variation in these cells was observed which requires further investigation. In conclusion, we have isolated a cartilage derived progenitor/stem population from patients with severe OA. This suggests a pool of viable cells from degenerate cartilage and challenges the dogma that the tissue is irrecoverable.

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The use of specific PEEK nanotopographies to modulate osteogenic behaviour in primary osteoprogenitor cells

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INTRODUCTION: Poly(aryl-ether-ether-ketone) (PEEK) is a semi crystalline polymer which exhibits properties which make it an attractive choice for use as an implant material, such as natural radiolucency (which it is possible to vary dependant on the amount of barium sulphate added during manufacturing) and MRI compatibility as well as good chemical and sterilization resistance[1]. Recently we have been able to injection mould highly defined Nanotopographies with PEEK and these are currently being assessed for their potential to alter osteogenic behavior of cells.

METHODS: Three different nanopatterned substrates were fabricated in an Engel victory 28 tonne injection moulder; planar, ordered square (SQ) and disordered square (NSQ) topographies were produced in both PEEK and polycarbonate (control material). PEEK processing parameters were set as prescribed by the polymer manufacturer and supplier (Invibio Biomaterials Solutions, UK). Oxygen plasma treatment was carried out using a Gala instruments plasmaprep 5 Asher. Changes to the topography due to the plasma treatment were assessed via AFM (Atomic Force Microscopy)). Additionally surface elemental composition changes as a result of plasma treatment were investigated with X-ray photoelectron spectroscopy (XPS).
Primary Osteoprogenitor cells were isolated from waste femoral heads from total hip replacements via a ficol isolation gradient and were plated at a seeding density 5600 cell/cm^2 on planar, NSQ and SQ PEEK substrates. The substrates had been oxygen plasma treated for 2min at 100W. Osteogenic behaviour was investigated by assessing a number of different stages of osteogenesis. Cell proliferation was measured at 7, 14 and 21 days using Alamar blue. Alkaline phosphatase (ALP) staining and assessment of enzymatic activity was carried out at 7, 14 and 21 days. Mineralisation was measured with Alizarin Red S and Von Kossa staining at 21 days. Additionally the Alizarin stain was dissolved with Cetylpyridinium Chloride Monohydrate (CPC) and quantified using a plate reader.

RESULTS: Our results indicate that that the Oxygen plasma treatment has addressed PEEKs underlying issues with poor cell adhesion[2] (we did not use non plasma treated PEEK substrates in this study due to the very poor level of cell adhesion we have previously observed on them) .We do observe decreased osteogenic behaviour on cells cultured on the SQ nanotopography however there appears to be little difference in osteogenic behaviour between cells on planar and NSQ.

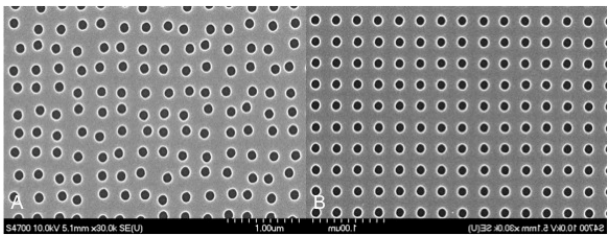

Fig. 1: Scanning Electron Microscope (S.E.M) images of our nanotopographies. On the left the NSQ nanotopography(A) and on the right the SQ nanotopography (B).

DISCUSSION & CONCLUSIONS: Our work shows that cells cultured on the SQ nanotopographies demonstrate reduced osteogenic behaviour compared to cells cultured on the Planar and NSQ nanotopographies and there was little difference in behaviour between cell on the Planar and NSQ nanotopographies. Previous work carried out with the NSQ nanotopography incorporated into poly carbonate demonstrated cells cultured on the NSQ nanotopography demonstrating increased osteogenic behaviour compared to those cultured on planar[3]. Since with PEEK, unlike poly carbonate, we have to Oxygen plasma treat the nanotopogrphies to deal with the materials characteristic low cell adhesion[2] it is possible that this treatment is masking the effect of the topography. As a result we are currently experimenting with reduced durations and intensities of plasma treatment.

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ACKNOWLEDGEMENTS:. Invibio Biomaterials Solutions for supplying PEEK.

BonyPid™: Osteoconductive and antimicrobial outcome in patients with Gustilo III open fractures: Six months follow up results

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INTRODUCTION: Gustilo type III open fractures are associated with high infection rates in spite of instituting a standard of care (SOC) consisting of intravenous antibiotics, irrigation and debridement (I&D), and delayed wound closure. Locally-delivered antibiotic has been proven to assist in reducing infection in open fractures. The aims of this study are to determine the effectiveness and safety of BonyPid™, a new implantable and biodegradable antibacterial bone void filer in initiating bone growth and preventing bacterial infections in open fractures.

METHODS: The osteoconductive antibacterial BonyPid™ used is a synthetic bone void filler (comprised of ≤1 mm β-tricalcium phosphate granules) coated by a thin layer (≤20 µm) of PolyPid nanotechnology formulation. Upon implantation, the coating releases doxycycline at a constant rate for a predetermined period of 30 days. One BonyPid™ vial of 10 grams contains 65 mg of formulated doxycycline. After approval, sixteen subjects with Gustilo type III open tibia fractures, were implanted with the BonyPid™ immediately on the first surgical intervention (I&D), followed by external fixation and systemic antibiotic treatment. Patients had periodic laboratory, bacteriology and radiology follow-up of 6 months.

RESULTS: Immediate soft wound closure was done in 6 (38%) subjects following implantation. Out of 10 remaining subjects, 3 needed soleus muscle transfer-skin grafting and 7 required delayed primary closure; by skin grafting (5) or suturing (2). Early callus formation seen at 8-12 weeks post-surgery, followed by bone healing seen from 16 weeks onwards. No infections were developed at the target fracture site. . Only one BonyPid™ implantation was needed with no subsequent I&D in the target tibia fracture. Safety of implantation was remarkable, with only one deep infection at a fibular open fracture without BonyPid™ implantation. One BonyPid™ related adverse event caused delay in skin healing due to excessive granules in the superficial soft tissues.

Fig. 1: 49Y. Male, Gustilo IIIB, 1.5 Hrs. post injury

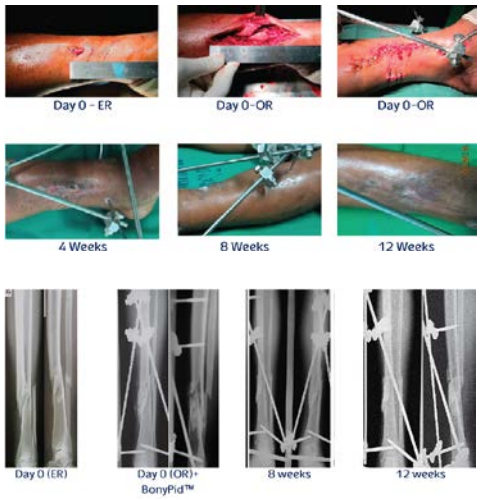
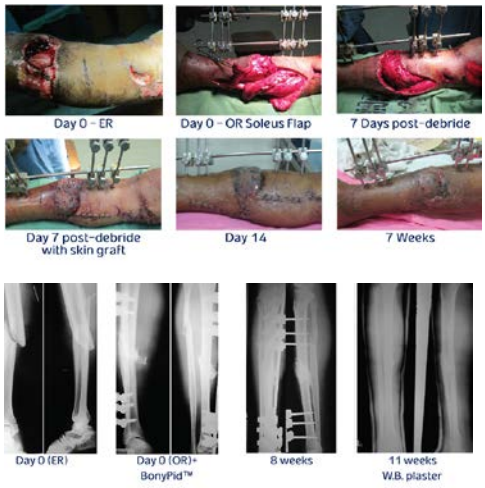


Fig. 2: 30Y. Male, Gustilo IIIB, 132 Hrs. post injury



DISCUSSION & CONCLUSIONS: BoniPid™ is a synthetic bone void filler coated with a new and unique nano-technology that delivered antibiotic, therefor can be applicable locally into contaminated bone voids.

BonyPid™ was found to be highly effective in promoting early callus formation, resulting in early bone healing of contaminated severe open-bone fractures. BonyPid™ is safe for immediate implantation into contaminated bone voids. Results support that BoniPid™ provides an effective way for treating open fractures.

Bioreactor-based engineered models for basic research and clinical translation

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The use of bone marrow- or adipose tissue-derived mesenchymal stromal cells (MSC) for treatment of genetic or immunologic pathologies, as well as in the emerging field of regenerative medicine, typically requires cell expansion by sequential passages in monolayer (2D) cultures. The process generally leads to large cell numbers, but is known to be associated with a progressive decrease in early progenitor properties of MSC. In this study, we propose a novel paradigm for the efficient expansion of human MSC within porous, three-dimensional (3D) scaffolds under direct alternate perfusion, which totally bypasses the 2D culture step. The resulting engineered tissues can be either dissociated to recover the expanded MSC for use in cellular therapy, since reproducibly exhibited higher clonogenic properties and more efficient multilineage differentiation capacity as compared to 2D grown cells, or employed directly for clinical uses due to their reproducibly osteogenicity, as assessed by direct ectopic implantation of the 3D scaffold-MSC constructs [1]. Moreover, the MSC-based engineered tissues can be loaded with additional cell types (i.e. osteoclastic progenitors, hematopoietic, endothelial) for the development of advanced 3D in vitro co-culture models to study physiological interactions among several cell types [2-4].

In summary, the multifaceted use of bioreactor-based models is relevant not only for the streamlining and standardization of MSC expansion and graft manufacturing, thereby facilitating the translation of bone tissue engineering strategies into the clinic, but also to support the development of multi-cell co-culture models in a controlled 3D environment, as paradigm for engineered stem cell niches.

ACKNOWLEDGEMENTS: The perfusion bioreactor device used in the above studies is now commercially available by Cellec Biotek AG.

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Mesenchymal stem cell homing into the intervertebral disc: A chemotactic induced response

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INTRODUCTION: Stem cell homing into damaged tissues may be seen as a promising therapeutic method for tissue regeneration. Homing may be defined as the migration of cells towards an attractant signal, e.g. a chemokine. Degenerative intervertebral discs (IVD) have an inherent poor repair capacity and therefore a homing approach could be used to induce/facilitate tissue regeneration. The present work hypothesizes that mesenchymal stem cells (MSCs) have the ability to home into degenerative intervertebral discs and that secretion of specific chemokines is involved in this process.

METHODS: Bovine caudal discs with or without end-plates (EP) were cultured under either low frequency (0.2Hz)–high glucose (DMEM 4.5g/L) conditions ("physiological"/LF-HG) or high frequency (10Hz) –low glucose (DMEM 2g/L) conditions with a 22G needle punch ("degenerative"/HF-LG + punch) for 7 days using our IVD bioreactor. Conditioned medium was used for semi-quantitative proteomic analysis, ELISA analysis, and for chemotaxis assays. ***Homing experiment*:** PKH-labeled human MSCs (1 x10^6) were applied to each disc on days 8, 10 and 12 of culture and were cultured under static conditions during this period until day 14. Transverse sections of the discs were created and visualized using a fluorescent microscope to count the MSCs homed into the disc. ***Chemotaxis assay*:** Media from "degenerative" and "physiological" discs were loaded into a Boyden Chamber with a concentration gradient formed in lower chamber and with MSCs in upper chamber. Chamber was incubated for 6 hours at 37^0C/5% CO_2. Membrane was stained with toluidine blue to enable counting of MSCs. ***Proteomic and ELISA analysis:*** During degeneration period, medium was replaced with PBS + 4.5g/L glucose between days 4-6. Medium was lyophilized and then sent for proteomic analysis performed by Proteomics facility, University of Geneva. Chemokines found in proteomic analysis were further investigated using specific bovine ELISA kits.

RESULTS: Greater MSC homing occurred for discs pre-cultured under "degenerative" conditions compared to discs cultured under "physiological" conditions with or without the presence of the end-plate (Figure 1a). The efficiency of the homing varied between both animal discs and MSC donor. A reason for the greater homing was the increased chemotactic potential of the degenerative media samples during the pre-culture period (Figure 1b). Furthermore, two chemokines CCL5 and CXCL6 were identified in the media, with a greater concentration of these chemokines found under degenerative conditions (Figure 1c and d).

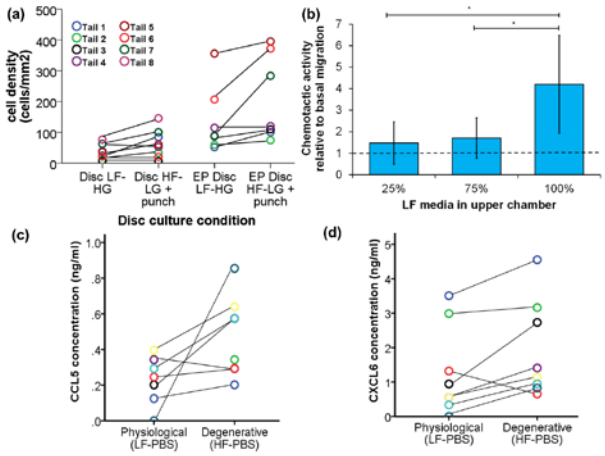

Figure 1: (a) MSC homing between discs from individual tails under "physiological" and "degenerative" conditions with or without EP (b) Boyden chamber analysis of degenerative media and ELISA analysis of (c) CCL5 and (d) CXCL6 chemokines in the media.

DISCUSSION & CONCLUSIONS: The present study demonstrates that MSC homing can occur into degenerative intervertebral disc and that it is driven by the release of chemokines from the tissue. However, it needs to be elucidated whether CCL5 and/or CXCL6 are specific for the disc or other tissues and whether systemic administration of MSCs can be used to regenerate the tissue.

ACKNOWLEDGEMENTS: We would like to acknowledge the funding provided by AO SPINE Research Network (AO SRN) for this project.

Importance of collagen-binding integrins for the matrix remodelling by tendon stem/progenitor cells

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INTRODUCTION: The establishment of appropriate interactions between tendon cells (TSPC) and extracellular matrix proteins is essential for tendon homeostasis and healing. Cell-extracellular matrix bounds are predominantly mediated by integrins and are required for the normal cell functions including the matrix remodelling. Therefore, we questioned in our study the importance of collagen I-binding integrin receptors for the remodelling capacity of TSPC. For this purpose, we first analysed the integrin expression changes in three dimensional (3D) collagen gels and second, we applied a stable knockdown to assess the effect of integrins on TSPC remodelling ability.

METHODS: TSPC were isolated and characterized from several human Achilles tendon biopsies according to [2] and maintained in DMEM/Ham's F-12 culture media supplemented with amino acids, FCS and L-ascorbit-acid-2-phosphate. First, the obtained TSPC were used for adhesion assay on non- or collagen I-coated surface as described in [3]. Next, the cells were cultured on collagen gels in order to assess their matrix remodelling ability. The changes in gel area were monitored over a period of 5 days by microscopy. Cell morphology inside the gels was visualized by phaloidin-AF546 staining. Last, previously established lentiviral transduction system was used to deliver stable shRNA, specific for individual integrin subunits [3]. The integrin expression and knockdown validation was performed by quantitative PCR analysis.

RESULTS: The adhesion assay demonstrated a positive effect of collagen I matrix on TSPC adhesion since 100% of the cells were attached after 120 min. Investigation of TSPC grown in 3D collagen gels revealed that the cells maintained the classical bipolar cell morphology. Moreover, these cells had the ability to remodel the collagen I gels and contacted 90% of their area after 120 hours. Analysis of the collagen-binding integrins in TSPC showed changes in their expression in 3D as integrin a1 and a2 were decreased, whereas the expression of integrin a11 was upregulated three folds in comparison to the 2D culture. Then, we established efficient integrin knockdown in TSPC for the three collagen I-binding integrins. This was validated by PCR examination, demonstrating that the mRNA levels of all integrins were reduced more than 70%. Finally, our analysis on the effect of integrin knockdown on TSPC matrix remodelling revealed a significantly diminished collagen gel contraction in a2 and a11 integrin-deficient cells. In contrast, a1 knockdown did not affect TSPC remodelling ability as these cells demonstrated contractility similar to the control TSPC.

DISCUSSION & CONCLUSIONS: Our results demonstrated that TSPC possess a higher adherence affinity towards their native matrix and when maintained in 3D collagen gels, the cells exhibited the ability to contract the matrix by 90%. In a 3D environment, TSPC integrin expression was altered as integrin a1 and a2 were downregulated and integrin a11 was upregulated. Based on our result that knockdown of a2 and a11 integrin lead to impaired matrix remodelling, we suggest that these two integrin receptors are of significant importance for the TSPC function in 3D collagenous environment.

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Combination of a new injectable multiphasic bone substitutes based on gel-coated Osprolife HA/TTCP granules with bone marrow concentrate: an *in vitro* and *in vivo* study in sheep

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[1] *Eurocoating S.p.A, Via Al Dos de la Roda 60, Pergine Valsugana, Trento, I.* [2] *Osteoarticular Regeneration Laboratory* [3] *BITTA Laboratory* [4] *Preclinical and Surgical Studies Laboratory and* [5]*Orthopaedic and Trauma Clinic III, Rizzoli Orthopaedic Institute, Bologna, I*

INTRODUCTION: The next-generation of bone graft substitutes aims at improving their handling properties and their clinical efficacy [1-2]. A new method based on bone marrow concentrate (BMC)-derived mesenchymal stromal cells (MSC) combined with injectable calcium phosphates bioceramics was designed in order to stimulate bone regeneration . The aim of this study was to investigate the *in vitro* and *in vivo* behaviour of a new injectable multiphasic bone substitutes (MBS) based on gel-coated Osprolife HA/TTCP granules (gel-HA/TTCP) (Eurocoating SpA) with ovine BMC (oBMC).

METHODS: Ovine BMC was obtained using the IOR-G1 device (Novagenit Srl). The concentration and viability of nucleated cells (NC) were analysed in the bone marrow and after isolation and NC recovery was calculated. BMC was evaluated for MSC progenitors content, performing the CFU assay and cells were expanded *in vitro* until passage 10 in order to evaluate cell kinetics. Then, *in vitro* and *in vivo* experiments were performed combining 200µL of BMC diluted in 200µL of gel cross-linking solution with 0.5cc of gel-coated granules loaded in syringe. In *vitro* experiments were performed maintaining the product in culture for assessing cell viability at time 0, 72h, and two-weeks using LIVE/DEAD staining kit. The distribution of viable cells in the product was also studied through confocal microscopy. In *vivo* study was carried out in an ovine model. Under general anaesthesia, 4 sheep underwent surgical implants of 4 gel-HA/TTCP and 4 gel-HA/TTCP +oBMC specimens, randomly, in lateral femoral condyles. Six weeks later, the specimens were retrieved and microtomographic (Skyscan-1172) and histomorphometric investigations were done.

RESULTS: The total NC recovery in the BMC ranged from 13.7 to 88.8% and viability was higher than 95% in all samples after IOR-G1 concentration. Cell kinetics was comparable in MSC obtained *both* from bone marrow and BMC. Despite a great variability, the mean value of MSC progenitors was maintained after the concentration/separation of BM and a range between 80 and 7200 of MSC progenitors was contained in 200µL of BMC used for *in vivo* surgeries. Cells remained viable hereafter the combination with the gel-coated granules and viability was maintained over time, as *in vitro* evaluation demonstrated. Confocal imaging showed the cells suspended in the gel after 2-weeks of culture. Preliminary histomorphometric results *on vivo* biopsies showed a good histocompatibility of gel-HA/TTCP granules, with or without oBMC, with a similar bone response.

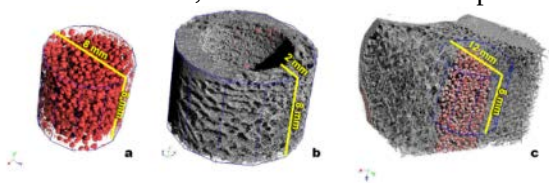

Fig. 1: VOI used for 3D analyses: (a) MBS; (b) implant site; (c) both VOI associated. Mat.V/TV: residual substitute volume.

Table . BV/TV: host bone volume; nBV/TV: new bone volume within and around grafts.

	gel-HA/TTCP	*gel-HA/TTCP+oBMC*
Mat.V/TV %	17.0 ± 4.8	16.2 ± 4.4
nBV/TV%	3.2±0.6	3.1±1.3
BV/TV %	37.6±4.7	

DISCUSSION & CONCLUSIONS: Injectable MBS based on gel-coated Osprolife HA/TTCP granules were proved to be biocompatible and their easy combination with BMC can lead to a more clinically successful "single-step procedure" for bone regeneration.

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ACKNOWLEDGEMENTS: The work is part of "CaP Project" cosponsored by Eurocoating SpA and Provincia Autonoma di Trento (Regional Public Authority). The authors wish to thank Novagenit S.r.l. for providing the IOR-G1 device for the BM concentration.

Mesenchymal Stem Cell Transplantation on Rabbit Tuberculous Spondylitis Lesion: Analysis on Osteoblast Activity Via CBFA-1, ALP and OPN Biomarker

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INTRODUCTION: *Mycobacterium tuberculosis*' debris does not affect Mesenchymal Stem Cell's growth in vitro. This research observes *Mycobacterium tuberculosis*' growth toward the expression of marker Core Binding Factor Alpha-1 (CBFA-1), secretion of Alkaline Phosphatase (ALP), and Osteopontin (OPN) in vivo.

METHODS: Six rabbits with Spondylitis Tuberculosis were divided into two groups: first group having positive Culture (C), PCR (P), and Histopathology (H) hence n = 3; while the second group with only positive P, H considered as the control group. Both groups underwent intervention of treatment, MSC transplantation and anti tuberculous drugs. After six weeks, CBFA-1, ALP, and OPN were evaluated. The result was tested statistically to obtain osteoblast's activity total score.

RESULT: ELISA results for ALP and OPN of the rabbits' blood were nil. Immunohistochemistry, ALP, and OPN results for both treatment and control groups were all positive. CBFA-1 immunofluorescence results on the first group were all positive (3/3), while only 2 of 3 samples from the control group gave positive results. The mean scores for first and second group respectively, are 160 and 145.

Table 1. Evaluation of osteoblast marker's result

	Treatment group (n=3)		Control group (n=3)		P
	n=3	mean ± SD	n=3	mean ± SD	
ALP from blood	3	0	3	0	Na
ALP absorbansion	3	0,062 ± 0,0134	3	0,109 ± 0,094	Na
ALP from lession	3	3/3	3	3/3	Na
OPN from blood	3	0	3	0	Na
OPN absorbansion	3	0,051 ± 0,009	3	0,050 ± 0,013	Na
OPN from lession	3	3/3	3	3/3	Na
CBFA-1 from lession	3	3/3	3	2/3	Na
Final skor	3	160	3	145	

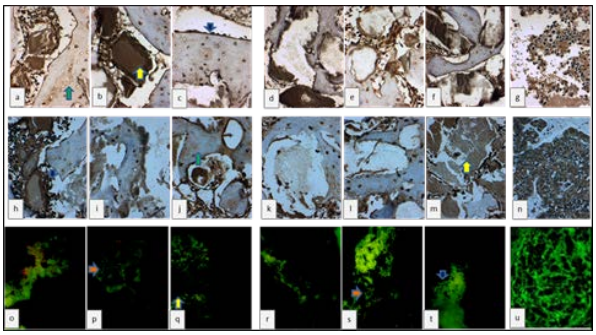

Fig. 1: Overview of osteoblast marker from spondylitis tuberculosis's rabbit. a, b, c: IHC ALP control group. d, e, f: IHC ALP treatment groups. g: IHC ALP positive control from sarcoma tissue. h, i, j: IHC OPN control group. k, l, m: IHC OPN treatment group. n: IHC positive control from breast cancer tissue. o, p, q: IF CBFA-1 control group. r, s, t: IF CBFA-1 treatment group. u: IF CBFA-1 positive control. Looks lined osteoblast cells (blue arrows), skafol hydroxy apatite (yellow arrows), positive IHC (green arrows), positive IF (brown arrows)Table 2. Result of healing percentage

DISCUSSION AND CONCLUSION: Existence of *Mycobacterium tuberculosis* gave different effects on osteoblasts' activities in expressing and secreting their markers. CBFA-1 expression and OPN secretion were not inhibited by *Mycobacterium tuberculosis* on both groups, while ALP secretion were somehow inhibited. Scoring results show that the existence of *Mycobacterium tuberculosis* seems to reinforce osteoblasts' activities.

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Mesenchymal stem cell effects on microbiological and histopathological alterations in spondylitis tuberculosis rabbit's healing process

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INTRODUCTION: Mesenchymal Stem Cell (MSC) has the potency to modulate immune response and to repair tissues. The existence of MSC in *Mycobacterium tuberculosis* microscopical environment is hoped to reduce its proliferating activity. This research aims to observe the direct MSC transplantation effect on the vertebral body defect in spondylitis tuberculosis rabbit on microbiological and histopathological aspects of bone healing in vivo.

METHODS: Fourteen spondylitis tuberculosis rabbits were divided into two groups which are the treatment (n=7) and the control group (n=7). The treatment group underwent total treatment Subroto Sapardan alternative 6 (TTSSA6) intervention procedure, scaffold and MSC transplantation and anti tuberculosis drugs administration, meanwhile control group underwent the same procedure as treatment group without MSC transplantation. Th1 and Th2 along with microbiological and histopathological examination on the tissue lesion from both group were analysed from blood after 6 weeks of incubation. The results were tested statistically and the healing score was calculated.

RESULT: Six weeks after MSC transplantation, Th1 was increased from 4.79% (SD= 2.35) into 30.90% (SD = 30.23) and Th2 was decreased from 42.74% (SD = 10.23) into 29.26% (SD = 34.95). Th1/Th2 population was increased from 0.12 (SD = 0.08) into 5.84 (SD = 7.80). Microbiological examination showed healing of 3 out of 7 treatment rabbits group (3/7, 42.9%) and 4 out of 7 control rabbits group (4/7, 57.1%); (p = 0.500). Histopathological examination showed healing of 2 from 7 treatment rabbits and also 2 from 7 control rabbits group (2/7, 28.6%); (p = 0.720). Hence the healing score of both the treatment group (n=7; mean score 268.57; SD = 15.74) and the control group (n=5; mean score 264.00; SD = 16.73) were all above 105 (healed) (p = 0.595).

Table 1. Result of Th1 and Th2 examination

	Before MSC tranplantation		After MSC tranplantation		P
	%	SD	%	SD	
Th1	4.79	2.35	30.90	30.23	Na
Th2	42.74	10.23	29.26	34.95	Na
Ratio of Th1/Th2	0.12	0.08	5.84	7.80	Na

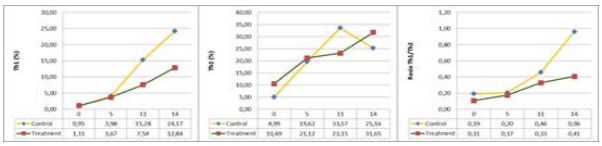

Fig. 1: Graph changes in population of Th1 and Th2 from blood samples spondylitis tuberculosis rabbit's

Table 2. Result of healing percentage

Modality examination	With MSC		Without MSC		P
	n	%	n	%	
Microbiological	3 (3/7)	42,9	4 (4/7)	57,1	0,500
Histophatological	2 (2/7)	28,6	2 (2/7)	28,6	0,720

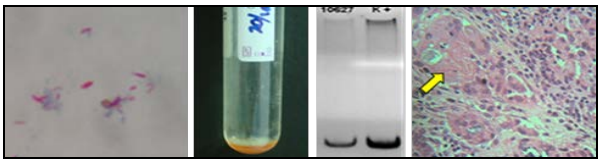

Fig. 2: Image of result of AFB staining, culture, PCR dan histophatology test from tissue lession spondylitis tuberculosis rabbit's

DISCUSSION AND CONCLUSION: Microbiological and histopathological examination on tissues lesion shows a good healing process from both treatment and control group which show that there is no effect of MSC transplantation to the immune response system on the infection of *Mycobacterium tuberculosis* which is shown on the alteration of positive into negative results from 2 out of 7 rabbits on both groups. MSC transplantation does not have significant effect on ST rabbit's healing process microbiologically and histopathologically shown by the same total healing score.

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Mesenchymal stem sell transplantation on rabbit spondylitis tuberculous lesion: analysis on ossification process through osteoblast cell count, osteocyte count and calcium level on lesion

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INTRODUCTION: Mesenchymal Stem Cell (MSC) differentiates into osteoblast then osteocyte during the process of ossification. *Mycobacterium tuberculosis* (MTb) is proven not to affect MSC growth in vitro. This research aims to observe the ossification on micro environment containing live MTb which was transplanted with MSC in vivo rabbit.

METHODS: Six Spondylitis Tuberculosis (ST) rabbits were divided into two groups: the first group having positive Culture(C), PCR(P), and Histopathology(H) hence n=3; while only P, H were positive for the second group (n = 2). Both group underwent intervention of treatment, MSC transplantation, and anti tuberculous drugs. After six weeks, ossification process was evaluated by counting the number of osteoblast, osteocyte and level of calcium on lesion. The results were then tested statistically and ossification score was obtained.

RESULTS: Mean number of osteoblast on the first group were 207.00 cells (SD=31.00) and for the second group were 220.33 cells (SD=73.46). Mean osteocyte number intra lesion on first group were 18.33 cells (SD=30.04) and 31.00 cells for the second group (SD=26.87). Mean level of calcium on the first group was 2.94% (SD=0.89) while on the second group was 2.51% (SD=0.13). Total ossification score on the first and second group respectively were 31.00 and 25.67.

Table 1. Count result of osteoblast, osteocyte and calcium level on treatment and control group

Variable	Treatment Group		Control Group		P
	n=3	mean ± SD	n=3	mean ± SD	
Number of Osteoblast	3	220,33 ± 73,46	3	207 ± 31	Na
Number of Osteocyte	3	18,33 ± 30,04	3	31,00 ± 26,87	Na
Calcium level	3	2,94 ± 0,89	3	2,51 ± 0,13	Na

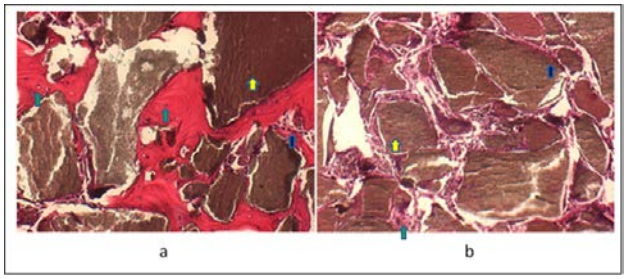

Fig. 1:Observation to hematoxicilin eosin stain with 400x magnification. Picture a shows slide from rabbit group that was given MSC into the defect, there is scaffold with bone island and bone formation. Picture b shows slide from infected rabbit without MSC was given, there are inflammatory cells without formation bone island

DISCUSSION & CONCLUSIONS: MTb is able to suppress osteoblast differentiation to osteocytes while stimulates calcium metabolism intra-lesion to inhibit the ossification process to produce immature bone. The microscopic environment containing live MTb yields better ossification process.

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Influences of *mycobacterium tuberculosis* exposures in the bony bridge formation of mesenchymal stem cell transplantation to rabbit with spondylitis tuberculosis

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INTRODUCTION: Fusion is defined by the incorporation of bone-to-bone or between the parts of bones. Mesenchymal stem cell (MSC) differentiation into osteoblast is not disturbed in relation to *Mycobacterium tuberculosis's* debris exposure in the microscopic environment in vitro. This research aims to observe the influences of live *Mycobacterium tuberculosis* on bony bridge formation on Spondylitis Tuberculosis rabbit's defect which is transplanted with MSC.

METHODS: Six spondylitis tuberculosis rabbits were divided into two groups which were culture (C), PCR (P), and histopathologic (H) positive as the exposed group (n=3) and PCR and histopathologic (PH) positive as the control group (n=3). Both groups underwent total treatment Subroto Sapardan alternative 6 (TTSSA6) treatment, anti tuberculosis drugs administration, scaffold and MSC transplantation. Clinical examination was carried after 6 weeks incubation time and bony bridge through the manual palpation (MP) method was examined. Bone intra defect area percentage (BIDAP) was calculated, bone area per field view (BAFV), and lateral bone defect area (LBDA) were measured based on microscopic examination from histopathology preparation while antero-posterior projection callus area (APPCA) and lateral projection callus area (LPCA) were measured based on x-ray image using Image *J software*. The results were tested statistically and used to determine the fusion score.

RESULT: MP from both control and exposed group showed 1 positive MP rabbit. Mean BIDAP score for exposed group 30.00% (SD = 14.00) and control group 40.67% (SD = 12.50), mean BAFV score for exposed group 0.05 mm^2 (SD = 0.02) and control group 0.07 mm^2 (SD = 0.02), mean LBDA score for exposed group 0.155 mm^2 (SD = 0.067) and control group 0.230 mm^2 (SD = 0.07). Mean APPCA score for exposed group 34.30 mm^2 (SD = 5.61) and control group 25.77 mm^2 (SD = 9.79), mean LPCA score for exposed group 25.87 mm^2 (SD = 5.61) and control group 23.71 mm^2 (SD = 8.34). Mean fusion score for exposed group 77.67 and 120.6 for control group. These results showed that there were 2 of 3 rabbits (2/3) in the exposed group had fusion disturbances and 1 out of 3 rabbit (1/3) had better fusion; while in the control group there was 1 of 3 rabbits (1/3) had delayed fusion, 1 of 3 (1/3) had normal fusion and 1 of 3 (1/3) had better fusion.

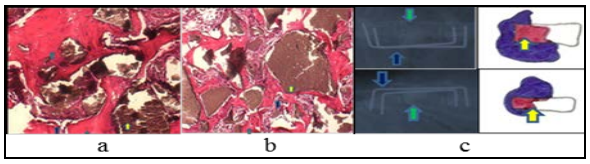

Fig. 1: Observation of HE staining from the exposed group (a) and the control group (b). c. X-ray and callus shading anteroposterior

DISCUSSION AND CONCLUSION: *Mycobacterium tuberculosis* existence does not affect bone rigidity manually. This result differs from the result obtained based on the three histopathologic aspects showing the impression of *Mycobacterium tuberculosis*'s existence that suppresses formation of new bone intra-defect proven by the smaller bone area formed in the exposed group compared with the control group. Fusion disturbance by *Mycobacterium tuberculosis* exposure was also stated in several literatures emphasizing the suppressing effects of *Mycobacterium tuberculosis* to osteoblast's activity which inhibit the formation of new bone necessary in the bony bridge formation hence, fusion. The presence of *Mycobacterium tuberculosis* bacteria histopathologically and radiologically interfere with the formation of new bone in the infection defect of spondylitis tuberculosis rabbit, but manually palpation of the relationship between bone shows that it is still in the fusion.

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Expression of cartilage stem cell markers is dependent on time in culture

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INTRODUCTION: Our laboratory has isolated stem cells from human, bovine and equine articular cartilage utilising fibronectin adhesion assay. These clonal populations have been expanded and demonstrated maintenance of chondrogenic phenotype to over 50-population doublings[1]. Although we can enrich for stem cells and expand this population, to date there is no individual stem cell marker. However, markers located in mesenchymal stem cells have been identified in articular cartilage, including CD44, CD49e, CD105 and CD166[2]. Localisation of these markers to stem cells in articular cartilage would enable selective isolation facilitating further characterisation of the stem cell population

METHODS: Full depth cartilage explants were taken from immature bovine metacarpal-phalangeal joints. Immunofluorescence was carried out on cryosections using antibodies for the cell surface markers CD29, CD44, CD49e, CD105 and CD166. To further investigate and quantify expression of these stem cell markers, superficial zone cells were isolated and immunolabelled immediately following cell isolation. To assess the changes in expression, stem cell markers were cultured for 14 days, to provide a proxy for dedifferentiated cells. Superficial zone chondrocytes were immunolabelled at day 3, 7 and 14. Cells were detached using accutase and immunolabelled using directly conjugated CD29FITC, CD44FITC, CD49eRPE, CD105APC and CD166RPE. Immunolabelling was quantified using flow cytometry.

RESULTS: This study identified stem cell markers CD44, CD49e, CD105 and CD166 localised to the articular cartilage surface with CD29 expressed throughout the tissue. The restricted expression of these markers predominantly to the superficial zone in immature bovine is indicative of a resident stem cell population as previously described. Cell surface marker expression was absent or reduced following cell isolation and upregulated following monolayer culture (figure 1). The differences observed are indicative of cell surface marker cleavage during cell isolation and subsequent cell adhesion and proliferation. The majority of cell surface receptors exhibited a unimodal increase in expression indicative of a homogeneous population. Cell marker expression increased up to 7 days in culture and decreased from 7 to 14 days, with the exception of CD105 labelling which increased from day 7 to 14 (figure 1).

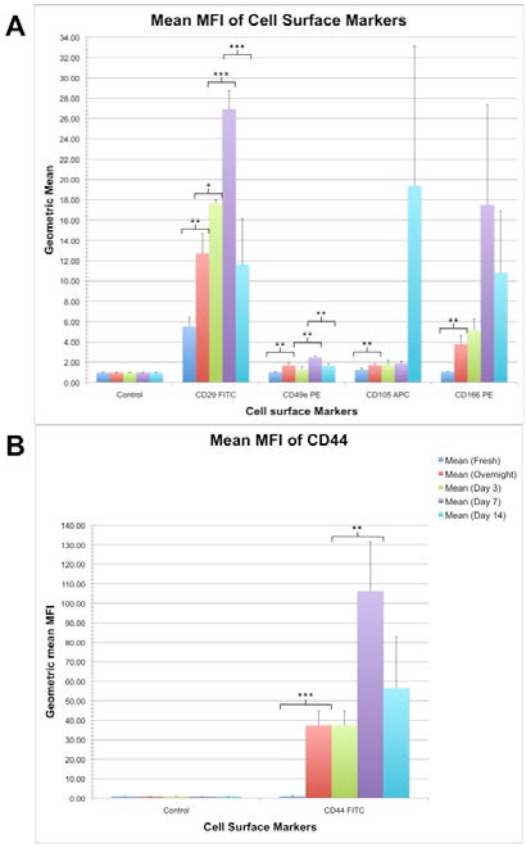

Fig. 1: Graphs illustrating the geometric mean fluorescent intensity (MFI) of cells immunolabelled at each time point. A: Labelling for CD29, CD49e, CD105 and CD166. B: Labelling for CD44.

DISCUSSION & CONCLUSIONS: *These data demonstrate that stem cell markers are expressed in the superficial zone of articular cartilage and that their expression in monolayer is dependent on time in culture. These results emphasise the importance of analysing cell surface marker expression throughout culture. Isolation of these cells must be supported by further characterisation before selection for in vitro and in vivo studies.*

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Is NOV/CCN3 a potential new regulator of joint homeostasis?

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INTRODUCTION: NOV/CCN3 is a member of the CCN family of matricellular proteins which have important roles in development, wound healing and disease. Their common modular structure enables them to modulate various signalling pathways including BMP, Wnt, TGFs, Notch and integrins[1]. We have shown that NOV is required for normal joint development and function as its disruption in the targeted mouse mutant Nov^{del3} leads to joint abnormalities in the embryo and adult[2], suggesting that NOV may have a role in joint homeostasis.

Joint homeostasis is essential for tissue maintenance and its failure leads to joint dysmorphogenesis and degenerative disease. Stem cells are thought to be important for joint homeostasis and repair, acting as a source of progenitors to replenish lost or damaged cells and are thus essential for joint health, contributing to the protection of the joint from degenerative diseases such as OA. Interestingly, NOV has been implicated in OA with altered expression in experimentally-induced OA in mice[2] as well as in human OA[3].

To determine whether NOV is required for normal joint homeostasis and if its disruption predisposes the joint to OA, we characterised the expression of NOV in the adult mouse joint and studied the consequences of its disruption in Novdel3-/- mice during ageing.

METHODS: NOV expression in the adult mouse joint was characterized by immunohistochemistry. A detailed comparison of the joints of Nov^{del3} homozygote and wild type (WT) males and females at 6 and 12 months of age was determined by X-ray, histology and immunohistochemistry.

RESULTS: NOV protein was detected in multiple tissues of the adult joint including the articular cartilage, meniscus, synovial intima and ligament entheses. NOV was found in the superficial layer of the articular cartilage and meniscus (which are both locations of resident stem cells) and in a subset of cells in the calcified cartilage and in core of the meniscus.

X-ray and histological analysis revealed that Nov^{del3}-/- males exhibited severe degeneration at 12 months of age affecting all tissues of the joint: erosion of the articular cartilage, enlargement of the meniscus, osteophytic outgrowths, ligament degeneration and expansion of fibrocartilage within the joint. Changes to the extracellular matrix composition were also seen. In comparison, Nov^{del3}-/- females exhibited a much milder phenotype. A significant decrease in articular cartilage cell density was seen at 6 months, prior to severe degeneration of the joint, and at 12 months in both male and female Nov^{del3}-/- mice.

DISCUSSION & CONCLUSIONS: We have identified NOV as a new potential regulator of joint homeostasis. NOV is expressed in multiple structures of the joint including the articular cartilage and synovium. Disruption of NOV in the targeted mouse mutant Nov^{del3} causes severe abnormalities in the adult joint, demonstrating its requirement for tissue homeostasis. The decreased articular chondrocyte density in Novdel3-/- mutants indicates that NOV may play a role in maintaining articular cartilage cells, and may thereby contribute to protecting the joint from OA.

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Osteogenic-like behaviour of adipose derived stem cells in selected scaffolds obtained by 3D-printing

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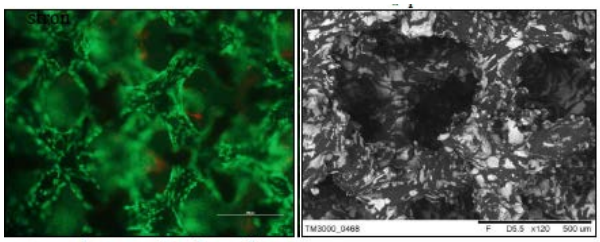
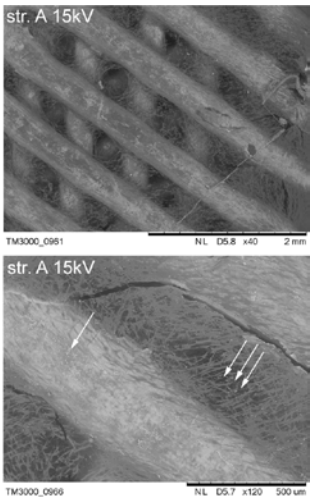
INTRODUCTION: Adipose derived stem cells (ADSC) have been widely accepted as promising adult stem cells to be used in regenerative medicine. There are strong evidence showing their capacity to differentiate into various phenotypes. For this reason they are, inter alia, be considered to be suitable for the generation of tissue engineered bone. In the present study we show examples of successful distribution and osteogenic differentiation of human ADSC in the three-dimensional PCL-based and Ti-based scaffolds obtained by 3D-printing.

METHODS: PCL-based scaffolds were manufactured with Bioscaffolder® by plotting the melted composite (PCL enriched with TCP particles) layer by layer, with lay-down pattern of the fibers: 00/600/1200 with shifted layers. Titanium-based scaffolds were produced as lattice structures from Ti-6Al-7Nb powder by Selective Laser Melting. They were modified by the immersion process in simulated body fluid to obtain HAp/Ca-P layer on their surface. ADSC were pre-induced by culture with vit C containing medium and seeded onto the scaffolds at passage 2. After 24 hours, differentiation medium enriched with dexamethasone and β-glycerphosphate was applied and the cells were cultured for 14, 21 or 42 days. Cell morphology and distribution was visualized (fluorescence and SEM). Cell proliferation was controlled by means of DNA assay (PicoGreen) and cell metabolic activity (Alamar Blue or XTT). Selected markers of osteogenic phenotype were investigated at the level of gene (RT-PCR) or protein (FACS) expression.

RESULTS: ADSC proliferated efficiently on the scaffolds which was shown by about 10-fold increase in metabolic activity after 14 days. Live/dead fluorescent staining revealed that cells covered the whole surface of scaffolds at day 14 and filled scaffold pores at day 42. SEM analysis showed the onset of extracellular matrix (ECM) deposition at day 14, which increased at day 21 and 42.



Images of ADSC distribution on titanium scaffolds - SEM (left) and fluorescent (right) observations



ECM produced by ADSC on PCL-based scaffolds

This observation was further confirmed by fluorescent staining of hydroxyapatite. Up-regulation of Runx2, collagen I and osteopontin genes expression was already observed after 14 days of culture. Cells detached from the scaffolds after 14 days produced osteonectin protein, which was evidenced by FACS analysis. Interestingly, osteogenic genes up-regulation on the PCL-based 3D scaffolds was significantly higher as compared to the 2D control.

DISCUSSION & CONCLUSIONS: ADSC differentiate and produce ECM characteristic for osteogenic cells in 3D scaffolds produced from the materials widely used for bone tissue engineering

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The addition of osteoclastic cells activates devitalized engineered hypertrophic cartilage to form bone

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INTRODUCTION: Devitalized engineered tissues with the capacity to induce bone formation are of high clinical and commercial interest. In this work we investigated whether osteoclastic cells derived from human peripheral blood co-cultured with devitalized hyperthropic cartilage (HC) produced *in vitro* by adult human mesenchymal stromal cells can induce matrix remodeling into bone tissue.

METHODS: HC was devitalized by successive freeze/thaw cycles and cultured alone as control or together with freshly isolated CD14+ monocytes in the presence of osteoclastogenic factors (MCSF; RANK-ligand) for up to 23 days. Moreover, after 1 day of *in vitro* culture, samples from both groups were implanted ectopically in nude mice for 8 weeks. Analysis consisted of biochemistry, protein assays, histology and microtomography.

RESULTS: *In vitro* results indicated matrix degradation through a significant loss of glycosaminoglycans only when HC was cultured with osteoclastic cells. In addition, supernatants of co-cultures contained significantly higher amounts of chemoattractant (MCP-1 192-fold; SDF-1 4-fold), angiogenic (IL-8 556-fold; VEGF-A 5.4-fold) and matrix degrading (MMP9 13534-fold; MMP13 8.5-fold) factors as compared to controls. Only co-cultured HC generated frank bone through endochondral ossification *in vivo (Fig.1)*, with a 3.5-fold higher mineralized volume as compared to controls.

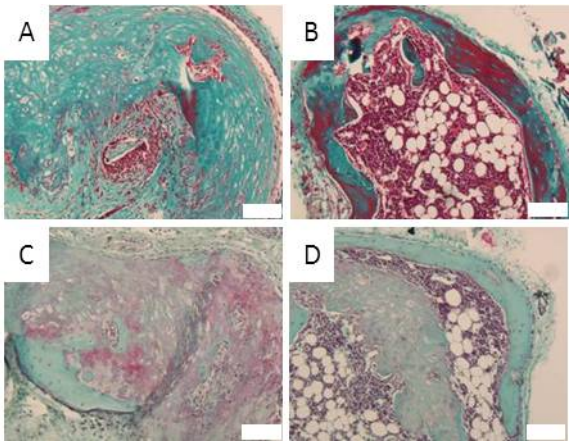

Fig. 1:Masson Trichrome (A, B) and Safranin-O (C,D) histological assessment of explants of devitalized HC without (A;C) or with the presence of osteoclastic cells (B;D) experimental groups (white scale bar:100um).

DISCUSSION & CONCLUSIONS: Collectively, the addition of osteoclastic cells on devitalized HC primed the onset of remodeling leading to bone formation. Ongoing experiments aim to identify some crucial factors released from the HC matrix in co-cultures which are capable to recruit key cell populations involved in ossification. The activation/stimulation of off-the-shelf, devitalized engineered tissues using easily available autologous cells could represent a novel paradigm in regenerative medicine.

Isolation and characterization of exosomes derived from human platelet lysate

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INTRODUCTION: Bone tissue regeneration involves integrative activity of native tissues, mesenchymal stromal/stem cells (MSC) and growth factors (GFs). One of the main sources of GFs is represented by human platelet lysate (PL). PL is a hemoderivate presently used both in *in vitro* studies, where it is being established as a safe and efficient MSC culture supplement [1], and in orthopaedic surgery [2]. The rationale for the use of PL on bone lesions is based on the significant reduction of endogenous GFs observed in nonunion sites [3]. PL may be used as a special source of GFs mirroring what happens physiologically in the native haematoma following bone injury. Despite the encouraging benefits of the use of PL in *in vitro* as well as in bone-related clinical studies, it is still unknown if PL efficacy is due to free bioactive molecules or GFs included into exosomal-like vescicles and released following platelets activation. Exosomes have recently received much attention as these are a subclass of nanovesicles (30-100nm) which derived from the endosomal compartment of most cell types. They contain a wide range of functional proteins, mRNAs and microRNAs [4], providing a novel paracrine signalling mechanism during important physiological and pathological process. In this study, we isolated and characterized exosomes from human PL, and investigated their effect on MSC proliferation and osteogenic differentiation *in vitro*.

METHODS: Exosomes were isolated from samples of human PL by differential ultracentrifugation. Their purity was assessed by electron microscopy and evaluating CD63 expression by Western blot analysis. To test the effect of exosomes on MSC proliferation, bone marrow-derived MSC were cultured in presence of two different exosomes concentrations (5µg and 50µg) or with the corresponding PL for 7 days. At day 3 and 7 after seeding, cell growth was assessed by Alamar blue assay. In addition, we analysed the effect of exosomes on MSC osteogenic potential, evaluating the ability of exosome-treated cell to deposit extracellular matrix by Alizarin red staining and the expression of specific bone-related genes (e.g. Collagen type I, bone sialoprotein, Osteocalcin) by Real-time PCR.

RESULTS: Exosomes were isolated from human PL by serial low speed centrifugation followed by ultracentrifugation. Electron microscopy revealed the presence of vesicles within the expected size range of exosomes (30– 100nm) which expressed the specific exosomal marker CD63 by Western blot analysis. MSC treated with PL-derived exosomes showed a significant and dose-dependent increase of cell proliferation. Furthermore, we investigated exosome influence on MSC osteogenic potential. Preliminary results indicated that exosomes do not have any significant effect on extracellular calcium deposition as well as on the expression of specific osteogenic genes.

DISCUSSION & CONCLUSIONS: In the present study, we demonstrated that exosomes can be successfully isolated and purify from human PL. Following treatment of MSC with different concentration of PL-derived exosomes or the corresponding PL, we observed that PL-derived exosomes increased cell proliferation more than PL and in a dose-dependent manner. This suggests that PL-derived exosomes may contain factors which promote cell growth. Based on these preliminary observations, PL-derived exosomes might be used as a culture supplement for robust MSC cultivation *in vitro*. In addition, they could represent a potential "cell-free approach" to apply in tissue regeneration, where evidence of permanent engraftment and transdifferentiation of transplanted cells sufficient to repair the damaged organ is still lacking.

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The transpedicular approach for the study of intervertebral disc regeneration strategies: in vivo characterization

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INTRODUCTION: Present delivery approaches of therapeutic agents such as growth factors, stem cells and/or bioactive hydrogels within the intervertebral disc (IVD) need to be through injection, via the anulus fibrosus (AF). However, it has been demonstrated that even a small needle puncture of the AF leads to further degeneration and disc herniation. The study purpose was to *in vivo* characterize the transpedicular approach as an alternative route to study intervertebral disc degeneration (IDD) and IVD regeneration strategies in a sheep model. The qualitative criteria used to evaluate our technique were feasibility, accuracy of the wire position in the nucleus pulposus (NP), surgery technique related adverse effects.

METHODS: *In vivo* animal study using 48 lumbar spinal segments of 12 sheep. In each sheep the transpedicular approach to the NP was performed under fluoroscopy, followed by nucleotomy using a shaver resector through the 2 mm tunnel. The endplate was repaired using a porous polyurethane scaffold press-fit at the endplate edge. Intraoperative fluoroscopy images, X-Ray and MRI images preoperatively and after 1, 3 and 6 months were acquired and analyzed. Complications were recorded.

RESULTS: The transpedicular approach was feasible in all animals of this study. The NPs of vertebrae from L1 to L5 were accessible through the transpedicular approach and the intradiscal K-wire placement was achieved in all animals without major complications. MRI images showed the loss of NP signal intensity in discs that underwent nucleotomy compered to controls.

DISCUSSION & CONCLUSIONS: The transpedicular approach described is feasible *in vivo*, repeatable after only a short learning period and can be safely performed without significant morbidity. This animal model allows the study of IDD physiopathology and the investigation of *in vivo* disc regeneration techniques such as those based on the use of mesenchymal stem cells.

Effects of bone marrow derived cells on pain and structural changes in a mono-iodoacetate rat model of osteoarthritis

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INTRODUCTION: Mesenchymal stem cells (MSCs) are promising candidates for osteoarthritis (OA) therapy. In OA joints multiple intra-articular tissues, such as cartilage and synovium, are affected and currently no therapy to stop or cure OA is available. MSCs are promising because they are the body's natural healers, they can differentiate to generate repair tissue and they have the capacity to secrete immunomodulatory and trophic factors [1-2].
We studied the effects of intra-articularly injected bone marrow derived MSCs of rats, as well as freshly isolated rat bone marrow mononuclear cells (BMMNCs), on pain and structural tissue damage in a rat OA model in-vivo.

METHODS: OA was induced unilaterally in 24 Wistar rats by an intra-articular injection of 300 µg mono-iodoacetate (MIA) in the knee. After 3 weeks the rats were randomly divided into three groups: 1. control, 2. rat MSCs, 3. rat BMMNCs. 50 µl Saline was used as a control and as vehicle for all other injections. MSCs were given at a dose of $1x10^6$ cells per joint and BMMNCs were given at a dose of $10x10^6$ cells per joint. Before and four weeks after treatment, pain was assessed by measuring hind limb weight distribution using an incapitance tester as an index of joint discomfort as described previously [3]. Subchondral bone was analyzed by µCT and cartilage degeneration was analyzed by histology.

RESULTS: At baseline the limbs that received MIA bore 50.6±1.6% of the weight and the contralateral limbs 49.4±1.6%. Three weeks after MIA injection, weight distributed to the affected leg was significantly reduced compared to baseline (45.8±5.4% vs 50.6±1.6%, P=0.002), indicating pain sensation. Both therapies were well tolerated by the animals. Animals treated with MSCs distributed significantly more weight to the affected limb after treatment than before treatment 51.2±5.0% vs 46.5±4.1%, P=0.003). Comparing the treatment groups after four weeks, the group with MSC treatment had the highest average percentage of weight distributed to the affected limb, albeit no significant differences between the groups were demonstrated. MIA injected knees had significant cartilage damage and subchondral bone alterations compared to contralateral knees. Rats treated with MSCs displayed the least MIA induced changes, albeit not statistically significant different from the other treatment groups.

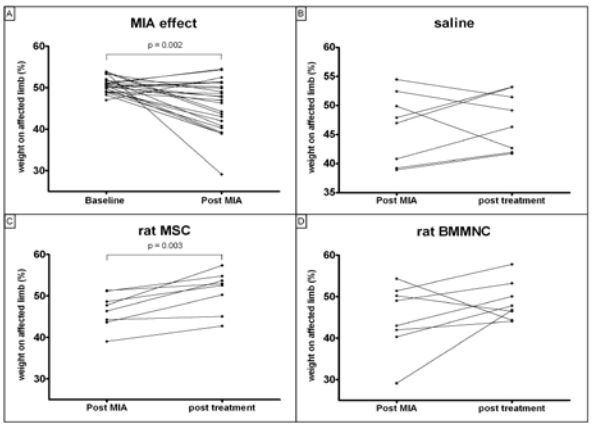

Fig. 1: Effects on pain of A: MIA, B: saline in OA, C: rat MSCs in OA, D: rat BMMNCs in OA.

DISCUSSION & CONCLUSIONS: This is the first study evaluating the effect of cell therapy on pain in a small animal OA model. Using intra-animal measurements we demonstrated a reduction of pain in animals treated with MSCs, not with the freshly isolated mononuclear fraction of bone marrow. Due to a high variation between the animals, differences between groups were not significantly different. Treatment after development of OA could not influence structural changes. Our study shows the importance to further study and optimize cellular therapies as a treatment for OA.

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The collagen component of biological bone replacement materials promotes bone formation by human mesenchymal stem cells

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INTRODUCTION: Synthetic bone replacement materials are attractive for the regeneration of a variety of bone defects. Seeding of these biomaterials with mesenchymal stem cells (MSCs) may further improve bone repair due to the osteogenic potential of these cells. However, human MSCs showed considerable donor variability in ectopic bone formation assays on synthetic bone substitutes which may limit clinical success. The study addressed whether bone formation variability of MSCs is cell-intrinsic or if it depends on the biomaterial and may be overcome by collagen-containing biological bone replacement materials.

METHODS: MSCs were isolated from bone marrow aspirates harvested from the femur shaft or from the iliac crest of 9 patients (mean age 58±19 years). Ectopic bone formation of MSCs was analysed on collagen-containing (bHA-C, eHA-C) and collagen-free (bHA; eHA) biological biomaterials of bovine and equine origin and on synthetic ß-TCP (Table 1). Granulated biomaterials of a defined particle size (0.25-1 mm) were seeded with MSCs from each donor and implanted subcutaneously into immune-deficient SCID mice. Bone formation was evaluated after 8 weeks by histomorphometry. The origin of new bone was determined by in-situ hybridization using human-specific and murine-specific probes.

Table 1. Characteristics of the bone replacement materials.

Bone substitute material	Porosity (%)	Pore diameter	Collagen content (%)	Origin
bHA-C	50-70	150-600 µm	33	bovine
bHA	75-80	100-400 µm 10-20 nm	0	bovine
eHA-C	75-80	100-400 µm 10-20 nm	40	equine
eHA	75-80	100-400 µm 10-20 nm	0	equine
ß-TCP	60	100-500 µm	0	synthetic

RESULTS: Histology of 8 week explants demonstrated a significant influence of the bone substitute material on donor variability of ectopic bone formation with best results seen for eHA-C (15/17) and ß-TCP (16/18). MSCs from one donor formed no bone with any biomaterial. Significantly more neo-bone was built on eHA-C compared to bHA, eHA, and ß-TCP. Neo-bone formation was superior on collagen-containing compared to collagen-free and synthetic bone substitutes, with low influence of species-origin. Newly built bone was of human origin as shown by in-situ hybridization.

DISCUSSION & CONCLUSIONS: Variable success of MSC-based bone tissue engineering displays the heterogenic character of the MSC populations. This heterogeneity is influenced by donor-specific parameters like age, gender or disease, by the cell harvest technique and by the progenitor cell content of expansion cultures. This study demonstrated that ectopic bone formation by human MSCs strongly depended on the biomaterial, whereby the switch from synthetic to biological bone replacement substitutes alone was not sufficient to resolve cell-intrinsic limitations of any MSC population. The initiation of bone deposition via induction of osteogenesis was highly effective on synthetic ß-TCP and on biological eHA-C. However, more bone was formed on eHA-C, suggesting that the collagen-component may be essential for matrix deposition over time and/or may boot biomaterial resorption in order to allow its replacement by new bone.

In summary, biological collagen-containing biomaterials are promising tools for MSC-based bone tissue engineering strategies.

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In vitro degradation, biocompatibility, and physical structure of composite scaffold incorporating bioactive phytomolecule icariin for bone regeneration

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INTRODUCTION: Icariin (ICI), a principal flaornoid glycoside in *Herba Epimedii* which has been reported to be a potent enhancer of bone healing[1]. Many studies have further demonstrated that ICI promoted osteoblasts differentiation and proliferation, and facilitated the process of matrix calcification; meanwhile they reduced the motility and bone resorption activity of isolated osteoclasts[2]. This study provide a new mind to design inducible bone scaffold by incorporating ICI into scaffold for bone regeneration.

METHODS: This study fabricated a novel scaffold composited with poly(lactic-co-glycolic acid) (PLGA), tricalcium phosphate (TCP) and ICI at low, middle, high doses (P/T/ICI-L, P/T/ICI-M, P/T/ICI-H groups, and P/T served as control) by low-temperature rapid prototyping technology. Structure, mechanical properties were characterized. The osteopromotive effects of P/T/ICI was evaluated using MC3T3-E1 osteoblast. *In vitro* degradation properties were examined by incubiting scaffolds in saline at 37 ℃ for 15 weeks.

RESULTS: Scaffolds had high porosity and perfect connectivity. Massive micropores distributed on the pore wall of the scaffold which ranged from 2.5 to 90 μm.（*Fig. 1*） The Young's modulus of P/T/ICI group (from 70 to 80 MPa) was higher than that in P/T group (around 50 MPa) ($P < 0.5$). *In vitro* degradation study showed weight changes, volume, and morphology of scaffolds, Ca^{2+} and lactic acid concentrations of medium. High dose ICI induced slower degradation during 15 weeks. At the end of degradation, P/T/ICI groups maintain initial shape. But P/T group collapsed sharply in 12 weeks, and the surface of scaffolds became soft and smooth. The MC3T3-E1 proliferation on P/T/ICI scaffold from day 1 to 7 demonstrated that P/T/ICI promoted MC3T3-E1 proliferation. P/T/ICI significantly enhanced ALP activity ($p < 0.01$), indicating an enhanced osteogenesis differentiation of osteoblast. Two days after MC3T3-E1 seeded to the scaffolds, expression of OC and BSP mRNA was observed increase in the P/T/ICI group, though no expression of OPN was observed in all groups.

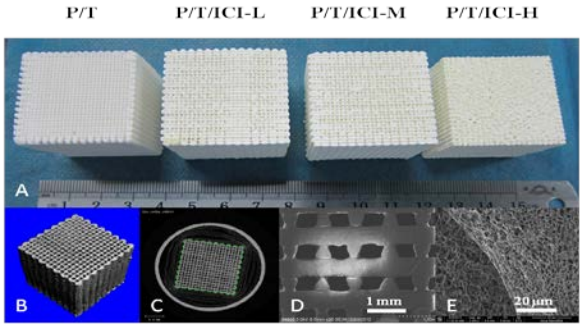

Fig. 1 Morphology of scaffolds (A) 3-D and 2-D images of scaffolds by micro-CT (B, C). Macro-pores and micro-pores of scaffold by SEM (D, E).

DISCUSSION: Micropores biomimiced the construction of human cancellous bone were fabricated by volatilizing of solvent in scaffolds.The special micro-pores structure of scaffolds not only affected the cell movement but also had a slight effect on the proliferation by increasing amount of accessible surface area of scaffold[3]. These unique properties would physically benefit cell seeding, migration and tissue ingrowth. The higher ICI incorporated, slower degradation could be observed during 15 weeks. ICI might chemically bind to scaffold increased a proper initial mechanical properties and degradation stability.

CONCLUSIONS: Current *in vitro* study showed that the innovative P/T/ICI scaffold had bioactivity to accelerate and induce the proliferation and differentiation of osteoblast. Further research will be run *in vivo* study.

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Effect of a novel bioactive porous PLGA/TCP/Icariin composite scaffold for boen defect repair in a rabbit femur segmental defect model

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INTRODUCTION: A novel osteoconductive poly lactic-co-glycolic acid (PLGA) / tricalcium phosphate (TCP) composite scaffold incorporating icariin was fabricated by low-temperature rapid-prototype biospinning technology and prepared for enhancing bone segmental defect repair. Icariin is a major flavonoid glycoside extracted and purified from Epimedii Herba and is proved to have osteogenesis effect [1]. The purpose of this study was to examine the *in vivo* osteogenic effect of icariin to be released from PLGA/TCP/Icariin scaffold into the bone tunnel after core decompression in the steroid-associated osteonecrosis (SAON) in rabbits.

METHODS: The SAON model was induced according to an established protocol [2]. Two weeks after SAON induction, the rabbits were anesthesiaed with pentobarbital sodium, then the core decompression surgery was performed at the distal femora, the cylinders of PLGA/TCP (P/T) and PLGA/TCP/Icariin (P/T/I) scaffords were implanted into the bone tunnel. After 2, 4 and 8 weeks of scaffold implantation, micro-CT evaluation and biomechanical analysis were performed to dertermine the osteogenesis inside the surgical bone tunnel.

RESULTS: At week 2, 2D images of micro-CT showed scattered new bone formation within the bone tunnel in both groups. At week 4 and 8, there were detectable new bone formed in the bone tunnel. 3D structure images of the new bone formed at the centre of scaffold with 2mm in diametersl at week 2, 4 and 8 were quantified. Micro-CT based histomorphometric analysis confirmed that the bone tunnel was almost empty in both groups at week 2. At week 4, the new formed bone was and its volume only 3.37% in P/T group and 5.66% in P/T/I group ($p<0.05$). At week 8, the bone volume increased to about 3.65% in P/T group, but decrease to about 4.86% in P/T/I group ($p<0.05$). Biomechanical analysis showed that the compression stiffness and the energy of healing bone tunnel were significan higher in P/T/I group than those in P/T group ($p<0.05$).

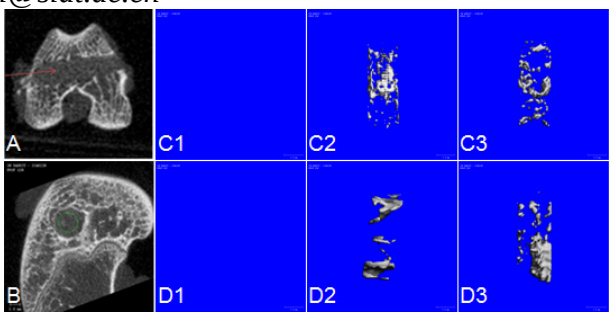

Fig1 A: 2D micro-CT image of bone tunnel (arrow) in a coronal view; B: 2D micro-CT image at sagittal view ;C and D: representative micro-CT 3D images of the new bone formed within the bone tunnel of 2mm in diameters of P/T group (C1-3) and P/T/I groups (D1-3) at week 2, 4 and 8.

DISCUSSION : Icariin was able to exert beneficial effect on preventing SAON in rabbits with inhibition of both intravascular thrombosis and extravascular lipid deposition [3]. In vitro studies demonstrated that icariin stimulates proliferation of rat bone marrow stromal cells, enhances the osteogenic differentiation of marrow stromal cells and osteoblasts by promoting alkaline phosphatase activity, osteoalcin secretion and calcium deposition level [4].

CONCLUSIONS: This study proved icariin could enhance the osteogenesis in bone defect repair, but needs further research proof.

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