Wnt signaling in bone development and regeneration

Christine Hartmann

Institute of Experimental Musculoskeletal Medicine, Dept. of Bone and Skeletal Research, Medical Faculty, University of Münster, Germany.

INTRODUCTION & DISCUSSION: The Wnt family comprises 19 different members in vertebrates encoding secreted ligands that activate and signal through diverse pathways distinguishable by their intracellular components. The best-studied pathway is mediated by β-catenin encoded by the *Ctnnb1* gene. Signaling through a LRP5(6)/frizzled receptor complex results in stabilization of cytoplasmic β-catenin and its nuclear translocation where i acts in combination with members of the Tcf/Lef family as a transcriptional co-activator [1]. Over the past years multiple roles for Wnt-signaling in bone development have been reported: its involvement in lineage-decision of skeletal precursor cells, differentiation of skeletal cells such as chondrocytes, osteoblasts and osteoclasts, in bone homeostasis and regeneration.

Chondrocytic differentiation appears to be the default pathway of skeletal precursor cells. In embryogenesis β-catenin is required to suppress the chondrogenic potential of osteoblast- and synovial joint lineage precursors, which are not yet committed [2-4]. Stabilization of β-catenin in osteoblast precursors during embryogensis promotes the expansion of precursors but at the same time blocks their maturation [4]. In addition, Wnt/β-catenin signaling in chondrocytes regulates the expression of the pro-osteoblastogenic factor *Indian hedgehog* [5]. During postnatal development genetic ablation of *Ctnnb1* in osteoblastic precursor cells results in a cell fate shift towards adipocytes [6], while inactivation in more differentiated osteoblasts results in increased osteoclastogenesis due to downregulation of the anti-osteoclastic factor *osteoprotegerin* (*opg*) and increased *Rankl* expression [7,8]. In addition, β-catenin plays a □□□□-autonomous role in osteoclasts [9-11]. Last but not least β-catenin activity in hypertrophic chondrocytes locally regulates osteoclastogenesis at the chondro-osseous front primarily via *Rankl* suppression [12] (Houben A and Hartmann C, unpublished).

Collagen 10a1-expressing, hypertrophic chondrocytes have been identified as a source for trabecular and endosteal osteoblasts [13,14]. Differentation these chondrocyte-derived osteoblasts requires β-catenin activity in hypertrophic chondrocytes (Houben A and Hartmann C, unpublished).

The Wnt/β-catenin pathway influences the *in vitro* differentiation potential of mesenchymal stem cells (MSCs) in a differentiation stage-dependent manner: pathway activation in uncommitted MSCs inhibits osteoblastic differentiation [15-18], while in osteoblastic committed MSCs its activation promotes differentiation but interferes with final maturation [19-21].

Bone regeneration during fracture healing recapitulates the key steps of embryonic bone development, but involves in addition an early inflammatory response [22]. Here again the Wnt/β-catenin pathway plays varying roles at the different stages of fracture healing [23-27].

Evolution of the mineralized animal skeletons: Formation of bone hydroxyapatite via amorphous Ca-carbonate and Ca-phosphate

WEG Müller and XH Wang

ERC Advanced Investigator Grant Research Group at the Institute for Physiological Chemistry, University Medical Center of the Johannes Gutenberg University Mainz, Duesbergweg 6, D-55128 Mainz, GERMANY (wmueller@uni-mainz.de)

INTRODUCTION: By learning from nature, our group introduced novel biomaterials which have the potential to be suitable for bone tissue engineering. Building on the established facts that all metazoan organisms evolved from a common ancestor, the sponges (phylum: Porifera), as well as the necessity that all organisms larger than 2 cm need to be stabilized by a skeleton we investigated the strategies of mineralization, used by basal metazoans, for the fabrication of bone implants in human. *The evolutionary steps*: The phylogenetically oldest sponge taxa are the siliceous sponges, followed by the calcareous sponges; later, the corals, echinoderms, also having calcareous skeletons, evolved, and finally, the vertebrates appeared with their calcium phosphate/HA skeletons.

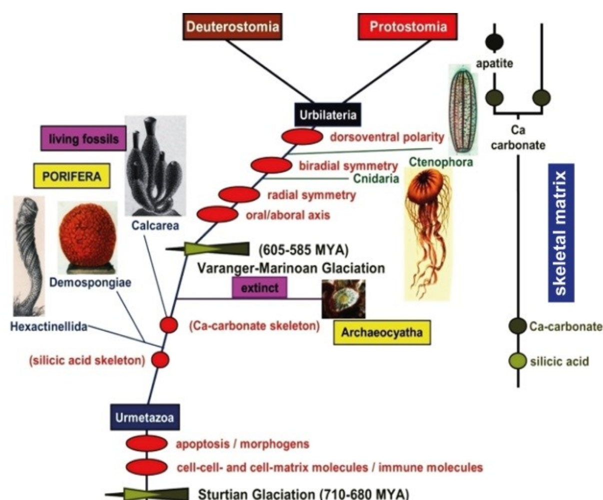


Fig. 1: Evolution of the skeletal mineral from the siliceous sponges, via the calcareous sponges to the Ca-phosphate containing skeletal animals and final to the hydroxyapatite-formed vertebrates.

THE EVOLUTIONARY STEPS TO VERTEBRATE BONE: Stage 1: Siliceous scaffold. Biosilica, a biocompatible, natural inorganic polymer that is formed in siliceous sponges to build up their inorganic skeleton, has been shown to be a morphogenetically active mineral and to induce mineralization *in vitro* and *in vivo*. **Stage 2: Amorphous Ca-carbonate (ACC) scaffold.** In human bone, amorphous calcium carbonate (ACC) is enzymatically formed as a precursor of the crystalline carbonated apatite/hydroxyapatite (HA). We describe that the metastable ACC phase can be stabilized by inorganic polyphosphate (polyP). Both *in vitro* and *in vivo* data revealed that ACC functions as a morphogenetically-active mineral (bio-seed). **Stage 3: Amorphous Ca-phosphate (ACP) scaffold.** PolyP allowed the synthesis of amorphous Ca-polyP hybrid particles with a size of 50 nm. Those Ca-polyP particles cause a strong upregulation of the expression of the genes, involved in bone formation and provide the ortho-phosphate substrate for bone mineralization.

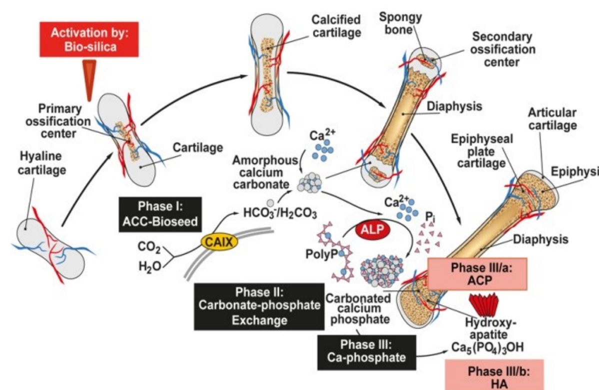


Fig. 2: Schematic presentation of the endochondral ossification and the proposed phases of bone mineral (hydroxyapatite/HA) deposition. After (Phase I) enzymatic formation of ACC (amorphous Ca-carbonate) and subsequent carbonate-phosphate exchange to ACP (amorphous Ca-phosphate) (Phase II) the ACP is transformed from the amorphous (Phase III/a) to the crystalline phase, the bone HA (Phase III/b).

The biology of heterotopic endochondral ossification and approaches to therapy

M Pacifici

The Children's Hospital of Philadelphia, Translational Research Program in Pediatric Orthopaedics, Philadelphia, Pennsylvania, USA

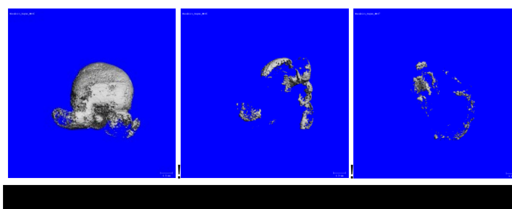
INTRODUCTION: Heterotopic ossification (HO) consists of formation and accumulation of endochondral bone at extraskeletal sites, causing major health problems and even premature death[1]. Fybrodysplasia Ossificans Progressiva (FOP) is a congenital and severe condition involving extensive and pervasive HO. FOP is caused by activating mutations in *ACVR1*, and HO is usually preceded –and likely promoted- by local flare-ups and inflammation. Trauma, invasive surgery, deep burns or protracted immobilization can induce non-congenital forms of HO. Anti-inflammatory drugs are often used as preventive HO treatments, but are not very effective[2]. Surgery is often used in non-congenital HO, but it can be dangerous and may actually trigger another HO cycle. Thus, there is urgent need for new and effective therapies. Recent studies from our research groups have identified synthetic retinoid agonists as novel, effective and seemingly safe treatments for both forms of HO.

METHODS: FOP was modelled in transgenic mice expressing *ACVR1 R206H* or *Q207D* mutants. Trauma models consisted of subcutaneous or intramuscular implantation of a scaffold containing the pro-chondrogenic protein rhBMP2. Drugs were given systemically by gavage, and extent of HO was assessed by µCT, histochemistry and histomorphometry.

RESULTS: By being an endochondral process, congenital or acquired HO initiates with recruitment of progenitor cells to the inflamed or injured site. The cells undergo chondrogenesis and lay down cartilage tissue that undergoes maturation and hypertrophy and is eventually replaced by endochondral bone. Thus, we reasoned that retinoid agonists could represent effective anti-HO agents because they have long been known to be anti-chondrogenic[3], thus blocking the initial phase of the HO process. Mice in which HO had been induced by injury or transgene expression were given synthetic retinoid agonists selective for the nuclear retinoic acid receptor alpha (RARα) or RARγ by daily gavage. Control companions were given vehicle. By 2 to 4 weeks post-HO induction, control mice had developed extensive HO at the affected sites. However, mice treated with RARγ agonists displayed much reduced HO levels, but RARα agonists were moderately effective[4]. In congenital models, HO was often extensive and hampered skeletal growth and limb mobility. These defects also were greatly ameliorated by drug treatment [5].

Because anti-inflammatory drugs are often used as prophylactic agents, we asked whether they would help or hinder the anti-HO action of retinoids. Thus, we tested a combination therapy in the subdermal mouse model of HO and found that prednisone enhanced the anti-HO action of retinoid agonists (Fig. 1), though it had some side effects.

Fig. 1: Representative µCT images of subdermal HO in control mice or those treated with retinoid



agonist alone or in combination with prednisone. Doses are in mg/kg/day over a 12 day treatment.

DISCUSSION & CONCLUSIONS: The data clearly show that retinoid agonists can strongly inhibit congenital and acquired forms of HO. The effectiveness of the drugs likely reflects the fact that they block chondrogenesis and canonical BMP Smad1/5/8 signaling, and may even dampen recruitment of inflammatory cells at the HO site. Their potency is moderately enhanced by co-treatment with anti-inflammatory drugs, thus expanding their therapeutic range.

ACKNOWLEDGEMENTS: Data presented here are from original studies and reports with colleagues at CHOP, the University of Pennsylvania and Regeneron. Financial support was received from the NIH and the US Department of Defense.

Endochondral ossification in regenerative medicine

Eric Farrell,

Department of Oral and Maxillofacial Surgery, Orthodontics and Special Dental Care, Erasmus MC, University Medical Centre, Rotterdam, The Netherlands

INTRODUCTION: Repair of critical sized bone defects that will not heal spontaneously is a costly endeavour for patient and society alike. Treatment of large bone defects still requires the surgical harvesting of bone from another anatomical location of the patient causing increased pain, multiple surgeries, longer hospital stays and as a result high associated costs. Clearly a less invasive approach would be desirable to treat such injuries. While the use of BMPs in certain circumstances is successful, complications resulting from off label use and generally high doses have necessitated the search for alternatives. Within the field of regenerative medicine there are many such options being researched, including various combinations of cells, materials and bioactives to induce defect repair or even de novo bone formation. We and others have demonstrated the ability of different cell types, in our case the mesenchymal stem cell, to initiate the process of endochondral ossification in vivo following various in vitro cell priming regimes. This is a very promising approach, since a relatively simple in vitro priming initiates an extremely complex series of cell processes in vivo, ultimately resulting in the formation of marrow containing bone both ectopically and orthotopically. However there are still several hurdles to overcome in order to bring such an approach to the clinic; scale up, reproducibility and reduced cost to name but a few. At present there is still much we do not understand about how the process of endochondral ossification occurs, particularly with regard to this regenerative medicine based approach using adult marrow stromal cells. I will present the approaches we are taking to better understand how such bone formation occurs and how we might scale-up this approach to generate larger quantities of bone in shorter amounts of time thereby reducing cost. Our research focuses on understanding the role of the host/recipient in the formation of new bone in order to properly engage the various body systems (vascular, immune etc) in the generation of new bone tissue. Advancing our knowledge of how new bone is formed will allow us to develop new therapies optimised to engage the patient’s own biology to accelerate and enhance repair. This should include considering the role of the immune system and disease states in MSC mediated endochondral ossification as well as specific extracellular matrix components and secreted factors critical for this process.al.

Mesenchymal stem cell heterogeneity: Diversity in the endogenous synovial stem cell compartment

Kavitha Sivasubramaniyan[1], Wendy J. Koevoet[2], Eric J. Farrell[3], Maria Sande[1], Jan A.N. Verhaar[1], Gerjo J.V.M. Osch[1, 2]

[1]Department of Orthopaedics, [2]Department of Otorhinolaryngology, [3]Department of Oral and Maxillofacial Surgery, Special Dental Care and Orthodontics, Erasmus MC, Rotterdam, The Netherlands

INTRODUCTION: Mesenchymal stem/stromal cells (MSCs) render promise as cell-based therapies for articular cartilage repair. Superiority of synovium as a potential source of MSCs for cartilage repair has been demonstrated, but the cellular heterogeneity associated with endogenous synovial MSCs is not yet clearly understood. In our study, we define distinct endogenous human synovial MSC subsets that differ in their immunophenotype, function and anatomical localization.

METHODS: Freshly isolated cells from synovium of 9 patients undergoing total knee replacement were stained with a panel of markers and analyzed on a FACS canto II flow cytometer or sorted on a FACS Jazz cell sorter. The sorted cells were cultured, phenotypically characterized and subjected to chondrogenic differentiation. The anatomical localization of the different MSC subsets in the synovium was verified by immunohistochemistry.

RESULTS: Flow cytometric analysis and soring demonstrated that a combination of CD45, CD31, CD73 and CD90 can isolate two distinct MSC subsets in the primary synovium. These MSC subsets did not express CD45 or CD31 but expressed CD73 and a sub-population of these CD73+ cells expressed CD90. CD45-CD31-CD73+CD90- MSCs were significantly more chondrogenic than CD45-CD31-CD73+CD90+ MSCs. Interestingly, CD73+CD90- and CD73+CD90+ MSCs had distinct anatomical localization; CD73+CD90- cells were found in the intimal layer lining the joint cavity whereas CD73+CD90+ cells were located in the sub-intimal layer, in the perivascular region. In addition, primary bone marrow MSC specific markers including CD271 and SUSD2 were expressed only in the sub-intimal MSCs and not in the intimal MSCs. Preliminary studies suggest that the reduced chondrogenic ability of CD73+CD90+ cells could be reversed by the addition of BMP2, showing discrete chondrogenic factor requirement by distinct MSC subsets. This is linked to the differential receptor expression in these MSC subsets.

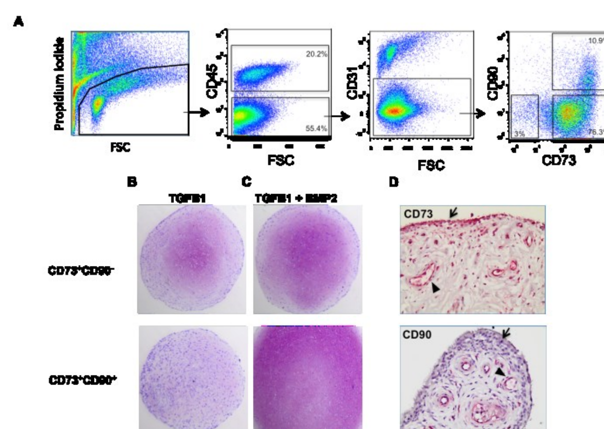


Fig. 1: *A) Synovial cells were stained with CD45, CD31, CD73 and CD90, gated on propidium iodide negative live cells, followed by gating on CD45-CD31- subset, then analyzed for expression of CD73 and CD90. Sort windows were set as shown in the CD73 vs CD90 plot and cells were sorted with BD FACS Jazz. B &C) After FACS sorting, cells were cultured and subjected to chondrogenic differentiation. (Thionin staining). D) Both CD73 and CD90 are expressed on perivascular MSCs (marked by arrowhead). MSCs in the lining intimal layer (marked by arrow) expressed CD73 and are negative for CD90.*

DISCUSSION & CONCLUSIONS: In summary, we introduce markers which can isolate distinct MSC subpopulations in synovium. CD73+CD90- MSCs in the intimal layer are adjacent to the cartilage in anatomical localization while CD73+CD90+ MSCs are relatively away from the cartilage. However, further studies are needed to utilize these cells for therapeutic purposes, as little information exists about their participation in cartilage repair *in vivo*.

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Fate of dental epithelial stem cells *in vivo* injected in the mouse incisor

G Orsini[1], P Pagella[2], L Jimenez-Rojo[2], M Procaccini[1], A Putignano[1], TA Mitsiadis[2]

[1] *Department of Clinical Sciences and Stomatology, Polytechnic University of Marche, Ancona, Italy.* [2] *Orofacial Development and Regeneration, Institute of Oral Biology, University of Zurich, Zurich, CH*

INTRODUCTION: The continuously erupting rodent incisor represents a unique organ system for studying the cell biology of odontogenesis. The posterior area of the incisor is characterized by the presence of cervical loops (CLs) in which the labial side contains dental epithelial stem cells (DESCs), which are able to form all the dental epithelial cell populations. Recently, we have developed a useful method for the *in vivo* administration of DESCs in the mouse CL area [l]. The aim of the present work is to test whether this newly developed method can be a suitable model to monitoring stem cells behaviour *in vivo*, by following their fate at different time points after administration.

METHODS: DESCs encoding Green Fluorescent Protein (GFP) were administered in the incisor CLs area of immunocompromised RAG1-/-mice at 8–12 weeks of age, using the “bone window technique” [l]. The mice were sacrificed after 7, 12, 30 and 45 days and hemimandibles dissected, decalcified and embedded in paraffin. The specimens were further processed for immunohistochemistry and immunofluorescence analyses and observations were performed under light microscopy and confocal electron microscopy.

RESULTS: We have applied an experimental model to locally administer DESCs encoding GFP. The system consists of drilling a window in the alveolar bone overlying the apex of the mouse incisor, allowing local injection with relatively large concentrations of DESCs into the apical cervical loop. Immunofluorescence and immunohistochemical results demonstrated that the bone window technique allows the administration of DESCs that can be traced not only in situ (bone hole), but also at a certain distance from the site of administration, within the dental epithelial lineages. Indeed, GFP positivity was observed in the different dental epithelial layers such as ameloblasts and stratum intermedium, at all the different time points.

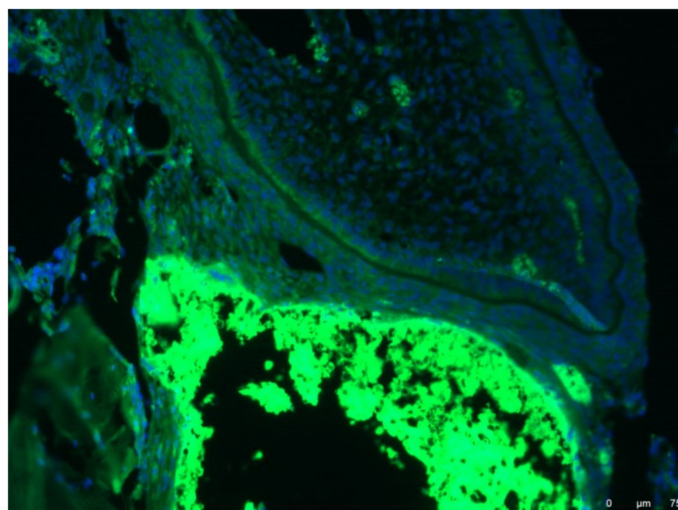

Fig. 1: drilled hole overlying the mouse incisor cervical loop (CL).

DISCUSSION & CONCLUSIONS:

This newly described approach has been demonstrated to be useful to trace the *in vivo* fate of DESCs after their administration. DESCs have shown to have an integration capacity within the epithelial dental lineages, giving rise to the different epithelial layers of the incisor. These findings contribute to the knowledge that epithelial stem cells display remarkable differentiation potential and can acquire epithelial lineage *in vivo*. Current and future studies will be driven to further explore whether dental epithelial stem cells of the cervical loop are limited in giving rise to distinct dental epithelial cell populations, or whether they can multilineage differentiation potential. These facts could contribute to establish innovative treatment protocols after traumatic or pathological injuries.

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gene expression profiling shows the desregulation of multiple hormone signaling in osteoporosis that could be therapeutically reversed by the treatment of strontium gluconate

J Li[1,2], **YH Liang**[1], WJ Peng[1], TH Chen[1], ML Zhou[1], and GQ Zhou[1]*

[1] *The Centre for Anti-ageing and Regenerative Medicine, Shenzhen University, Shenzhen, China,* [2] *Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, Shenzhen University, Shenzhen, China*

Introduction: Bone loss can be the result of various factors and causes including the imbalance of multiple hormones such as estrogen, glucocorticoid, parathyroid hormones, Vitamine D and leptin[1]. However, the interactive roles of these hormones have been poorly understood on bone tissues and cells. Stontium is well known to promote bone formation by stimulating osteogenesis of osteoprogenitors through influencing multiple cytoking and growth factor-mediated signaling pathways. Its role in regulating hormone signaling, however, has not been documented. To this end, this study reports our recent findings in gene expression profiling analysis of osteoporotic bone tissues induced by the overdose of glucocorticoid with or without oral administration of strontium gluconate (GluSr).

Subjects and Methods: A total of 16 sprague-dawley rats, aged between 7-8 weeks, were induced for GIOP by subcutaneous injection of glucocorticoid, 5.0mg/kg, 3 times/week. At the same time, half of these rats were treated with GluSr for oral administration, 400mg/kg.d. An additional 8 rats were left intact as control group. After treating 12 weeks, the microarchitecture of the trabecular bone from distal femur and proximal tibiae was analyzed with micro-CT scanner. Compact bone of long bone was cut into small pieces for RNA extraction. Total RNA sample was quantified and Agilent Array platform was employed for microarray analysis.

Results: Images of micro-CT show that trabecular bone microstructure in the distal femur and proximal tibiae of GIOP rats become less dense and more porous than GIOP rats treated with GluSr and control rats (Fig.1A-C). The BMD value of distal femur of GC, GC+GluSr, and CTRL

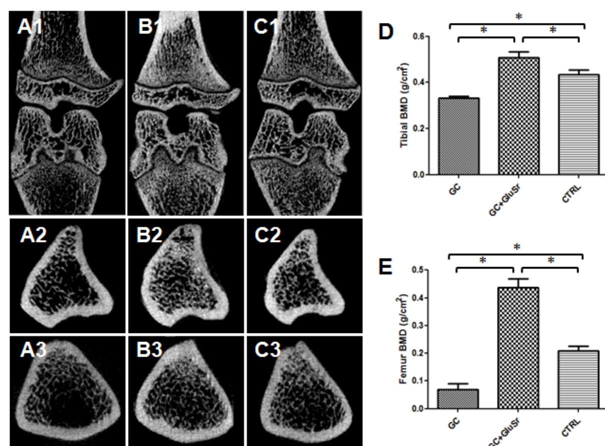

Fig. 1. micro-CT features and the bone mineral density of the tibia and femur of osteoporotic rats with or without GluSr treatment in comparison with healthy controls. Ultrastructure of bone of GC (A), GC+GluSr (B), and CTRL (C) were observed in coronal (A1-C1), transverse of distal femur (A2-C2) and proximal tibiae (A3-C3). (D) BMD of proximal tibiae. (E) BMD of distal femur.

groups were 0.09±0.01, 0.44±0.04, 0.21±0.03 g/cm^2, and the BMD value of proximal tibiae were 0.33±0.02, 0.51 ± 0.04, 0.43 ± 0.04 g/cm2, respectively (Fig.1D). Interestingly, after the GIOP rats treated with GluSr, the BMD value striking increased and even significantly higher than CTRL groups. The distinguishable gene expression among samples of hormones-related signaling pathways were listed as figure. 2A-B. A total of 30 genes were changed with up or down regulation, which are highly relevant to glucocorticoid receptor (GR), estrogen receptor (ESR), parathyroid (PTH), leptin, Vitamin D receptor (VDR), Ca_2^+ signaling pathways.

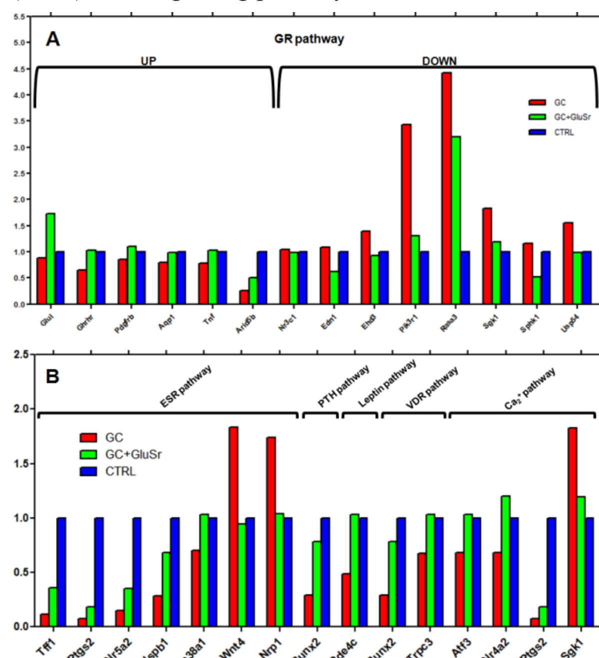

Fig. 2. A portion of differentially expressed genes in glucocoid-induced rats (red) , treated with GluSr (green) and controls (blue), grouped in GR, ESR, PTH, Leptin, VDR, and Ca_2^+ pathways.

Dissussion and Conclusions: Hormones play a critical role in regulating mineral metabolism and bone mass. Our study provides preliminary evidences that various hormone signaling are interactively influenced in bone tissue and cells, particularly osteoblasts. Furthermore, this is the first study to show that hormone signaling pathways are regulated or even reversed by the anti-osteoporosis Sr-containing reagent, probably implicating its potential value of Prophylactic intervention against chronic osteoporotic conditions. Further work on revealing the function of particular genes newly identified is undergoing.

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Mesenchymal stem cells: where is the stem..?

B. Péault

Centre for Regenerative Medicine and Cardiovascular Science Centre, University of Edinburgh, UK and Orthopaedic Hospital Research Center and Broad Stem Cell Research Center, University of California, Los Angeles, USA

Mesenchymal stem cells – MSCs – have been very popular among cell therapists and tissue engineers, as shown by the use of MSCs in over 500 clinical trials. This success is justified by the diverse positive contributions exerted by MSCs toward organ repair as tissue progenitors, pro-angiogenic and immunosuppressive cells and supportive niche cells for lineage-committed stem cells. Mesenchymal stem cells are also, importantly, remarkably easy to derive and expand since MSC extraction is a mere primary culture of unselected dissociated cells. Moreover, MSCs can be grown from virtually any vascularized organ, leaving a choice of convenient, abundant and dispensable sources of these cells such as adult adipose tissue and fetal appendages at birth.

On the negative side, indirect selection by adherence and proliferation in culture has long obscured the biologic characteristics of innate mesenchymal stem cells. MSCs being by essence long-term cultured cells, the native embryonic origin, identity, lineage affiliation, tissue distribution, frequency and – importantly – actual role of these cells in normal tissue homeostasis and repair remained unknown decades after the initial discovery of MSCs. In the past ten years, the very identity of native mesenchymal stem cells has been progressively uncovered, revealing a perivascular origin for these elusive regenerative cells. We will review and discuss experimental evidence demonstrating that MSCs isolated from distinct organs share blood vessel associated ancestors.

The prospective identification of innate MSCs now opens the possibility of using highly purified – and, in some instances, uncultured – precisely characterized perivascular cells for cell therapies, in place of their heterogeneous, culture selected conventional progeny. We will also review the medical use of customary, in vitro derived mesenchymal stem cells, and put in perspective recent attempts and future plans to achieve tissue regeneration using their perivascular native counterparts.

Chondral cell differentiation

Brian Johnstone, PhD

Department of Orthopaedics and Rehabilitation, Oregon Health & Science University, OR, USA

The variation in chondrogenic capacity among adult human-derived stem/progenitor cell populations is an important consideration in tissue engineering. Beyond the obvious outcome that poorly chondrogenic cells make little extracellular matrix, our latest work suggests variation in intrinsic chondrogenicity will influence experimental results. For example, the baseline chondrogenic capacity influences a stem cell's response to a physiologic low oxygen environment (physioxia) in 3D culture. Biologic replicate of human bone marrow-derived stem cells (MSCs) and articular cartilage-derived progenitor cells (ACPs) were categorized as high- or low-GAG based on a threshold defined by their total GAG production relative to that of healthy human articular chondrocytes in the same 3D pellet conditions at 20% oxygen (hyperoxia). While physioxic culture increased GAG production across all MSC preps and the majority of ACP clones, physioxia was of greater benefit to biologic replicates that exhibited very low GAG production at baseline in hyperoxia, driving a greater fold change than for clones that started with high GAG production and chondrogenic capacity in hyperoxia. However, even with this significantly higher fold-induction, the pellets of low-GAG cell preparations of both cell types were still poorly chondrogenic in comparison with matched high-GAG pellets. Furthermore, MSCs and ACPs of high chondrogenicity upregulate protein expression of the articular chondrocyte phenotype and downregulate the hypertrophic phenotype in physioxia; however, only ACPs consistently attenuate hypertrophic markers at the tissue level in the physioxia while MSCs retain high type X collagen protein regardless of oxygen tension. Thus, ACPs may overcome the historical challenges of MSC hypertrophy in tissue engineering applications.

We then developed conditions to create larger, scaffold-free cartilage 3D implants from various cell types, using pre-selected cell preparations of high chondrogenicity. Multiple biological replicates of bone marrow-derived MSCs, articular chondrocytes (ACs) and ACPs derived from healthy human adult articular cartilage, were guided toward self-organization through cell condensation. Discoid tissue was produced from all three cell sources. Regardless of oxygen tension and consistent with pellet culture, MSCs produced neocartilage tissue of a hypertrophic phenotype. In comparison with culture in hyperoxia, AC neocartilage cultured at physioxia exhibited a significant increase in chondrogenic gene expression, proteoglycan production, and mechanical properties with a concomitant decrease in collagen content. ACP-derived neocartilage produced tissue with significantly enhanced mechanical properties and collagen content relative to HAC-derived neocartilage. Interestingly, they had much lower differential responses between physioxia and hyperoxia. Regardless of oxygen tension, neocartilage from ACPs exhibited anisotropic organization of native cartilage with respect to a pericellular matrix when compared with AC-derived neocartilage; however, only ACs produced abundant surface-localized lubricin. To date, few methods utilizing adult human cells in scaffold-free approaches to tissue engineering have been reported. Guiding human-derived cells toward condensation and subsequent culture in physioxia promoted the articular cartilage tissue phenotype for ACs and ACPs, but less so for MSCs. The advantage of ACPs over ACs is that they can be cloned and are highly expandable while retaining chondrogenicity. Ultimately, the ability to generate tissues of the articular cartilage phenotype utilizing a scaffold-free approach from a single cell origin may provide the functional properties and therapeutic level of neocartilage destined for autologous repair.

Autogenic mesenchymal stromal cells (MSC) are superior to allogenic MSC in regeneration of large bone defects

AE Rapp[1], R Bindl[1], M Rojweski[2,3], J Kemmler[1], H Schrezenmeier[2,3], I Müller[4], A Ignatius[1]

[1]Institute of Orthopaedic Research and Biomechanics, University of Ulm, Ulm, Germany; [2]Institute of Clinical Transfusion Medicine and Immunogenetics, German Red Cross Blood Transfusion Service, Baden Wuerttemberg-Hessen, Ulm, Germany; [3]Institute of Transfusion Medicine, University of Ulm, Germany; [4]Clinic for Paediatric Haematology and Oncology, Bone Marrow Transplantation Unit, University Medical Centre Hamburg-Eppendorf, Germany

INTRODUCTION: Mesenchymal stem cells (MSC) are promising tools for the regeneration of large bone defect. While the benefit of autologous MSC on for bone regeneration is widely acknowledged, the efficacy of allogeneic MSC has been poorly investigated so far and available studies report inhomogeneous results[1-3]. As the use of allogenic MSC would overcome the limited availability of autogenic cells, further investigations on the use of allogeneic SMC are necessary. This study compared the potential of allogeneic and autologous human MSC (hMSC) to regenerate large bone defects in an animal model that mimics the human immune system.

METHODS: In humanized NOD/scid-IL2$R_y^{c-/-}$ mice, which had established a human immune system after engraftment with human hematopoietic stem cells, a 1 mm defect, stabilized by an external fixator, was created in the right femur. The defect was left untreated or filled with either allogeneic or autogenic hMSC in a collagen type-1 matrix. The animals were killed after 3, 10 or 35 days. The healing outcome was analysed by µCT, histmorphometry and immunohistochemistry for human β2-microglobulin, human CD8, PECAM (CD31), Runx2 and Osteocalcin.

RESULTS: Staining for human β2-microglobulin confirmed the presence of transplanted human cells in the defect region. Newly formed bone in the defect region in both, allogenic and autogenic treated mice did not stain positive for the human marker. µCT analysis after 35 days showed a significantly higher bone volume in the defect region of mice that received autologous MSC compared to allogeneic MSC (+132%) or untreated defects (+205%). Histomorphometry confirmed this finding. Consequently, staining for osteogenic markers on day 10 (Runx2) and 35 (osteocalcin) was more intense in mice treated with autologous MSC. To detect adverse immune reactions, we stained for CD8+ T-cells. 3 days after surgery, CD8+ cells were detected near the implant in mice that received allogeneic MSC, while positive cells were absent in mice with autologous treatment. The same observation was made on day 10. Staining for PECAM revealed newly formed vessels in the surrounding of the collagen gels in both treatment groups with no obvious differences. On day 35 however, more stained structures were found in mice treated with autologous MSC compared to allogeneic MSC, indicating increased angiogenesis. Furthermore the distribution of the vessel-like structures was different, in autogenic treated mice, the vessels were distribute throughout the defect region, while they were at the margins of the defect region in allogeneic treated mice.

DISCUSSION & CONCLUSIONS: Our results indicate a higher efficiency of autogenic hMSC for bone regeneration compared to allogeneic hMSC, as treatment with autogenic hMSC led to a significantly higher bone formation compared to empty defects or defects treated with allogeneic hMSC. We found no signs of a strong adverse immune reaction in animals that received allogeneic hMSC; albeit CD8+ cells were detected. There are hints that T cells and interferon-gamma might be associated with inhibition of bone formation in allogeneic settings[4]; however this has to be investigated further. It is still unclear, how the implanted cells contribute to bone regeneration. We found signs for enhanced angiogenesis and osteogenesis after autologous treatment. Together with the absence of bone stained positive for human β2-microglobulin, this indicates an indirect action of the implanted cells via trophic factors rather than a direct contribution. In conclusion, our results demonstrate a superior efficacy of autogenic hMSC treatment compared to allogeneic hMSC in supporting bone healing.

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The osteogenic differentiation of mesenchymal stromal cells is enhanced by the BMP2 variant L51P in the presence of intervertebral disc-derived cells

A Tekari[1], R May[1], DA Frauchiger[1], HJ Sebald[2], LM Benneker[3], B Gantenbein[1]

[1] *Tissue and Organ Mechanobiology, Institute for Surgical Technology & Biomechanics, University of Bern, CH.* [2] *The Spine Center, Thun, CH.* [3] *Department of Orthopaedic Surgery & Traumatology, University of Bern, Inselspital, CH.*

INTRODUCTION: Discectomy and spinal fusion represents the gold standard treatment for spinal disorder to relieve pain. Fusion can be hindered, however, for yet unknown reasons that lead to non-union with pseudo-arthrosis. We previously showed that intervertebral disc (IVD)-derived cells hinder the ossification process of human bone marrow-derived stromal cells (hMSC) [1]. Within this study, we hypothesized that BMP-antagonists secreted by IVD cells are the responsible factors for such inhibition and that this can be reversed by addition of L51P. L51P is an engineered BMP2 variant [2] that has been recently demonstrated to be a generic antagonist of a variety of BMP-inhibitors that controls osteoinduction of bone [3,4].

METHODS: The experimental work was ethically approved and written consent of patients was obtained. hMSCs, primary nucleus pulposus (NPC) and annulus fibrosus cells (AFC) were obtained from patients undergoing spinal surgery, isolated and expanded in monolayer cultures up to passage 3. IVD cells were seeded in 1.2% alginate beads (4Mio/mL) and separated by culture inserts from hMSCs in a co-culture (CC) set-up. The allogenic CCs were paired in 11 repeated experiments. MSCs were kept in 1: osteogenic medium (positive control, ±alginate beads), 2: osteogenic medium+NPC (±100ng/mL L51P), 3: osteogenic medium+AFC (±100ng/mL L51P) and 4: basal medium (negative control) for 21 days. Relative gene expression of bone-related markers was quantified with qPCR, and histological staining for calcium deposition and Alkaline Phosphatase (ALP) assay were performed. The endogenous expression of three common BMP-antagonists in IVD cells (passage 1) was evaluated by qPCR, immunohistochemistry and flow cytometry.

RESULTS: Osteogenesis of hMSCs was hindered as shown by reduced alizarin red staining in the presence of NPC and AFC. However, L51P added to CCs of hMSCs with either NPC or AFC induced mineralization by blocking the activity of the IVD cell's secreted BMP-antagonists (Fig. 1).

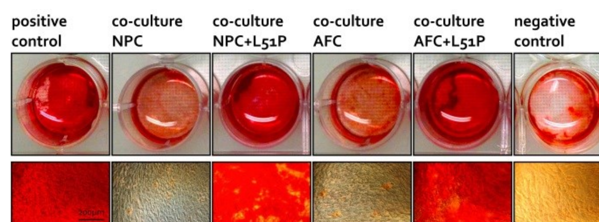

Fig. 1: Osteogenic differentiation of hMSC is inhibited in CC with NPC and AFC as shown macroscopically (top row) and microscopically (bottom row) at 10x magnification. L51P blocks the inhibitory effect of IVD cells in CC of NPC or AFC and restores the osteogenic differentiation.

It was noted that L51P caused a general reduction in ALP activity in all experimental groups. ALP activity was significantly up-regulated in positive control, and in CCAFC+L51P relative to negative control, suggesting osteogenesis in these groups. Gene expression analysis confirmed these observations. IVD cells expressed BMP-antagonists, namely noggin, gremlin and chordin as measured by transcript and protein levels.

DISCUSSION & CONCLUSIONS: The IVD cells secrete BMP-antagonists, which are responsible for bone non-union. The concept of antagonizing endogenous BMP inhibitors with L51P may represent a promising clinical option to augment and accelerate bone regeneration during

ACKNOWLEDGEMENTS: This study was supported by funds from the Lindenhof Foundation "Funds Research & Teaching" (project #15-05-F), by Hansjörg Wyss Medical and the Swiss National Science Foundation (project #310030_153411). Eva Roth assisted in the biochemical assays.

Growth environments and cues for engineering bone tissue *in vitro*

El Haj AJ

Institute of Science and Technology in Medicine, Guy Hilton Research Centre, Keele University UK

INTRODUCTION: Engineering bone tissue for use in Orthopaedics poses multiple challenges. Providing the appropriate growth environment which will allow complex tissues such as bone to grow is one of these challenges. There are multiple design factors which must be considered in order to generate in vitro a functional tissue for replacement surgery in the clinic. Complex bioreactors have been designed which allow for different stress regimes such as compressive, shear and rotational forces to be applied to 3D engineered constructs but ultimately we need simplified prototypes which can be standardized for scale up. Combined with biological directional cues, we aim to provide the right conditions to grow bone tissue ex vivo as models or for implantation for orthopaedic repair.

METHODS: Human bone marrow derived MSCs are grown on multiple material substrates and cultured either *in vitro* within a well plate or placed within an *ex vivo* chick femur epiphyseal defect. A hydrostatic stimulation regime has been developed with a pressure range of 0-280 KPa at a frequency of 1 Hz for 1 hour daily. Examples of other bioreactors such as the magnetic force bioreactor have been tested for comparison on different configurations. Osteogenic differentiation *in vitro* is identified by increased bone marker expression and amplified mineralisation. Biological cues such as Wnt proteins and growth factors have been patterned to enable spatial differentiation cues within the bioreactor environment.

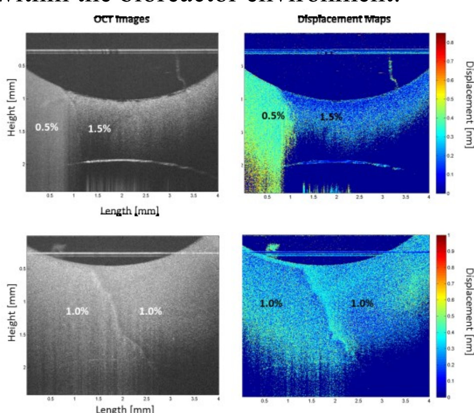

Fig. 1: Monitoring hydrogels during mechanical stimulation in the hydrostatic force bioreactor. Relative displacement maps were generated by elastography algorithms. Colour represents displacement.

RESULTS: Our results have demonstrated the interplay between the biological cues and the mechanical environment. Creating mechanical environments which can be monitored [1] combined with biological cues such as Wnt in spatial orientations[2] can provide new bone tissue morphogenesis. Further examples of the different models and growth environments will be presented.

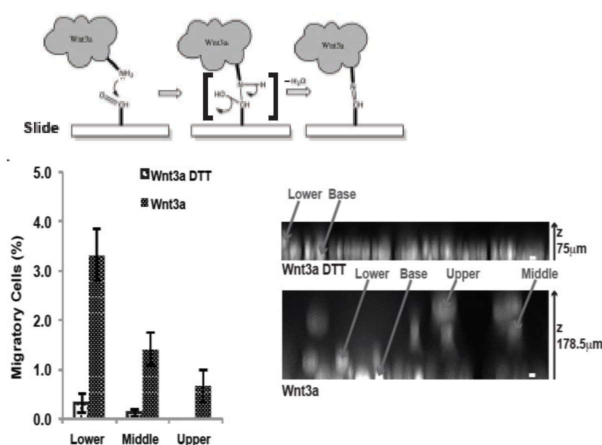

*Fig 2 : hMSCs cultured on the active Wnt3A surfaces coated with collagen gel were stained for DAPI to determine cell number. Gels were imaged as z-stacks and the number of cells in each layer was counted: lower (up to 72µm / 46% gel), middle (up to 132µm, 85% gel) and upper layers (up to 179µm, 100% gel). Values represent average cell counts, error bars represent SEM, * denotes $p<0.05$.*

DISCUSSION & CONCLUSIONS: Mimicking the biological niche conditions involved in tissue growth, repair or development to regenerate tissues requires complex engineering of biological cues in spatial and time directed manner. Engineering these niche environments in 3D requires novel designs of simple growth bioreactors for standardised production. We are aiming to define protocols which can be used as biological models or as repair strategies for regenerative medicine.

ACKNOWLEDGEMENTS : BioDesign EUFP7-NMP.20102.3-1;262948; MRC UK Regenerative Medicine Programme- Niche and Delivery Hubs

Additive Biomanufacturing – The rationale to change the current paradigm by changing the question from "what can we do with this method?" to "how can we change this technology platform to achieve what we need for Skeletal Tissue Engineering & Regenerative Medicine".

Dietmar W Hutmacher

| QUT Chair in Regenerative Medicine| Institute of Health and Biomedical Innovation | Queensland University of Technology | 60 Musk Avenue, Kelvin Grove QLD 4059 |

Nature provides an outstanding blueprint for scientists, engineers and architects who seek to learn from the natural geometries and structures formed throughout millions of years of iterations. World-renowned Spanish architect Antoni Gaudi is among those prodigious innovators who pursued inspiration in the natural world and achieved an unprecedented biomimetic design approach, which revolutionized the way in which architecture was understood in his time. Nature uses fibre reinforcement to transform weak structures into outstandingly mechanically robust ones and hard and soft structural natural composites discovered in biology have spurred motivation for the design of advanced synthetic materials. Many examples of bio-inspired hard materials based on natures design of bone, dentine, seashell nacre can be found in the literature, however far less attention has been devoted to soft tissues such as articular cartilage, breast and heart valves as well as ocular tissues formed by stiff and strong collagen fibres intertwined within a weak hydrogel matrix of proteoglycans.

The combination of a bioinspired & biomimetic strategy translating natures approach into soft network composites has remained largely unexplored in science, technology, engineering and mathematics (STEM) disciplines. *By bringing this novel natural design perspective of fibre reinforcement into the field of biomaterials science & tissue engineering (BS&TE) the talk will deliver fundamental and applied research* concepts in cross-disciplinary areas of regenerative medicine, bioengineering, advanced manufacturing, materials science, biology and biomechanics; and delivering innovations in design & fabrication of soft and hard tissue replacement materials for tissue engineering applications with a focus on Skeletal Tissue Engineering & Regenerative Medicine

Water-based polyurethane 3D printed scaffolds with controlled release function for customized osteochondral tissue engineering

K.-C. Hung[1], C.-S. Tseng[2], L.-G. Dai[3], S.-h. Hsu[1,4]

[1] *Institute of Polymer Science and Engineering, National Taiwan University, Taiwan, R.O.C.*
[2] *Department of Mechanical Engineering, National Central University, Taiwan, R.O.C.*
[3] *Department of Orthopedics, Shuang Ho Hospital, Taipei Medical University, Taiwan, R.O.C.*
[4] *Center of Tissue Engineering and 3D Printing, National Taiwan University, Taiwan, R.O.C.*

INTRODUCTION: Conventional three-dimensional (3D) printing may not readily incorporate bioactive ingredients for controlled release because the process often involves the use of heat, organic solvent, or crosslinkers that reduce the bioactivity of the ingredients.[1] Here we develop customized scaffolds with cell aggregation capacity and controlled release function based on polyurethane (PU) elastomer and natural polymer. We show that the waterborne process can retain the bioactivity of encapsulated growth factor or drug. Self-clustering of mesenchymal stem cells (MSCs) within the 3D printed scaffolds is followed by the tissue formation as the embedded bioactive compound is timely released from the scaffolds without giving any exogenous induction medium. We further prove that scaffolds printed from the ink are effective in regenerating rabbit cartilage defect. The platform may be modified for bone tissue engineering.

METHODS: The biodegradable PU elastomers were synthesized from a water-based process. The soft segment was poly(ε-polycaprolactone) diol and polyethylene butylene adipate diol. The hard segment was isophorone diisocyanate, 2,2-bis(hydroxymethyl) propionic acid and ethylenediamine. 3D scaffolds were printed from a feed containing PU, hyaluronan (HA), and Y compound. The expression levels of chondrogenic, hypertrophic, and fibrotic marker genes for MSCs grown in the scaffolds were analyzed by qRT-PCR. The contents of glycosaminoglycan were determined by dimethylmethylene blue assay. The capacity for chondral regeneration of the scaffolds was evaluated in a rabbit chondral defect model.

RESULTS: Water-based 3D printing of compliant and bioactive tissue engineering scaffolds is achieved by a growth factor-free process from PU dispersion mixed with HA and Y compound. These scaffolds promote the self-aggregation of MSCs and, with timely release of the bioactive ingredients, induce the chondrogenic differentiation of MSCs and produce matrix for cartilage repair. Moreover, the growth factor-free controlled release design may prevent cartilage hypertrophy. Rabbit knee implantation supports the potential of the novel 3D printing scaffolds in cartilage regeneration (Fig. 1).

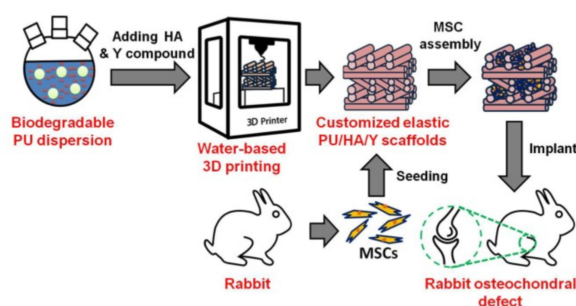

Fig. 1: Flow chart for the fabrication of PU/HA/Y scaffolds and histological examination of regenerated cartilage.

DISCUSSION & CONCLUSIONS: Compliant and bioactive scaffolds were printed from the water-based ink containing PU, natural polymer, and soluble factor. MSCs seeded in the scaffolds were self-assembled into MSC aggregates and underwent chondrogenesis effectively. This unique platform may have potential in customized tissue engineering.

ACKNOWLEDGEMENTS: This work was supported by grants from the Ministry of Science and Technology, Taiwan, R.O.C.

Introduction of a new biodegradable composite implant for bone repair: poly(trimethylene carbonate)-hydroxyapatite made by stereolithography

Guillaume O.[1], Geven M.[2], Eberli U.[1], Zeiter S.[1], Grijpma D.[2], Alini M.[1] and Eglin D.[1]

[1]-AO Research Institute, AO Foundation, Davos, CH.
[2]-Department of Biomaterials Science and Technology, University of Twente, Enschede, NL.

INTRODUCTION: Stereolithographic process of scaffolds with controlled internal structure and degradation, and with incorporation of osteoinductive ceramic has seldom been achieved. Poly(trimethylene carbonate) (PTMC) based resin loaded with nano-hydroxyapatite (nHA) were recently produced to create implants using stereolithography (SLA)[1]. In this study, films and scaffolds were fabricated and assessed for their osteopromotive effect *in vitro* and *in vivo*.

METHODS: PTMC-methacrylate resin mixed with nHA at 0, 20 and 40% w/w were prepared and films and scaffolds were produced using SLA. Human bone marrow stromal cells (hMSCs) were seeded on films and cultivated for 4 weeks in osteogenic media and differentiation was assessed by quantification of alkaline phosphatase activity (ALP) and by mineral deposition using alizarin red staining (ARS). Subsequently, *in vivo* experiment was conducted by creating 4 calvarial defects of 6 mm Ø on 8 rabbits (agreement 19A/2015). After cleaning and washing, the defects were either left empty (control group) or PTMC and PTMC/nHA at 20 and 40% w/w scaffolds (Ø 6 mm x H 3.5 mm) were inserted in the cavities. Following 6 weeks of implantation, osseointegration was assessed by X-ray scan and by histology (Giemsa-Eosin staining).

RESULTS: *In vitro* hMSCs were able to attach and to proliferate similarly in all biomaterials (Fig 1A) and expressed high ALP and ARS when cultivated on PTMC 20 and PTMC 40 (Fig 1B and C). Following implantation, the incorporation of 40% w/w of nHA in PTMC significantly increased the osseointegration of the implant compared to PTMC 20% (quantified at 70% *vs* 45% respectively).

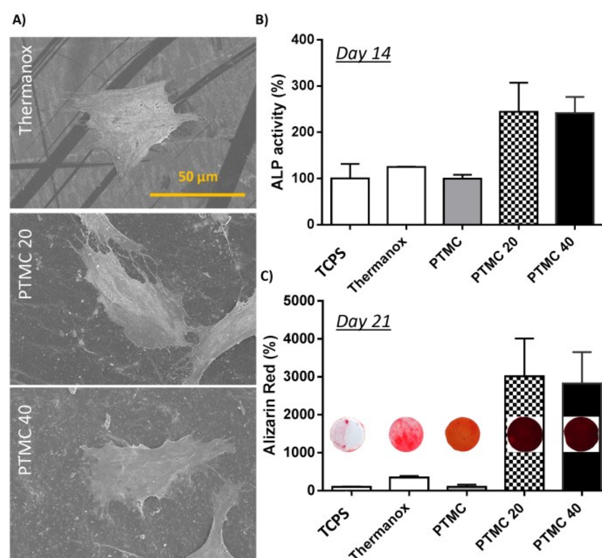

Fig. 1: Illustration of hMSCs adhesion on the biomaterial surfaces (SEM observation, A) and investigation of osteogenic differentiation using early marker (ALP activity, B) and late marker (AR staining, C) analysis after 14 and 21 days of hMSCs culture on the different films. ALP and ARS are expressed in percentage compared to the control TCPS (100%).

DISCUSSION & CONCLUSIONS:

For the first time, we reported the fabrication of PTMC/nHA-based SLA implants for bone repair. This composite biomaterial exhibited excellent biocompatibility and osteopromotive effect.

ACKNOWLEDGEMENTS: NSFC-DG-RTD Joint Scheme (Project No. 51361130034) and the European Union's 7th Framework Program under grant agreement n° NMP3-SL-2013-604517.

Multi-scale mineralized collagen-polycaprolactone composites for craniofacial bone tissue engineering

D.W. Weisgerber[1], C. Flanagan[2], X. Ren[3], S.J. Hollister[2], J.C. Lee[3], M.B. Wheeler[1], B.A.C. Harley[1]

[1] University of Illinois at Urbana-Champaign, Urbana, IL, USA. [2] University of Michigan, Ann Arbor, MI, USA. 3 Greater Los Angeles VA Healthcare System, Los Angeles, CA, USA.

INTRODUCTION: Craniomaxillofacial (CMF) defects present unique, unmet challenges for the field of tissue engineering. Typically large in size and marked by significant bone loss, these defects are often treated via autogenous bone transplant. Biomaterials for CMF repair must balance considerations regarding mechanical competence and load bearing, the need to fit complex 3D defects unique to each patient, as well as bioactivity and biotransport to support cells within a large construct. We are developing a multi-scale biomaterial that is mechanically competent for large, load-bearing bone defects which also supports cell bioactivity and tissue biosynthesis. We have integrated mineralized collagen-GAG scaffold with micron-scale porosity into a mechanically-robust, polycaprolactone (PCL) frame with mm-scale porosity. We report the osteogenic nature of the collagen scaffold as well as the regenerative capacity of the multi-scale PCL-collagen composite via porcine mandible defect and rabbit calvarial defect models.

METHODS: PCL frames were fabricated by selective laser sintering of a powder precursor of polycaprolactone and 4 wt% hydroxyapatite [1]. Mineralized collagen scaffolds (and non-mineralized scaffold controls) were fabricated via lyophilization from precursor suspensions of collagen, GAG, and calcium phosphate [2]. Collagen-PCL composites were generated by infiltrating the suspension into the PCL frame prior to lyophilization. Scaffolds were seeded with hMSCs and cultured in vitro, with osteogenic differentiation evaluated via Western blot, RT-PCR, ELISA, and histology. Alternatively, collagen-PCL composites (vs. PCL or non-mineralized scaffold controls) were implanted in 10 mm dia. mandible defects in 6mo Yorkshire pigs (or rabbit calvarial defects [3]), with bone infiltration assessed via microCT and histology.

RESULTS: Mineralized collagen scaffolds instruct osteogenic MSC differentiation independent of use of conventional osteogenic media or supplemental BMP-2. The mineralized scaffold constitutively activates canonical (Smad1/5/8) and smad-independent (ERK1/2, Akt, p38 MAPK) BMP receptor signaling paths in hMSCs. PCL-collagen composites show a significant (up to 6.8 ± 0.4 MPa) increases in elastic modulus (6000-fold vs. scaffold only) and ultimate stress (vs. PCL frame) which can be specified by the PCL frame geometry. Incorporation of the PCL frame does not reduce the osteogenic capacity of the CGCaP scaffold. In vivo, the composite (without MSCs, BMP2) promotes porcine mandible repair (Fig. 1).

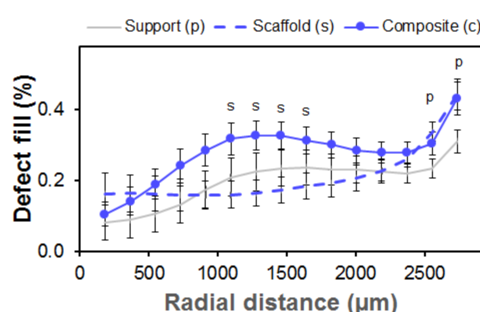

Fig. 1: Significantly increased radial bone infill (6 wks) in porcine mandible defect for collagen-PCL composite vs. PCL (p) and scaffold (s) alone.

DISCUSSION & CONCLUSIONS: We report a strategy to create a PCL-collagen composite that combines a mineralized collagen scaffold that promotes MSC osteogenesis in the absence of osteogenic supplementation with a mechanically-robust, patient-customizable PCL frame. Mineralized scaffolds show enhanced bone repair in a rabbit calvarial defect vs. non-mineralized scaffolds [3]. Here we show collagen-PCL composites show improved bone infill in a porcine mandible defect. Ongoing efforts are evaluating the efficacy of the composite in critically sized (25mm dia.; 10mm thick) mandible defect.

ACKNOWLEDGEMENTS: Funding provided by the AO Foundation (S-15-54H).

Engineered bone for the repair of large bone defects: The challenge of implanted MSC survival

Delphine Logeart-Avramoglou

Osteo-Articular Bioengineering and Bioimaging Laboratory, UMR7052 CNRS, Paris Diderot University, Paris, France

INTRODUCTION:
The repair of large bone defects due to trauma and to pathological bone withdrawal/resorption still represents a major challenge for orthopedic surgeons. Stem-cell-based-bone tissue engineering (TE) approaches are currently developed by combining osteoprogenitors (such as multipotent stromal cells derived from bone marrow (BMSC)) with a supporting substrate with the aim of obtaining new bone tissue. The efficacy of bone TE in experimental and clinical studies, although promising, remains inferior to that of autologous bone grafts. The underlying reasons for the limited success of TE constructs are not yet fully understood but, one of them may be the in vivo massive death of transplanted cells observed after engraftment into tissue constructs.
Delivered BMSC are believed to promote tissue repair through direct participation after differentiation and incorporation into new tissue and/or through the paracrine activity of the cells that modulates immune response, induces angiogenesis, or promotes wound repair. Whatever the mechanisms of action of BMSC in bone repair, the poor viability of the cells after administration remains a major impediment to their biological functionality.
Among the possible factors responsible for such massive cell death, the hostile environment that BMSC faced upon implantation is considered as a prime reason. When loaded on material constructs devoid of pre-existing vascular network, BMSCs encounter an ischemic environment with low oxygen tension and deprivation of nutrients and, consequently, a considerable bioenergetic challenge.
To meet this challenge, various approaches have been developed in order to sustain long term viability of the TE constructs up to the host vascular bed establishment. These strategies rely on the development of scaffolds that favour the cell viability or on approaches of BMSC preconditioning to alleviate the ischemia-mediated cell death.

The Influence of Mechanical Environment in Bone Healing

Vaida Glatt

Institute of Health and Biomedical Innovation at Queensland University of Technology, Brisbane, QLD, Australia

The management of bone defects and impaired fracture healing remains one of the most challenging clinical problems faced by orthopedists today. Several treatments exist to aid in the healing of large bone defects, including recombinant human bone morphogenetic protein-2 (BMP-2). Although BMP-2 has shown preclinical efficacy in animal models, the clinical effectiveness has been disappointing. The current practice of using extremely large amounts of BMP-2 has major concerns about the many possible side effects such as bone overgrowth in unwanted areas, bone resorption, implant dislodgment, with even cancer being reported[1].

Regeneration of bone requires a coordinated network of molecular signals, and is dependent upon the local mechanical environment playing a major role in the rate and success of healing. The mechanical environment itself is determined by the stiffness of the implant used to stabilize the fracture and weight-bearing, and as a result, if fixation is either too flexible or too rigid the healing might fail. In our previous studies we demonstrated that the local mechanical environment influenced the healing of 5 mm large bone defects in response to a standard dose of BMP-2 using a rat model[2]. Based on our preliminary experiments we hypothesized that the healing of large-segmental bone defects and fractures can be accelerated by the imposition of an appropriate mechanical environment. This concept arose based on evidence that flexible fixation stimulates endochondral bone formation. However, the same process would jeopardize bone consolidation by disrupting the formation of blood vessels of regenerating bone. Therefore, we proposed the regimen we named Reverse Dynamization (RD) where the defect is initially stabilized using a fixator with low axial stiffness, and subsequently increasing the fixator stiffness at the first signs of radio-opacity. In fact, by imposing RD, where the fixator stiffness was changed from low to high after 2 weeks, a time when bone was forming within the defect, this study demonstrated accelerated healing and remodeling through the modulation of the mechanical environment around the defect site[2].

Based on these observations, additional studies were performed using Reverse Dynamization in a rat model to extend it to a wider range of stiffnesses and BMP-2 concentrations to learn more about its scope and biology. The underlying hypothesis was that by using the appropriate stiffness parameters and timing, RD can enhance the healing of large segmental defects thereby minimizing the dose of BMP-2 required.

The results from this study showed that defect healing was influenced by the dose of BMP-2 suggesting that a lower dose of (5.5 g) BMP-2 was sufficient enough to enhance the healing of defects. Although the healing was slightly delayed, the quality of healed bone was equivalent compared to a high dose (11 g) of BMP-2. It also demonstrated that the mechanical environment plays a role when using a lower dose, as was evident from the presence of the radiopaque line at the end of treatment, which is a consequence of prolonged movements in the defect during the early stages of healing when lower stiffness fixators are used[3]. While further studies are essential, the results of this study indicate that the fixation stability could be used to maximise the regenerative capacity of bone healing while minimising the dose of BMP-2 required clinically.

Similar studies are being performed to test the effectiveness of Reverse Dynamization in a 1mm osteotomy rat model. Initial results showed superior healing outcomes when the RD regimen was used, and this was time dependent. Although additional studies will be required to confirm these findings, this data suggest that fracture healing could be accelerated through the manipulation of fixation stability, and it also introduces a potential clinical strategy to improve the healing outcome of unstable fractures, particularly for delayed non-unions through increased stabilization

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Direct use of freshly-isolated adipose-derived cells for fracture augmentation in a first-in-man phase I clinical trial

Franziska Saxer[1] and Arnaud Scherberich[2, 3] Atanas Todorov[2], Patrick Studer[1], Sylvie Miot[2], Simone Schreiner[1], Sinan Güven[2], Laurent AH Tchang[3], Martin Haug[3], Michael Heberer[2], Dirk J Schaefer[3], Daniel Rikli[1], Marcel Jakob[1], Ivan Martin[2].

[1]Clinic of Traumatology, [2]Department of Biomedicine and of Clinical Research, [3]Clinics of Plastic, Reconstructive and Aesthetic Surgery, University Hospital Basel, Basel, Switzerland

INTRODUCTION: Stromal Vascular Fraction (SVF) cells, freshly isolated from adipose tissue, are an abundant and easily accessible source of mesenchymal/endothelial progenitors. Previous studies have demonstrated their osteogenic and vasculogenic properties. Given the dysfunctionality of autologous bone in osteoporosis, we aimed at investigating safety and feasibility of a clinical implementation of the SVF for fracture augmentation in the elderly. To investigate the contribution of the implanted cells to bone healing, a similar approach was evaluated in a nude-rat femoral-defect model.

METHODS: Autologous human SVF-cells were intra-operatively isolated using an automated device (Celution®800CRS, Cytori, USA) and used as cellular component of hydroxyapatite(HA)-based composite grafts for the augmentation of low-energy proximal humeral fractures after locking-plate fixation in 8 elderly patients. The grafts were assessed for cell characteristics, viability and differentiation potential. Follow up was performed for 6 month. In case of plate revision or removal, a bone biopsy was taken from the grafted area (n=6) and analysed using microCT and histology. The safety of the approach was defined as the absence adverse reactions (AR), feasibility as the absence of protocol deviations. Similar constructs were implanted in a segmental femoral defect in immune-compromised rats after locking-plate osteosynthesis (RatFix, RISystem, CH), with cell-free grafts as control. Mechanical, microCT and histological analysis was performed after six weeks.

RESULTS: The intra-operative cell isolation from 272 ± 63 ml abdominal lipoaspirate yielded 121.4 ± 72 million SVF-cells, manufacturing of the graft was well feasible, the production standardized and reproducible, the intervention was prolonged by the manufacturing process with liposuction (≈ 60 min) and cell isolation (≈ 120 min). The procedure was safe, without AR during the trial or in the following up to 39 months. The duration of hospitalization and the course of rehabilitation were normal. MicroCT and histology of the repair tissue from clinical biopsies demonstrated formation of ossicles as early as 6 weeks postoperative (fig 1.), structurally disconnected and morphologically distinct from osteoconducted bone, suggesting the osteogenic nature of implanted SVF cells. In the animal model, only SVF cell-treated defects healed mechanically stable and displayed mature bone with osteocytes and vascular structures of human origin.

Fig. 1:

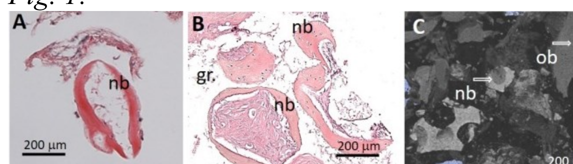

Fig. 1: Hematoxylin/eosin staining from a biopsy of the SVF-based graft after 6 weeks (A) and 6 months (B) with nb marking new bone (opposed to gr.=granules, ob=osteoconducted bone). Picture C shows the isolated bone formation within the pores of the whitish HA granules.

DISCUSSION & CONCLUSIONS: These trials strongly suggest that the non-expanded SVF without exogenous priming but within a fracture-micro-environment, can safely promote de novo generation of vascularized bone. The approach can be streamlined to optimize the utilisation of operating-room capacity. The efficacy of the proposed approach should now be evaluated in controlled trials with larger patient cohorts.

REGISTRATIONS/PERMITS:
ClinicalTrials.gov # NCT01532076, EKBB, Ref.#348/10, BAG Ref.# Bk2010-nTx-Z046-N0-V00, KVet Basel-Stadt, permission no. 2357

ACKNOWLEDGEMENTS: AO Start up grants S-12-08S / S-09-112S and SNF Project Grant No. 310030-156291 partially supported this study.

The new regenerative frontiers in cranio-mandibulo-facial surgery in primates: The pleiotropic inductive activities of the mammalian TGF-β3 isoform

U Ripamonti[1], R Duarte[2], RM Klar[1,2], R Parak[1,3], C Dickens[2], T Dix-Peek[2]

[1] *Bone Research Laboratory*, [2] *Dept. Internal Medicine,* [3] *Dept. Oral Biological Sciences, School of Oral Health Sciences and of Internal Medicine, Faculty of Health Sciences, the University of the Witwatersrand, Johannesburg*

INTRODUCTION: Contrary to results in rodents and lagomorphs, heterotopic implantation of recombinant human transforming growth factor-β_3 (hTGF-β_3) in the *rectus abdominis* muscle of non-human primates *Papio ursinus* results in the rapid induction of bone formation. [1] Mechanistically, this is set into motion by a series of profiled *bone morphogenetic proteins* (*BMPs*) and *TGF-βs* that are expressed at different time points temporally and spatially regulating the induction of bone formation. [1,2]

METHODS: 27 Chacma baboons *Papio ursinus* were implanted with doses of hTGF-β_3 loaded osteogenic devices in the *rectus abdominis*, mandibular and calvarial sites. Harvested tissues at 15, 30, 60, 90 days and up to 14 months after mandibular implantation were examined by quantitative reverse- transcriptase polymerase chain reaction (qRT-PCR) and compared to morphological data obtained from sections prepared using the EXAKT precision cutting and grinding system on undecalcified specimen blocks.

RESULTS: Implantation of 125 μg hTGF-B_3 in full thickness mandibular defects of *P. ursinus* resulted in an unprecedented *restitutio ad integrum* of the mandibular defects on day 30 with complete healing after long term studies. hTGF-β_3 failed however to engineer regeneration of calvarial defects which could be partially restored by adding pericytic/perivascular/myoblastic stem cells from morcellated fragments of *rectus abdominis* muscle. The morphology of incomplete calvarial repair on day 90 with bone formation pericranially and with lack of bone induction endocranially above the dura suggested a *radius* of activity set into motion by inhibitory mechanisms originating from the *dura mater* and/or the highly vascularized leptomeninges below. Such a diffusion molecular hypothesis was tested by surgically inserting a nylon fold impermeable membrane below the endocranium and above the dura and the arachnoids, segregating the molecular and cellular micro-environments of the calvarial defects from the dura. Segregation restored the endocranial induction of bone formation by hTGF-β_3 (Fig. 1) whilst segregated untreated defects showed limited, if any, induction of bone formation indicating the critical role of the *dura mater* in calvarial tissue induction. hTGF-β_3/treated segregated constructs showed significant induction of bone formation with *OP-1*, *BMP-2*, *Osteocalcin*, *RUNX-2*, *ID2* and *ID3* up-regulated.

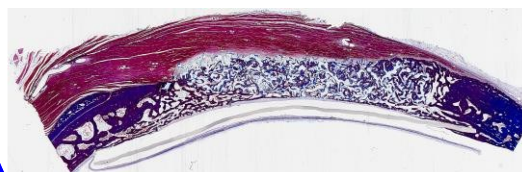

Fig. 1: (A) Segregated hTGF-β_3-treated calvarial defect with a nylon fold membrane: induction of bone formation across the defect.

DISCUSSION & CONCLUSIONS: In *P. ursinus*, calvarial segregation restores the induction of bone formation by hTGF-β_3 by blocking inhibitory signalling pathways from the underlying dura and leptomeninges below, with complete regeneration of large mandibular defects up to 14m post-implantation. The overall tight control of the induction of calvarial bone by hTGF-β_3 in *P. ursinus* is orchestrated by the *dura mater* which is disrupted by relatively high doses of the exogenously applied recombinant morphogen. In primates and in primates only, the TGF-β_3 gene and gene product singly yet synergistically and synchronously set into motion the induction of bone formation that now demands a paradigmatic shift in bone tissue engineering.

Can confining stimulation to the proliferative phase promote a sufficient healing outcome?

PM Tufekci[1], A Tavakoli[1], C Dlaska[1,2], M Neumann[2], M Shanker[1], S Saifzadeh[1], M Schuetz[1,2], DR Epari[1]

[1] *Queensland University of Technology, Brisbane, Australia,*[2] *Princess Alexandra Hospital, Brisbane, Australia*

INTRODUCTION: Mechanobiology is a research area which investigates the role of mechanical loads on the biological processes of fracture repair. Provided there is a sufficient mechanical stability, the overall course of bone healing is sensitive to the magnitude of interfragmentary movement due to the fixation used. It has been previously demonstrated that stimulation during early proliferative phase (3 weeks) can enhance bony callus formation but excessive movements during the late consolidation phase can delay healing. The aim of this study was to determine if stimulation confined only to the early proliferative phase is sufficient for timely healing. To answer this question we used a novel defect configuration capable of eliminating physiological loading experienced in a fracture gap.

METHODS: The defect model consisted of a 3 mm experimental fracture and 30 mm critical size defect separated by a 30 mm mobile segment of bone. A dual fixation was used to stabilise the model; the first fixation stabilised the proximal and distal fragments and the second (active device) anchored the mobile segment to the proximal fragment. The active device was used to axially manipulate the mobile segment relative to the proximal fragment. An *in vivo* study (Fig 1) was conducted using twelve skeletally mature sheep (3–4 years) separated into two groups (n=6); control and stimulatory. The stimulated group used axial compressive movements (1 mm, 500cycles/day @1Hz) as loading which commenced on the fifth postoperative day and continued until the third postoperative week. Both groups were subjected to weekly axial measurements (0.1 mm, 100 cycles/day @1Hz) for monitoring the healing pathway. MicroCT evaluation was used to quantify the bone volume within the periosteal and intracortical regions and *ex vivo* biomechanical torsional testing was used to assess the quality of the tissue formed.

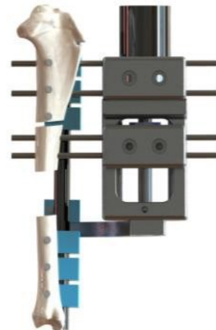

Fig 1: Schematic of dual fixation

RESULTS: Evaluation of the microCT images demonstrated advanced healing with significantly greater bone volume intracortically observed in the stimulatory group (448 mm^3) whilst the control group resulted in a poorer healing outcome and lower bone volume (210 mm^3) (Fig 2 left). This was supported by the biomechanical testing (Fig 2 right), which generated a superior torsional strength within the stimulatory group (19.8Nm) in comparison with the control (7.8Nm).

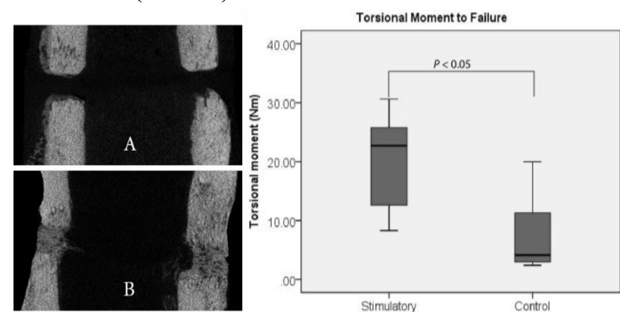

Fig 2: µCT of experimental fracture for control (A) and stimulatory group (B) (left). Ex vivo biomechanical torsional testing (right).

DISCUSSION & CONCLUSIONS: The novel experimental model has permitted the application of controlled loading within an experimental fracture. The lack of tissue formation and poor healing within the control group depicts an understimulation, validating the minimisation of functional loading. This study has demonstrated that confining stimulation to the proliferative phase is sufficient or timely healing. Further experimental work using a physiological loading pattern to verify these findings is required. In the long term this research can lead to optimized patient rehabilitation protocols and inform of the design of fixation strategies.

Improved fracture healing by short-term G-CSF treatment

M Herrmann[1], S Zeiter[1], U Eberli[1], M Hildebrand[1], K Camenisch[1], U Menzel[1], M Alini[1], S Verrier[1#], V Stadelmann[1]

[1] *AO Research Institute Davos, CH, #correspondence to sophie.verrier@aofoundation.org*

INTRODUCTION: Stem- and progenitor cell mobilization is a critical event in bone regeneration. This includes mesenchymal stem cells as a source of osteoprogenitor cells as well as endothelial progenitor cells (EPCs), which may promote neovascularization. In fact, impaired vascularization is considered as one of the most important causative factors for atrophic non-unions. Granulocyte colony-stimulating factor (G-CSF) mediated mobilization of CD34+ progenitor cells into the circulation is widely applied for peripheral stem cell donations and a positive effect of G-CSF administration on bone healing has been suggested. The aim of this study was to characterize the different cell populations mobilized by G-CSF and investigate the influence of cell mobilization before and after surgery on the healing of a critical size femoral defect in rats.

METHODS: First, the time course and cell populations mobilized by G-CSF treatment were determined. Rats were randomly assigned into four experimental groups, including an untreated control group and three groups receiving sub-cutaneous injections of G-CSF for 5 consecutive days, with blood analysis performed at day 1, day 5 and day 11 after the last injection. Measurements included total leukocyte counts and flow cytometry analysis. Second, bone healing was compared in a saline-treated control group, a group receiving 5 consecutive daily G-CSF injections before surgery and a group receiving 5 consecutive daily G-CSF injections after surgery. An empty 4.5mm critically-sized femoral defect was created in female adult Fisher rats and fixed internally. Bone healing was monitored by in vivo micro CT and histology.

RESULTS: Leukocyte counts show a peak increase at the first day after the last G-CSF injection (Fig. 1A). Analysis of different cell populations by flow cytometry revealed a cell-specific mobilization kinetic. We found that CD34+ progenitor cells were significantly enriched at day 1, and further increased at day 5 and day 11 (Fig.1B). Micro CT measurements revealed improved healing of the critically-sized bone defect in both G-CSF treatment groups with an increased bone volume in the fracture gap compared to untreated control animals (Fig.2). Although continuous bone formation was observed in all rats, healing was incomplete at the end of the observation period after 230 days, which was also confirmed by histology.

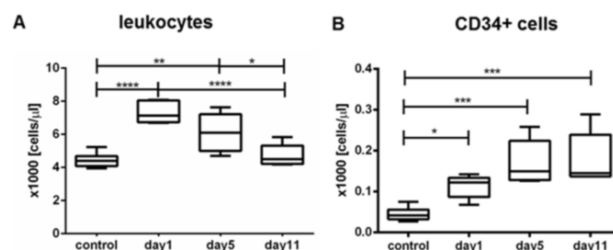

Fig. 1: *Blood cell values at different time points after 5 consecutive injections of G-CSF. Day 1 refers to the first day after the last injection.*

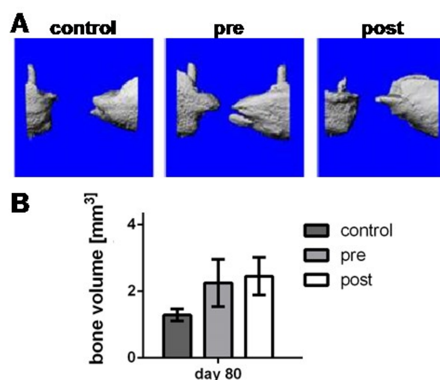

Fig. 2: *In vivo micro CT monitoring of fracture healing.* ***A.*** *Representative 3D renderings of segmented micro CT scans taken 80 days after osteotomy.* ***B.*** *Bone volume within femoral defects. Values are given as mean ± SEM.*

DISCUSSION & CONCLUSIONS: Our data shows that different cell populations are upregulated by G-CSF treatment in cell specific patterns. Although in this study no bridging of the critically-sized defect in the groups with G-CSF injections was reached, an improved healing was clearly shown. These results identify cell mobilization by G-CSF as a potential treatment option to facilitate the healing of large bone defects in combination with other treatment strategies.

ACKNOWLEDGEMENTS: The authors thank AOER/AO Foundation and AO Trauma for funding. The FACS AriaIII was kindly donated by the Innovationsstiftung Graubünden.

Choosing the right animal model for bone-related questions

MJ Allen

Surgical Discovery Centre, Department of Veterinary Medicine, University of Cambridge, Cambridge, United Kingdom

INTRODUCTION: Animal models continue to represent a critical component of both musculoskeletal discovery research and orthopaedic product/device evaluation and approval. This presentation will highlight the key factors that must be considered when considering an animal study in the context of bone-related questions. The goal of this review will be to challenge the audience to re-evaluate their current thinking and to present best practices in model selection, study design and the choice of clinically relevant outcome measures and time points.

DISCUSSION: *Model selection:* No matter the species being considered, every effort should be made to limit the use of animals in scientific research. There are tremendous potential advantages (financial and, more important, scientific) but unavoidable differences in body size, skeletal biology, skeletal structure (including skeletal size) and joint biomechanics may limit the validity of small animal models as screening systems for candidate new medical or surgical therapies. Large animal models such as sheep, goat and dog offer significant potential advantages in this regard, with more clinically relevant bone turnover rates, larger overall bone size and the possibility of using functional implants (e.g. joint replacements) that better simulate the intended clinical application in humans. However, the use of larger animals (especially companion animals) can be viewed as ethically contentious and may be outlawed in some jurisdictions. One of the key questions that needs to be addressed at the start of the pro cess is whether the study is intended as a feasibility/proof of principle, or as a definitive study for regulatory submission. Once this key step has been addressed, the decision-making process can move ahead.

Study design: Given the constraints of time, the presentation will focus on three specific examples in which animal models are used to address bone-related research questions: fracture healing, bone defect repair, and implant fixation. Each topic requires separate consideration, but there are some common themes that need to be addressed, including: (1) Has the experimental construct been validated (e.g. is the defect a critical size defect for the given species and age of animal)? (2) Is there an argument for/against doing multiple site surgeries and/or bilateral procedures? This is a very thorny issue with animal care and use committees, and would need strong justification and scientific support) (3) Are the biological processes in the animal model relevant to those in humans (e.g. if a pin is used as the sole stabilisation for a long bone fracture in rats, is this really mirroring the biology of fracture healing following surgical stabilisation with the plates or external fixators that are more typical in human surgery)? (4) Appropriate controls? What is the appropriate control for the experiment, is it ethical and how will the data be interpreted? (5) Use of a pilot study, to allow the research team to run through the logistical and technical aspects of the study, and to provide critical information that can be used to perform an appropriate power/sample size calculation.

Outcome measures and time points: One of the most frustrating aspects of reviewing literature relating to any aspect of bone research is the apparent lack of consistency in the selection of outcome measures and time points. To compound this, even when a standardised set of procedures is performed, it may not be reported adequately in the resulting manuscript. The combination of inconsistency and poor reporting contributes to the problem of irreproducibility, which the NIH has identified as a significant problem in animal studies throughout science[1]. Recent initiatives in terms of improved reporting will help in to improve transparency[2], but ongoing efforts are needed to define the most appropriate outcome measures for individual studies. With an extensive range of outcome measures available within the orthopaedic field, data collection can be maximised through the creative use of a combination of non-invasive, non-destructive testing (e.g. imaging) with the standard destructive outcome measures of mechanical testing and histopathology.

Reducing the use of animals by in vivo imaging of bone and blood vessels

R Müller[1]

[1] Institute for Biomechanics, ETH Zurich, Zurich, CH.

INTRODUCTION: With recent advances in skeletal tissue engineering and regenerative medicine there is a strong need for quantitative imaging of bone and blood vessels at the tissue and even cellular level. A number of new in vivo microstructural imaging modalities have been put forward recently allowing quantification with high precision and accuracy of these structures in a time-lapsed fashion in live animals. Although biomedical imaging technology is now readily available, few attempts have been made to expand the capabilities of these systems by adding quantitative analysis tools to assess transient structure-function relationships in a time-lapsed fashion. In the spirit of 3R, such longitudinal animal study designs not only allow to refine the methods used to measure biological in vivo function but also to directly reduce the number of animals needed for these studies, avoiding unnecessary cross-sectional studies. Using time-lapsed vivo imaging, each animal can serve as its own reference with respect to the changes observed at each time point. The aim of this contribution is to present recent developments in in vivo computed tomography (CT) imaging of bone and blood vessels in applications of tissue engineering and regenerative medicine.

METHODS & RESULTS: X-ray-based CT is an approach to image skeletal tissues and blood vessels in a hierarchical fashion providing multiscale imaging capabilities with isotropic resolutions ranging from a few millimeters (clinical CT), to a few micrometers (microCT) down to one hundred nanometers (nanoCT). A number of groups working in this field have demonstrated over the last two decades that X-ray-based tomographic imaging is a nondestructive and noninvasive procedure that allows precise 3D measurement of bone and blood vessels on all levels of hierarchy. The technique has been used predominantly in vitro but recently in vivo applications have gained more and more interest due the unprecedented resolutions in the order of 10 µm available in these in vivo systems. Due to the time-lapsed nature of the images, not only static but also dynamic morphometry can be performed to assess tissue remodeling and regeneration. With the recent introduction of computational tools that allow calculation of the mechanical microenvironment in these tissues, links between mechanical cues acting on ensemble of cells or even individual cells and the corresponding tissue adaptation and/or repair can now be monitored fully nondestructively in individual animals in vivo at relatively moderate cost and great ease of use. While studies in bone (Fig. 1) are straightforward due to the natural contrast of hard and soft tissue using X-rays, monitoring blood vessels in vivo is more challenging due to the required injection of contrast agents that provide good contrast, no extravasation and slow blood clearance (Fig. 2).

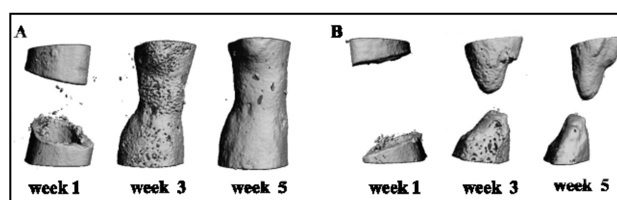

Fig. 1: Monitoring bone healing process in live mice. Reconstructions of the defect site and fracture callus for representative animals from 1.2mm defect group (A) and 2mm defect group (B).

DISCUSSION & CONCLUSIONS: Time-lapsed in vivo imaging allows longitudinal quantification of adaptation and regeneration of bone and blood vessels, thereby reducing the number of animals needed to show significant results. It is strongly recommended that quantitative imaging is used more often for in vivo animal studies in the area of tissue engineering and regenerative medicine.

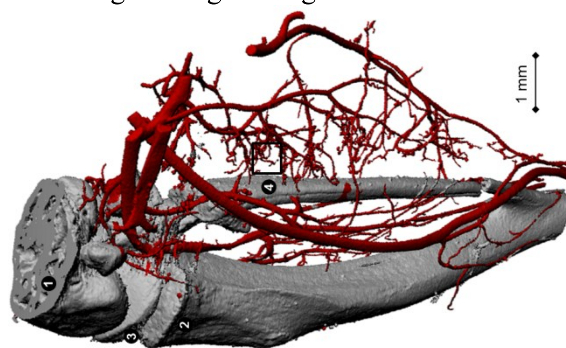

Fig. 2: Representation of blood vessels and bony structures in a mouse limb (10 µm resolution).

ACKNOWLEDGEMENTS: Financial support from the European Union (BIODESIGN FP7-NMP-2012-262948) is gratefully acknowledged.

injectable synthetic collagen beads hydrogel loaded with BMP2 promotes ectopic bone formation

S. Fahmy-Garcia[1,2]; D. Mumcuoglu [5]; L. de Miguel [5]; B.C.J van der Eerden [2]; D. Eglin [6]; S.G.J.M. Kluijtman[5]; G.J.V.M van Osch [1,3] and E. Farrell [4]
[1]Department of Orthopaedics, [2]Department of Internal Medicine, [3]Department of Otorhinolaryngology, Head and Neck Surgery, [4]Department of Oral and Maxillofacial Surgery, Special Dental Care and Orthodontics, Erasmus MC, University Medical Center, Rotterdam, The Netherlands. [5]Fujifilm Manufacturing Europe B.V, Tilburg, The Netherlands.
[6]AO Research Institute Davos, Davos, Platz, Switzerland

INTRODUCTION: The goal of bone tissue engineering is to use biomaterials, cells and signaling molecules capable to induce bone formation. Systems derived from polysaccharides and natural proteins are ideal scaffolds for tissue engineering since they mimic the extracellular matrices. In this study, we used synthetic collagen beads as developed by Fujifilm based on Cellnest (recombinant peptide based on human collagen I) loaded with BMP2, a well-known growth factor involved in bone regeneration, and included them in various hydrogels to generate an injectable system. The used hydrogels were thermoresponsive hyaluronic acid and two alginates types with different physico-chemical characteristics. The goal of this study was to determine the most biocompatible injectable collagen bead/hydrogel system in terms of host reaction, vascularization and bone formation in a cell free system.

METHODS: Twenty one male Sprague Dawley rats at 10 weeks old were used in this study. One of the alginate types or hyaluronic acid hydrogels containing synthetic collagen microspheres loaded with a constant concentration of rhBMP-2 were subcutaneously injected (n=6 per condition). At the end of 1-week, 4-week and 10-week period, six animals respectively were euthanized using CO_2. All implants were harvested and scanned using micro-CT to compare formation of mineralised tissue. For histological analysis, implants were paraffin embedded and processed for CD68, CD31, CollII, TRAP and H&E staining.

RESULTS: Injectable alginate gel with collagen beads containing BMP2 promotes ectopic bone formation. At 1-week cellular infiltration was visible, even though the hydrogel was not degraded yet. On the micro-CT scans, at 4-week mineralised tissue was observed in 4 of the 6 injected implants , especially at the edges or the areas where the hydrogel started to break down. At 10 weeks 5 implants were retrieved and bone formation was observed in all of them on CT. In addition, bone marrow presence was verified via histology. However, when thermoresponsive hyaluronic acid was used as hydrogel, although cellular infiltration was observed from the first week on, bone was not found within 10 weeks.

DISCUSSION & CONCLUSIONS: This work has shown that the use of collagen beads within alginate hydrogels in combination with BMP2 promote ectopic bone formation. However, when HA was used as hydrogel, most gel had disappeared within the first weeks and a high cell infiltration was observed, amongst which where macrophages suggesting a high degradation rate. Previous studies used hydrogel-beads combination with the addition of osteoprogenitor cells to trigger bone formation [1-2]. In our study, we obtained bone formation in a very high percentage of the constructs in a subcutaneous environment, which is very challenging using a cell free approach.
A major limitation of the commonly used release systems is the difficulty to modulate the release of the growth factors or signaling molecules to maintain their actions for a long time period. A combination of the hydrogel and recombinant collagen beads might help to solve this problem. Our data demonstrate the importance of the choice of the gel in this system. .

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Identification and isolation of live mesenchymal stem cells based on differentiation induced changes in mRNA expression

Bojun Li, Ursula Menzel, Claudia Loebel, Mauro Alini, Martin J. Stoddart

AO Research Institute, AO Foundation, Davos, CH.

INTRODUCTION: Osteogenesis of human bone marrow derived mesenchymal stem cells (hBMSCs) has been widely studied for bone tissue engineering. Recent studies show that the osteogenic differentiation of hBMSCs can be assessed by quantifying the ratio of two important transcription factors (Runx2/Sox9) [1]. In previous studies, these transcription factors were detected via destructive methods, either intra-cellular immunostaining or PCR. Here we demonstrate a new technique to observe mRNA expression of two genes in individual live cells using two fluorescent probes specific for Runx2 or Sox9 mRNA. Functionally homogenous cells can then be prospectively isolated based on the ratio of the two markers.

METHODS: hBMSCs were isolated from bone marrow aspirates by standard ficoll density gradient, followed by plastic adherence. Expanded cells (Passage 2-Passage 4) were plated at 10,000 /cm^2 and induced towards osteogenesis for 6 days. On the 6th day, media was replaced with fresh growth medium (GM) or osteogenic medium (OM) containing fluorescent Smartflare probes for Runx2-Cy3 and Sox9-Cy5, and the cells were left to incubate for 16 hours. The cells were then harvested by trypsinisation and sorted based on their relative Cy3:Cy5 fluorescence intensity compared to unstimulated controls. Gene expression analysis for a panel of osteogenic genes was performed. Sorted cells were further cultured to assess proliferation rate and osteogenic potential by way of alizarin red staining.

RESULTS: Cell differentiation in live cells could be monitored by investigating the relative ratio of Runx2 and Sox9 using non-destructive fluorescence based markers. Furthermore, cells sorted on day 7 using this ratio exhibited changes in osteogenic gene expression, proliferation rate and calcification potential compared to the unsorted population. One population (P1) appeared to have a later stage osteoblast phenotype, with low ALP, Runx2, Collagen I and the highest OC. This population also proliferated slowest, a characteristic of more differentiated cells, and calcified to the greatest extent.

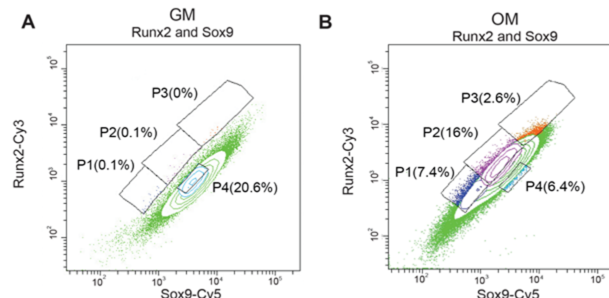

Fig. 1: Runx2 and Sox9 fluorescent probes can detect mRNA expression of two genes in individual live cells simultaneously and be used for cell sorting. A clear shift in profile was observed between cells in GM and OM. Cells in OM were sored into 4 groups (P1-P4).

DISCUSSION & CONCLUSIONS: A new method to observe mRNA expressions of two biomarker genes in individual live cells simultaneously, and isolate relatively homogeneous stem cells based on mRNA expression has been developed. Using this method we could observe and quantify the mRNA expression in live cells, and analyze cell differentiation. The cells also can be sorted based on mRNA expression for further studies. In contrast to previous isolation methods which are limited by available surface markers, isolation of cells using mRNA specific probes provides the opportunity to investigate intracellular markers, such as transcription factors Runx2 or Sox9, and isolate relatively homogeneous cell populations. Adapting the transcription factors investigated will allow this method to be utilized for other cell phenotypes

ACKNOWLEDGEMENTS: The Smartflare probes were kindly provided by Merck Millipore. This work was partly funded by the EU FP7-NMP-2010_LARGE-4 project BIODESIGN and part funded by the AO Foundation.

Hydrogels to control and recruit stem cells

SR Caliari, JL Holloway, SL Vega, JA Burdick

Department of Bioengineering, University of Pennsylvania, Philadelphia, PA, USA

INTRODUCTION: Hydrogels can be engineered for controlled biochemical and biophysical properties, to alter cellular behaviour (e.g., differentiation of mesenchymal stem cells (MSCs)), as well as for the controlled release of therapeutic agents (e.g., stromal cell derived factor-1α (SDF)). Towards the repair of bone, we have explored how gel properties alter the osteogenesis of MSCs when cultured either atop or within hydrogels across a range of mechanical and degradative properties. Additionally, we designed gels to release SDF local to a bone injury to recruit CXCR4+ cells for repair. In both cases, gels were engineered from hyaluronic acid (HA) and included crosslinks susceptible to matrix metalloproteinases (MMP) degradation.

METHODS: HA was modified with various reactive groups (e.g., norbornenes, maleimides) to form gels through either photoinitiated thiol-ene or Michael addition reactions and containing pendant RGD and MMP-sensitive crosslinkers (e.g.. VPMSMRGG).[1] Gels were characterized for mechanics (compression, rheological, AFM) and degradation (uronic acid). Towards understanding cellular interactions, MSCs were seeded atop or encapsulated within gels across a range of mechanics and with controlled degradation. Outcomes such as cell traction, viability, spreading, fate (e.g., ALP) and signalling (e.g., Yes-associated protein, YAP) were analyzed.[2] Gels were used for the encapsulation of SDF and bone morphogenetic protein-2 (BMP) and release was monitored with ELISA. Gels were implanted into rat cranial defects and characterized at 6 wks with radiography, micro-CT, and histology.[3]

RESULTS: HA gels with variable stiffness (1-20 kPa) were fabricated and supported viable MSC seeding and encapsulation (>90%). Higher stiffness gels degraded more slowly than lower stiffness gels. MSCs atop the gels increased their spreading, ALP activity, YAP nuclear localization and traction with increased mechanics, whereas cells within the same gels saw inverse trends with osteogenesis favoured in gels with lower mechanics or that were not degradable due to reduced spreading/traction (Fig. 1).

The release of SDF and BMP from the gels was dependent on the crosslink density and enzyme concentration and released molecules were active in *in vitro* studies. When implanted into the rat cranial defects, bone formation was greatest in the group with SDF/BMP (Fig.2), which was similar to BMP doses an order of magnitude higher delivered without SDF.

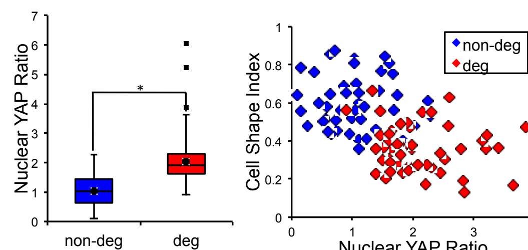

Fig. 1: Nuclear YAP ratio (left) and comparison to cell shape index (right) for MSCs within non-degradable and degradable hydrogels.

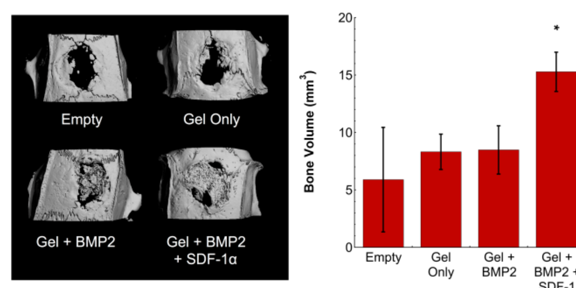

Fig. 2: 3D reconstructions (left) and quantification of bone volume (right) from micro-CT, 6 wks after implantation in a critical-sized rat cranial defect.

DISCUSSION & CONCLUSIONS: *Hydrogel properties (e.g., degradation, mechanics) influenced MSC fate and signalling differentially in 2D and 3D. When atop a hydrogel, increased mechanics enhanced traction and osteogenesis, whereas within a hydrogel, lower mechanics permitted spreading and osteogenic differentiation. Thus, hydrogel type and presentation is important towards guiding cell behaviour. The controlled release of SDF allowed for bone formation in the presence of BMP doses much lower than when BMP is delivered alone. These approaches illustrate the ability of engineered hydrogels to be used towards bone repair.*

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Synthetic bone fillers: how important is the micro and macrostructure?

M Bohner[1]

[1] *RMS Foundation, Bettlach, CH.*

INTRODUCTION: Synthetic bone fillers are broadly used to repair bone defects. Numerous studies have evidenced the importance of bone fillers architecture for this process. A roughness superior to 1 µm [1] and pores larger than 100 µm "macropores") [2] are considered to be ideal to accelerate and enhance bone formation. Additionally, a number of studies have underlined the importance of pores smaller than 100 µm ("micropores"), suggesting that multi-scale design approaches should be applied [3]. Nevertheless, past studies have delivered contradictory results, perhaps because the responses to architecture and composition are related [4]. The aim of my talk will be to review the data collected in two sheep studies [5-6], and reflect on the importance of the link between structure and bone healing. A total of 12 different materials were tested in [5-6], 11 out of β-tricalcium phosphate and one out of monetite (= anhydrous dicalcium phosphate). The materials had various micro and macrostructures, and implantation times included 2, 4, 6, 8, 12 and 24 weeks. A particular focus of my talk will be set on the nature of the tissues present within micropores and on morphological changes occurring during healing.

Healing of a critical size long bone defect through a serum free biomimetic cell-based approach

J. Bolander[1,2], W. Ji[1,2], J. Leijten[1,2], V. Bloemen[2,3], L. Moreira Teixeira[1,2], D. Lambrechts[1,2] and F. P. Luyten[1,2].

[1] Tissue Engineering Laboratory, Skeletal Biology and Engineering Research Center, KU Leuven, [2] Prometheus, Division of Skeletal Tissue Engineering, KU Leuven, [3] KU Leuven Campus Group T, Leuven.

INTRODUCTION: Cell-based constructs enable delivery of the critical number of progenitor cells required to heal complex large bone defects lacking intrinsic regenerative properties. Yet, the clinical success of such constructs has been limited, likely due to the presence of serum in the *in vitro* cell culture processes. Herein, we present a serum free regime for *in vitro* priming of human periosteum derived cells (hPDCs), crucial in postnatal fracture healing[1]. As a biochemical stimulatory factor, Bone morphogenetic protein -2 (BMP-2), was used due to its role in fracture healing[2, 3], and its potent effect on hPDC-mediated ossicle formation[4].

RESULTS: A serum free chemically defined media (CDM) was shown to maintain hPDC viability without inducing proliferation, as compared to standard serum conditions (GM). After pre-conditioning in CDM, the MSC CD-marker$^+$ decreased, most prominent in CD105: from 93% to 22%, whereas 73% became positive for CD34, further confirmed by mRNA transcript analysis, fig 1A. In addition, an increase in BMP receptor expression was observed. After 6 days of BMP-2 stimulation, elevated osteo-chondrogenic differentiation was depicted by *SOX9* and *OSX* mRNA transcript analysis, fig 1B, as well endogenous BMP-2-secretion. Dual positivity for SOX9 and OSX in single cells were confirmed, suggesting the onset of an intermediate osteo-chondrogenic differentiation pathway. In short, CDM pre-conditioning induced a more potent progenitor phenotype than conventional media *in vitro*. Upon assembly of the pre-conditioned cells into microaggregates, mimicking cellular condensations, the combined stimulation with BMP-2 further induced cell specification, fig 1C. Specifically, simultaneous aggregation and BMP-2 treatment led to osteo-chondrogenic differentiation *in vitro* where increased aggregate size (50, 100 or 250 cells/aggregate) further induced osteochondral cell specification. *In vivo* ectopic implantation displayed the formation of a hypertrophic cartilage intermediate after three weeks. When the *in vitro* primed intermediate tissue were transplanted to a critical size long bone defect model in nude mice, the formation of a fracture callus were seen after two weeks, which progressed to full bone bridging after four weeks, fig 1D and E.

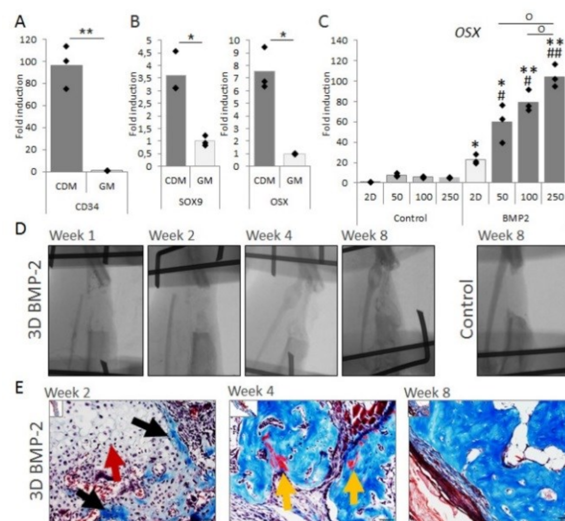

*Fig. 1: CDM pre-conditioning led to elevated expression of CD34 (A), and improved osteo-chondrogenic differentiation followed BMP-2 stimulation (B). Simultaneous stimulation by BMP-2 and aggregation led to further in vitro tissue differentiation, where an increased aggregate size further enhanced the effect (C). Endochondral fracture healing in a critical long bone defect was induced by the in vitro primed tissue as depicted by in vivo x-ray analysis and Masson's trichrome staining (red arrows: cartilage, black arrows: bone, yellow arrows: immature bone) (D and E). Significance: *: to 3D- and #: to 2D-control.*

CONCLUSIONS: Taken together, a synergistic stimulatory effect of serum free *in vitro* priming by pre-conditioning, aggregation and BMP-2 was seen. *In vivo*, transplantation of the *in vitro* primed intermediate tissue led to the healing of a critical size long bone fracture. Transplanted hPDCs actively contributed to *de novo* endochondral tissue formation. These findings provide an efficient and robust serum free cell-based alternative for the treatment of critical bone fractures.

Biomimetic nanostructured calcium phosphate scaffolds: osteoinduction and osteogenesis

A Barba[1,2], K Rappe[2], P Fontecha[2], A Diez-Escudero[2], Y Maazouz[1], M Espanol[1], C Öhman[3], C Persson[3], MC Manzanares[4], J Franch[2], MP Ginebra[1]

[1]Dept. of Materials Science and Metallurgy, Universitat Politècnica de Catalunya-BarcelonaTech, Spain. [2]Small Animal Surgery Department, Veterinary School, Universitat Autonoma de Barcelona, Spain. [3]Dept. of Engineering Science, Uppsala University, Sweden. [4]Pathology and Experimental Therapeutics Department, Universitat de Barcelona, Spain.

INTRODUCTION: Recently, some calcium phosphate (CaP) biomaterials have been shown to exhibit an intrinsic osteoinductive potential [1]. The mechanisms triggering the differentiation of MSCs to osteogenic cells are not well understood, although ionic exchange and micro-nanoporosity are believed to play a role [1]. Most commercial CaP bone substitutes are obtained by sintering at high temperatures (1000-1250ºC), which results in absence of nanostructure and low specific surface area (SSA). The possibility to obtain nanostructured CaP scaffolds, with controlled nanoporosity, together with tailored architecture, through low-temperature biomimetic routes opens up new possibilities in the design of osteoinductive bone substitutes with enhanced reactivity and protein entrapment capacity. The objective of this study was to evaluate the effect of nanostructure and macropore architecture on the intrinsic osteoinduction of a new family of biomimetic CaP materials in an ectopic site, and to correlate it with their osteogenic potential when implanted in bone.

METHODS: Nanostructured CaP scaffolds were obtained by a biomimetic process based on the hydrolysis of alpha-tricalcium phosphate (alpha-TCP) to calcium deficient hydroxyapatite (CDHA) in physiological conditions. Two different strategies were used to fabricate scaffolds with different architectures: i) Foaming of an alpha-TCP slurry (spherical concave macropores) [2]; ii) 3D Ink-jet printing (robocasting) using a self-setting alpha-TCP ink (prismatic macropores) [3]. Moreover, two different nanostructures were obtained: i) Fine (needle-like crystals, SSA=40 m^2/g) ii) Coarse (plate-like crystals, SSA=20 m^2/g). By combining the different parameters, four types of implants were compared: 1) Fine-Foam (FF) 2) Fine-robocasted (FR) 3) Coarse-Foam (CF) 4) Coarse-robocasted (CR).

The in vivo study was carried out in a standardized model of intramuscular (epaxial muscles) and intraosseous (5 mm Ø monocortical femur defect) implantation over 6 and 12 weeks in beagle dog (n=6). The presence of newly formed bone was assessed by backscattered scanning electron microscopy and quantified through microscopic computed tomography as a % of the total implant volume for intramuscular samples and as a % of the total cortical bone defect volume for the intraosseous samples.

RESULTS: In the intramuscular study, new ectopic lamellar bone formation was observed only in FF group (4/6 animals, % of bone volume = 2.06 ± 1.84) at 6 weeks and in FF (6/6 animals, 14.64 ± 6.15%) and CF (2/6 animals, 0.58 ± 1.32%) groups at 12 weeks. No ectopic bone formation was found in 3D-printed scaffolds. The osteogenesis results obtained in the intraosseous study are summarized in Table 1.

Table 1. Percentage of bone volume in the cortical bone defect

	6 weeks (%)	12 weeks (%)
FF	23.64 ± 5.04	54.92 ± 5.71
CF	12.95 ± 4.58	38.06 ± 4.62
FR	10.85 ± 3.38	29.12 ± 5.77
CR	9.15 ± 4.84	39.86 ±12.18

DISCUSSION & CONCLUSIONS: The in vivo performance of biomimetic CaP scaffolds with identical chemical composition (CDHA) is highly dependent on macropore geometry (Concave/Prismatic) and nanoscale surface topography (Needle/Plate-like crystals). A concave geometry of macropores combined with high SSA (needle-like crystals) stimulates the intrinsic osteoinduction, this resulting in an enhancement of the osteogenic potential when implanted in bone.

ACKNOWLEDGEMENTS: Spanish Government, project MAT2015-65601-R.

Why young companies will shape the MedTech World

S Lauber Fürst
Inartis Network, CTI's National Thematic Network (NTN) for the Life Sciences
Susanne.lauber@inartis-network.ch

WHY YOUNG COMPANIES: Breakthroughs in MedTech will be resulting from rapid convergence of the classical MedTech disciplines with Pharma and IT/Internet of things/digitalisation, all combined with the nanodimension of new materials. Young companies embracing the speed of the enormous growth of knowledge will be particularly able to exceed all the usual dimensions of transdisciplinary collaboration. Young MedTech companies will therefore spearheading this convergence, meaning the ongoing merger of life, physical, digital and engineering sciences. Internal critical preconditions will be the scientists' ability to engage in a true dialogue with other experts to quickly learn from each other, identify new opportunities and be effective from idea to market. External critical preconditions will be the ability of the regulatory bodies to define a solid and though adaptable framework to ensure quality and safety of those innovations.

THE ROLE OF INARTIS NETWORK: Inartis Network is the Swiss Life Science Community and a National Thematic Network (NTN) supported by CTI, the Swiss federal agency for the promotion and innovation. We are a motor and bridge builder for transdisciplinary and cross-industry innovation between business and academia, promoting "Innovations Made in Switzerland" through vibrant networking, initiating transdisciplinary and cross-industry R&D projects and events.

Life Sciences, and in particular MedTech, belong to the most dynamic and high-added value Swiss industrial and academic sectors, with innovation as driving force. Tomorrow's breakthrough innovations will not arise from within the traditional Biotech, Medtech or Pharma, but from transdisciplinary and cross-industry thinking and working.

Inartis Network is federating the dialogue between champions across the country and beyond. With our Expert Network, we drive innovation focusing on the areas of convergence, promoting interactions, and securing Swiss-wide synergies between the relevant disciplines and actors. We aim at lowering all possible hurdles to innovation and practice a no wrong door policy, which is the only possible mind-set if you want to successfully support young companies.

CONCLUSION: Young companies, able to capture the benefits from the rapid convergence will shape the MedTech world with disruptive innovations. To succeed, however, those companies need networks for quick and unbureaucratic support and a regulatory framework enabling and not blocking market access.

Public support on the steep and thorny way from spinoff to market success

Gabor Szekely

Medical Image Analysis and Visualization

Computer Vision Laboratory

Swiss Federal Institute of Technology

ETH-Zentrum Zurich

INTRODUCTION: The talk will shortly overview the numerous challenges and difficulties whicha Medtech startup company faces from its foundation to the successful introductionof its product on the market. The different tools and opportunities offered by public innovation support will be presented and their role and significancein this process will be analysed.

A novel product and market entry challenges for a young medtech startup

Xiang Li & Elias Bachmann
ZuriMED Technologies AG

INTRODUCTION: Although knee ligament reconstruction is performed almost a million times per year worldwide, a diverse range of techniques is employed in the clinic – a situation that almost always indicates that no technique is ideal. This suspicion is confirmed by clinical evidence, with highly variable long-term clinical outcomes after knee ligament reconstruction. Many knee surgeons are in favor of a bone-tendon-bone approach for higher performance in terms of faster and stronger healing, but at the cost of potentially severe pain at the graft extraction site. The hamstring grafting approach is widely used as well, which is less painful, but with lower performance attributable to slower healing with higher pullout risk.

We have developed a bone-tendon-bone conversion kit, that has the potential to “convert” a less painful “hamstring” graft into a higher performance BTB-like graft. This device uses a novel combination of advanced biomaterials that could accelerate graft healing while minimizing postoperative recovery time and pain. Our team originated in and is currently based at the Laboratory for Orthopedic Biomechanics at the Uniklinik Balgrist. ZuriMED Technologies AG was incorporated as a Balgrist and ETH Spin-off in September 2015, and got financed to further commercialize this device. Like most medtech startups in the early stage, our biggest challenge now is the regulatory pathway for market entry.

A materials approach to bone regeneration: From idea to market

Joost D. de Bruijn

Progentix Orthobiology BV, Bilthoven, The Netherlands; Institute of Bioengineering, Queen Mary University of London, UK; MIRA Institute, Twente University, Enschede, the Netherlands

INTRODUCTION: The use of growth factors or progenitor/stem cells for functional bone tissue regeneration have received much attention as potential alternatives to autologous bone grafting in the past decades. Some of the hurdles to overcome in these technologies include ensuring cell survival with the cell therapy approach and using potent but less supra-physiological concentrations of growth factors to minimize adverse reactions. To circumvent the necessity of cells or growth factors in bone tissue regeneration, we have developed a submicron structured calcium phosphate ceramic that is capable of inducing bone formation without the necessity of adding cells or growth factors. These instructive ceramics have shown excellent bone regeneration potential of large, critical sized bone defects. In this talk, an overview will be provided of the research performed to develop this new class of bone graft materials, including proof of efficacy and regulatory studies.rnal.

Biohybrid materials: inspired by nature to repair bones

G Perale[1,2]

[1] *Industrie Biomediche Insubri SA, Mezzovico-Vira, CH.* [2] *Department of Innovative Technologies, School of Applied Sciences and Arts of Southern Switzerland SUPSI, Manno, CH.*

INTRODUCTION: Evidence of clinical needs related to bone reconstruction dates back to ancient Egypt. A more rigorous scientific approach has been followed since 1889, when "modern" scientists started to focus their efforts on what can be defined as the early bone tissue engineering [1]. Nature here provides the key inspiration to new generation devices, where a composite approach is taking the lead by the smart combination of bio- and nano-technologies to replicate the intimate bone structure. The goal of a new approach is hence to combine the biocompatibility and tissue integration of natural materials with the possibility to tune mechanical and physical properties typical of synthetic ones: composite grafts best mimic the real nature of healthy human bone, being rigid and elastic, compact but porous, dense but viable to cells and vessels [2].

METHODS: A newly developed bone substitute, commercially named SmartBone®, was designed following a new concept of bottom-up composite approach, starting from bovine bone-derived mineral matrix mainly made of calcium hydroxyapatite ($Ca_5(PO_4)_3(OH)$), reinforced with bioresorbable aliphatic block-co-polymers (poly-lactide-ε-caprolactone) and RGD-containing collagen fragments as cell supporting biomacromolecules that increase cell viability and hydrophilicity, thus enhancing biocompatibility and osteointegration [2]. Medical grade components are mixed via a proprietary nanoemulsion physical-chemical process that allows obtaining the final device. 2 years of preclinical studies where followed by 4 years of clinical multi-centric studies, where bone regeneration in a wide range of defects from different patients was assessed by means of state-of-art imaging and histologic techniques [3].

RESULTS: Clinical and histologic evidences provided details on the *in vivo* behaviour of such a xeno-hybrid composite device: once grafted it soaks up blood, thus starting microcoagulation to occur inside the graft itself and hence enhancing graft integration. First weeks are then needed for cellular colonization of the graft, which is also enhance by the presence of collagen fragments that offer a viable environment for cells to spread onto; meanwhile, this time lag is also necessary for the degradation of the thin polymeric film, which progressively fades away leaving mineral structure for cells to consolidate and promoting the formation of new living bone (also by means of formation of new vessels); the following couple of months is needed for the integration of the graft with the native patient bone, thanks also to vascularization and new bone formation inside the graft. The remodelling process is hence completed.

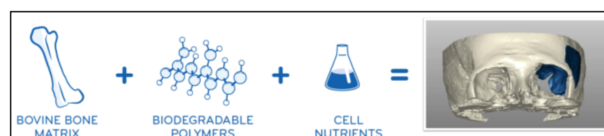

Fig. 1: choosing the right components to mimick the composite structure of natural bone to manufacture custom-made devices for regenerative surgery.

DISCUSSION & CONCLUSIONS: *Aim of an ideal bone graft is to allow its substitution by means of new growing healthy bone from the patient himself. Mother nature offers wide inspiration not only for intimate structure of innovative devices but also on regeneration processes and pathways that have to be walked down by these devices. The choice of composite approach runs along the track of bio-inspiration where, for bone engineering specifically, a composite mixture of natural minerals and synthetic polymers and signalling molecules allows mimicking at best the target tissue while also ensuring the right integration time. Success of such a bio-inspired approach is confirmed by the complete remodelling process, clinically evidenced by complete substation of grafted biomaterials with healthy human bone.*

The adaptive immune system impacts bone healing processes

K Schmidt-Bleek[1,2], C Schlundt[1,2], C Bucher[1,2], A. Serra[3], T ElKhassawna[4], S Wendler[1,2], H Wagner[1], HD Volk[2,5], GN Duda[1,2]

[1] Julius Wolff Institut, Berlin, Germany, [2] Berlin-Brandenburg Center for Regenerative Therapies, Berlin, Germany, [3] German Arthritis Research Center (DRFZ), Berlin, Germany, [4] Laboratory of Experimental Trauma Surgery, Giessen, Germany, [5] Institute of Medical Immunology, Berlin, Germany

INTRODUCTION: Delayed and disturbed bone healing remains a relevant clinical problem and will become even more relevant with the aging population in developed countries. Apparently, healing is challenged with an aged adaptive immunity, characterized by a distinctly different immune cell compositions in elderly compared to young, less experienced individuals. Recently, the influence of distinct immune cell subsets on the regenerative healing capacity of bone has become evident. The specific interplays of cellular components of the adaptive immunity with those of the bone system, however, have so far not been fully understood.

METHODS: Fracture healing has been investigated using either a fracture model (3 point bending) with an internal nail fixation or a mouse osteotomy model in which the left femur was stabilized with an external fixator (RISystem) with a 0.7 mm gap. Healing was observed for up to 28 days after surgery. The influence of the adaptive immune system on the regenerative bone healing process was investigated in WT Bl6 mice, in RAG1$^{-/-}$ mice lacking mature B and T cells, in JHT mice lacking T cells, and in WT Bl6 mice depleted of CD8+T cells using an antibody [anti-mCD8 AB (YTS169.4), Bio-XCell] injection (200 mg of mCD8 per injection for four consecutive days, with the last day being the day of surgery) [1]. Immune cell status, healing progress and healing success were monitored using x-ray, biomechanical testing, FACS, µCT, histology and immune histology.

RESULTS: Histological analyses of B and T cells revealed the participation of these cells in all fracture healing phases from early inflammation until remodelling [2]. Animals lacking B and T cells (RAG1$^{-/-}$) showed from early on a significantly altered healing process [3]. Without B and T cells organogenesis leads to stiffer bone structure indicating the involvement of immune cells in the organization of bone quality. To analyse whether this effect in intact bones depends on T or B cells, single knockout animals were investigated. Animals lacking T cells showed similar bone healing as RAG1$^{-/-}$ animals while animals lacking CD8+ T cells showed changes in their osteocalcin positive cells. These cells are responsible for the collagen I deposition, a major extracellular matrix component in bone. In RAG1$^{-/-}$ animals (lacking T and B cells) such changes in the collagen deposition could be linked to changes in the osteocalcin positive cell distribution and biomechanical callus composition.

DISCUSSION & CONCLUSIONS: A distinct interdependency of the skeletal and immune system becomes apparent in bone healing but recent findings also hint to an involvement of immune cells (specifically CD8+ T cells) in bone organogenesis. While the lack of T cells leads to less mature collagen deposition, the positive or detrimental effects of specific immune cell subsets remains, however, still unclear: CD8+ effector memory T cells have been proven to delay the healing process [1] and regulatory T cells are assumed to have a positive effect on the healing [4]. Yet, the specific involvement of the immune cells in the process of bone tissue formation and mineralization remains still unclear. Our results so far indicate, that a direct interaction of T cells and osteocalcin positive cells could explain some of the effects immune cell seem to have on the tissue quality of bone during healing and organogenesis.

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The role of angiogenesis in bone repair

Sabine Fuchs

Experimental Trauma Surgery, University Medical Center Schleswig Holstein, Kiel, Germany

Sabine.fuchs@uksh.de

INTRODUCTION: Vascularization is a critical factor in bone healing, not only by ensuring the supply with oxygen and nutrients. The current understanding implies a central function of the vascularization process in guiding bone repair at the cellular and molecular level. These processes are to a large extend controlled by the interaction of endothelial cells and bone forming cells, as well as circulating cells via paracrine factors, extracellular matrix components and direct cell communication mechanisms. The phenotype and functional properties of circulating cells, such as myeloid precursors, are determined by the interaction with endothelial cells and osteogenic cells mediating their differentiation towards inflammatory cells or osteoclasts during bone repair and remodeling. In addition, intermutual interactions of endothelial cells and osteogenic cells have a direct impact on the formation of new blood vessels but also on the differentiation and maturation of bone forming cells. Accordingly, a better understanding of these cellular and molecular processes might also result in new therapeutical approaches to improve vascularization. In this presentation we will highlight how co-cultures might be used to study the different mechanisms and pathways of cell interaction which mediate vascularization, bone repair or bone remodeling, respectively, but we also briefly refer to biomaterial based approaches creating vascularized tissue equivalents.

Such approaches might include the use of autologous cells isolated from an adult patient. In this context Outgrowth endothelial cells (OEC), derived from cultures of mononuclear cells from the peripheral blood, have been shown to form functional blood vessels after co-culture or co-implantation with osteogenic cells (Fuchs et al., 2010; Fuchs et al., 2009a; Ghanaati et al., 2011). Besides OEC, the initially diverse cell populations from the peripheral blood also contain cells such as early EPC contributing to the neovascularization in vivo, although these cells don't display the full spectrum of mature endothelial markers. These populations contain cells with a series of myeloid and M2 macrophage characteristics and are able to enhance the formation of vascular structures, when added to co-cultures of OEC and MSCs (Shi et al. 2014). These observations are in accordance with the current understanding of M2 macrophages known to be actively involved in endothelial repair. Nevertheless, myeloid precursor cells in principle have the potential to transdifferentiate into osteoclasts. In the co-culture approach the gene expression of myeloid and osteoclast markers seems to be modulated by endothelial as well as bone forming cells (Shi et al 2016) thus emphasizing the role these cellular interactions to control cellular differentiation during bone repair.

Small molecule Kartogenin promotes cartilage regeneration through activating IL-6-based mesenchymal stem cell proloferation

GQ Zhou, XLLi, T Liu, TH Chen, J Li, Y Zhu, YH Liang, ML Zhou

The Centre for Anti-ageing and Regenerative Medicine, Shenzhen University Medical School, Shenzhen, China; Shenzhen University, Shenzhen, China.

INTRODUCTION: Deregulation of the endogenous stem cells in cartilage tissues is considered to be a part of pathogenesis of osteoarthritis. Kartogenin (KGN) has been reported to exert stimulatory effects on chondrogenesis of mesenchymal stem cells by binding to filamin A that subsequently releases and activates transcription factor CBFβ and by up-regulate the Smad2/3 phosphosphorylation. In this study, we report that KGN is capable of stimulating the proliferation of primary cartilage derived progenitor cells (CPCs).

METHODS: Cartilage derived progenitor cells (CPC) were isolated from rat articular cartilage tissues and KGN stimulated-CPC proliferation was confirmed both *in vitro* and *in vivo*. Slow-cycling cells within cartilage were labeled and counted using RrdU and it specific antibodies. Cell cycle was analyzed using Flow Cytometry and RNA-seq was performed on CPC co-incubated with KGN for 5 days. IL-6 and Stat3 phosporylation were detected with ELISA kit and Western blotting. Cartilage repair/regeneration by oral administration and/or intra-articular injection of KGN was observed in rat knee joint injury models.

RESULTS: Our data shows that following 10 μM KGN treatment for a week, the percentage of G2-M phase cells in mitosis reached 9.6%, nearly twice of the control group, which was companied with the doubled total cell number. In the meanwhile, even after 4 weeks stimulation with KGN, Cells were proved to remain the expression of mesenchymal stem cell markers CD90(93%) and CD105(98%). As a control, no significant number change was observed in mature human T lymphocyte treated with KGN in the similar manner. Whole RNA-sequencing analysis of KGN-stimulated MSCs showed that significant expression changes of about 20 cell cycle-related genes upon KGN treatment for 72 hours. Among a number of genes found to be significantly changed by the KGN treatment are IL-6 and its receptor Gp130, which reach as much as 6 fold increase than the control. We further confirmed that the IL-6 level was significantly increased by KGN in both cytoplasm and supernatant media of CPC culture. We further demonstrated that the phosphosphorylation of Stat-3 was up-regulated at the same time. In-articular injection of KGN were also found to increase the number of BrdU-labeled slow-cycling cells. *In vivo* experimental evidences of the increased thickness in articular cartilage with KGN treatment was further confirmed in the rat model that were induced to have knee joint injury. IHC staining of the KGN treatment group showed up-regulate of Stat-3 phosphosphorylation in KGN-treated cartilage.

DISCUSSION & CONCLUSIONS: Based on the our in vivo and in vitro data, we propose that KGN may improve the number of endogenous cartilage stem cells by promoting their self-renewal in situ while inducing chondrogenesis and its use in cartilage regeneration and repair is definitely worth of further explored.

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In vivo bone marrow and cytokine dynamics in two experimentally injured tissues

L Leitão[1,2,3], CJ Alves[1,2], IS Alencastre[1,2], DM Sousa[1,2], E Neto[1,2,4], C Leitão[1,5], G Almeida-Porada[6], M Lamghari[1,2,3]

[1]Instituto de Investigação e Inovação em Saúde, Universidade do Porto (UP). Porto, PT. [2]Instituto de Engenharia Biomédica, UP, Porto, PT. [3]Instituto Ciências Biomédicas Abel Salazar, UP, Porto, PT. [4]Faculdade de Medicina, UP, Porto, PT. [5]Instituto de Biologia Molecular e Celular, UP, Porto, PT. [6]Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Winston-Salem, USA.

INTRODUCTION: Bone marrow (BM) multiple cell populations, namely hematopoietic cells (HSCs), mesenchymal stem cells (MSCs) and endothelial cells (EPCs), were reported to play a key role in tissue repair and regeneration[1]. Cytokine release was also described to be associated to these processes, providing cells signals to orchestrate in coordinated manner the different phases of tissue regeneration, from inflammation to ECM remodelling[2]. These findings were described in different experimentally injured tissues, including muscle, heart, kidney, skin, bone, liver and brain[1]. However, little is known whether different tissue insults, injury incidence and regeneration trigger similar temporal coordination of these BM multiple cell populations as well as the associated cytokines. Here we analysed the different subsets of BM cell populations and serum cytokines in two injury models: bone defect and skin/muscle laceration wound at different stages of tissue regeneration.

METHODS: Adult C57/Bl6 mice were submitted to skin/muscle laceration or to femoral bone defect. Of note, in the bone defect group to access to femoral bone and achieve bone injury, skin/muscle cuts were also performed. Thus, injury incidence in this group is higher when compared to skin/muscle laceration group. Non-operated animals were used as controls. Animals were sacrificed 1, 3 and 7 days post-injury. At each time point, the BM was harvested from the femurs and its different cellular populations were analysed by flow cytometry. These included the mesenchymal stem cells (Sca1^{+}; CD105^{+}; CD140a^{+}; CD11b^{-}; CD45^{-}; CD34^{-}), endothelial cells (CD105^{+}; CD31^{+}, CD11b^{-}; CD45^{-}; CD34^{-}) and hematopoietic progenitor cells (Sca1^{+}; CD117^{+}, CD3^{-}; CD4^{-}; CD8^{-}; CD11b^{-}; CD19^{-}; TER119^{-}; Ly6G^{-}; F4/80^{-}). Immune cell populations from the adaptive (CD8^{+} T cells, CD4^{+} T cells, CD4^{-}/CD8^{-} T cells and B cells) and the innate immunity (CD11b^{+} cells, macrophages and dendritic cells) were also analysed. Serum cytokines and chemokines relative levels were analysed at each time point using a commercially available proteome profiler.

RESULTS AND DISCUSSION: Both skin/muscle laceration and femoral bone defect groups showed alterations in the BM populations at the different time points upon injury. Each BM subset undergoes level´s variation in a time dependent manner. These alterations follow the same pattern in both groups. Interestingly, comparison of cell percentage of each BM subset between the two injury groups revealed no significant differences. Among several factors, pro and anti-inflammatory cytokines, chemokines and others have been described to play determinant roles in inflammation, MSCs homing and ECM remodelling. Cytokine analysis showed that G-CSF, MCP-1, CXCL1, CXCL13 and TIMP-1 were significantly triggered in both skin/muscle laceration and bone femoral defect when compared to non-operated animals. Surprisingly, in both groups, the levels of SDF-1/CXCL12 remained unchanged at the different time points after injury and are comparable to those of the non-operated animals. G-CSF, MCP-1, CXCL1, CXCL13 and TIMP-1alterations follow a time course pattern but with different magnitudes that are tissue injury dependent. In addition, in bone defect animals, cytokine levels are associated with long-lasting changes. Overall, these results suggest a reconfiguration of the BM cellular populations upon skin/muscle and bone tissue injury and during regeneration that seems to be tissue and injury incidence-independent but accompanied with differential systemic cytokine responses.

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Effective vascularization and efficient bone formation in osteogenic grafts requires VEGF dose control

A Lunger[1,2], MG Burger[1,2], A Grosso[1], P Briguez[3], JA Hubbell[3] , D Schaefer[2], A Banfi[1], N Di Maggio[1]

[1] Cell and Gene Therapy, Department of Biomedicine, Basel University Hospital, Basel, Switzerland; [2] Department of Plastic, Reconstructive, Aesthetic and Hand Surgery, Basel University Hospital, Switzerland; [3]Institute of Bioengineering, EPFL Lausanne, Switzerland

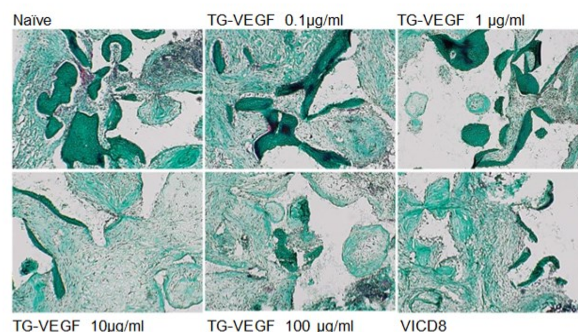
INTRODUCTION: Spontaneous vascularization of clinically relevant, large-size bone grafts based on bone marrow-derived mesenchymal stem cells (BMSC) is insufficient and requires therapeutic stimulation to ensure progenitor survival and bone formation. Vascular endothelial growth factor-A (VEGF) is the master regulator of angiogenesis. However, we found that, while its sustained over-expression by genetically modified human BMSC effectively improved vascularization of osteogenic grafts, it also impaired bone formation through excessive osteoclast recruitment [l]. Recently we found that delivery of VEGF for a limited duration of 4 weeks in the form of recombinant protein covalently bound to a fibrin hydrogel prevented excessive bone resorption while ensuring increased vascularization (Burger et al. unpublished results). Here we sought to investigate the role of VEGF dose on the coupling of angiogenesis and bone formation, in order to define a VEGF therapeutic window for vascularized tissue-engineered bone.

METHODS: Recombinant VEGF was engineered with a transglutaminase substrate sequence (TG-VEGF) to allow cross-linking into fibrin hydrogels [2]. Osteogenic constructs were prepared with primary human BMSC seeded on hydroxyapatite granules in a fibrin hydrogel containing 4 different TG-VEGF concentrations (0.1, 1, 10 and 100 µg/ml) and optimized to ensure the controlled release of the factor over 4 weeks [3]. Control grafts were generated with BMSC only or retrovirally transduced BMSC that constitutively express VEGF linked to the cell-surface marker truncated CD8 (VICD8). Histological analysis 1, 4 and 8 weeks after ectopic subcutaneous implantation in nude mice was used to determine vascularization (CD31 immunostaining), bone formation (H&E and Masson Trichrome) and osteoclast recruitment (TRAP staining).

RESULTS: *All VEGF doses effectively increased vessel density up to 5-fold already after 1 week and vascularization persisted at all later time-points. After 4 and 8 weeks, bone tissue development was enabled by 0.1 and 1 µg/ml of TG-VEGF as efficiently as with naïve BMSC alone. However, higher doses progressively impaired bone formation and 100 µg/ml caused a similar reduction as with VEGF-expressing genetically modified BMSC. The loss of bone formation correlated with increased osteoclast recruitment.*

Fig. 1: Masson's Trichrome staining shows that increasing VEGF doses gradually impair bone formation 8 weeks after in vivo implantation of osteogenic grafts.

DISCUSSION & CONCLUSIONS: These data suggest that VEGF effects on promoting vascularization and bone resorption can be uncoupled by short-term delivery of recombinant VEGF protein. However, VEGF effects on bone resorption are dose-dependent and a therapeutic window exists that enables both rapid vascularization and efficient bone formation. This could provide a clinically applicable strategy with several attractive features: 1) no genetic modification; 2) homogeneous and tunable factor doses; 3) limited and controllable duration of factor delivery.



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Adipose-derived stem cell seeded biominerizable nanocomposite for chest wall repair: suppression of inflammatory response in a murine model

J Buschmann[1], E Balli[1], SC Hess[2], WJ Stark[2], P Cinelli[3], S Märsmann[1,3], M Welti[1,4], W Weder[4], W Jungraitmayr[4]

[1]Plastic Surgery, University Hospital Zürich, Zürich, CH. [2]Institute for Chemical and Bioengineering, ETH, Zürich, CH. [3]Trauma Surgery, University Hospital Zürich, Zürich, CH. [4]Thoracic Surgery, University Hospital Zürich, Zürich, CH.

INTRODUCTION: Defects to the chest wall can occur after tumour resections or trauma caused by accidents, and appropriate chest wall reconstruction is therefore needed. Stability and integrity of the repaired chest wall should reach similarity to natural physiology. Addressing the treatment of critical size full-thickness chest wall defects, the ideal graft should be stable, fluid- and air-tight, biocompatible inducing no inflammatory reactions, biodegradable during the healing with non- toxic degradation products as well as rapidly integrating into the surrounding tissue. Here, we present the implantation of a biocompatible, biodegradable and easily vascularizable nanocomposite seeded with adipose-derived stem cells (ASCs) as a chest wall graft in a murine model. The cellular response towards this graft is compared to the cell-free graft.

METHODS: An electrospun poly(lactic-co-glycolide)/amorphous calcium phosphate (PLGA/aCaP) nanocomposite was seeded on both sides with murine ASCs and cultivated for two weeks before implantation as a chest wall graft. In addition, a cell-free analogous PLGA/aCaP scaffold was implanted on top of the cell-seeded scaffold towards the skin in order to be able to study not only direct cell-to-cell contact-based effects, but also to address paracrine effects caused by ASCs (control: cell-free scaffold alone). Histomorphometric analysis was performed at 4 and 8 weeks post-operation, respectively, to assess cell density of macrophages, lymphocytes and foreign body giant cells (Figure 1).

RESULTS: Inflammatory response towards the graft material was significantly reduced for macrophages, lymphocytes and foreign body giant cells in the presence of ASCs compared to cell-free scaffolds. Moreover, this anti-inflammatory action caused by ASCs was not only found on the side where direct cell-to-cell contact between seeded ASCs and local cell population was enabled and studied, but also on the scaffold side where predominantly diffusible factors secreted by ASCs were active (paracrine function).

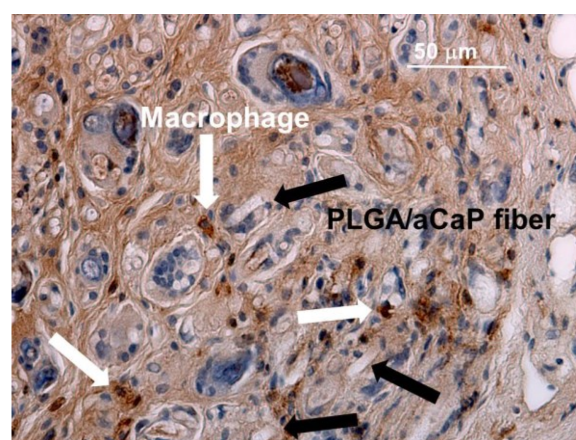


Fig. 1: F4/80 stained histological section at 8 weeks post-operation showing selected macrophages (white arrows) and electrospun PLGA/aCaP fibers of the degrading chest wall graft.

DISCUSSION & CONCLUSIONS: In clinics, the state of the art of repairing critical size chest wall defects is to use inert materials such as Goretex® which are not easily vascularizable and not biodegradable. Here, we present a biocompatible, biodegradable and well vascularizable nanocomposite for chest wall repair. In order to enhance integration of this graft material and accelerate wound healing, ASCs were seeded. A beneficial effect of these ASCs was that the inflammatory response towards the implant was significantly reduced. Therefore, such cell-seeded nanocomposites may be applied as chest wall grafts in clinics in the future.

ACKNOWLEDGEMENTS: We thank Gabriella Meier Bürgisser for her help with analysis of histological sections and Pia Fuchs for histological staining.

Clinical use of stem cells

Gun-Il Im

[1] *Donngguk University, Goyang, Koreas*

Orthopaedic medicine has been traditionally benefited from the innovation from other field of science. Development of metal and chemical engineering in the early 20th century contributed to current orthopaedic practice as various implants based on newly developed biomaterials were devised and applied for treating patient. Recent advancement in regenerative medicine has opened a new horizon in the orthopedics and may shift the paradigm in clinical practices in the future. The diseases which are currently managed by surgical treatment may be more effectively and more economically treated by less invasive procedures such as simple injection of cells.

Stem cell research has mostly inspired from the need to explore the new therapeutic possibility for intractable and lethal diseases. Although musculoskeletal disorders are basically nonlethal, the high prevalence of diseases and relative ease in performing clinical trial has facilitated the clinical application of stem cell in this field. On the other hand, there is a relative paucity of reliable clinical studies despite the plethora of in vitro and preclinical studies in the area of stem cell research for regenerative medicine in musculoskeletal system.

The stem cell therapy can be locally applied for the regeneration of bone, cartilage and tendon. Candidate disease modalities in bone regeneration includes large bone defect, nonunion of fracture, osteonecrosis. Focal osteochondral defect and osteoarthritis are current targets for cartilage regeneration. For tendon regeneration, bone-tendon junction problems such as rotator cuff tears are hot topics in clinical research. In this talk, the current status of stem cell application in clinical field is introduced along with future perspective from the author’s point.

ACKNOWLEDGEMENTS: This work was supported by a grant from the National Research Foundation (NRF) funded by the Korean government (2015R1A2A1A09002793)

BOOSTB4: a clinical study on pre- and/or postnatal stem cell transplantation for treatment of osteogenesis imperfecta

C Götherström[1,2], C DeVile[3], R Sakkers[4] O Semler[5], E Åström[6,7]
on behalf of the BOOSTB4 consortium

[1]Department of Clinical Science Intervention and Technology and [2]Center for Hematology and Regenerative Medicine, Karolinska Institutet, Stockholm, Sweden. [3]Great Ormond Street Hospital, London, United Kingdom. [4]University Medical Centrum Utrecht, Utrecht, the Netherlands, [5]Uniklinik Köln, Köln, Germany. [6]Astrid Lindgren's Children Hospital and [7]Department of Woman and Children's Health, Karolinska Institutet, Stockholm, Sweden.

INTRODUCTION: Osteogenesis imperfecta (OI) is a heterogeneous inherited condition and severe forms present already in utero. Persons with severe OI is affected throughout their lifetime with repeated, multiple fractures, short stature and orthopaedic problems, considerable pain and handicap. There is no curative or sufficiently effective symptomatic treatment for OI.

Preliminary clinical experience in two cases indicates that transplantation of fetal liver derived mesenchymal stem cells (MSC) before and after birth may ameliorate symptoms[1,2].
The main objective of the Boost Brittle Bones Before Birth (BOOSTB4) phase I/II multicentre study is to evaluate the safety and efficacy of pre- and/or postnatal MSC transplantation in severe vital forms of OI (type III and severe type IV).
METHODS: The study will include three groups:
1) Prenatal and postnatal transplantations in 15 patients, inclusion during pregnancy
2) Postnatal transplantation in 15 patients, inclusion before one year of age
3) Historical and prospective controls, 30-60 cases
Over twenty months, the patients will receive four postnatal infusions of same-donor MSC at 4-month intervals.

The primary outcome is safety for the fetus, child and pregnant woman. Secondary outcomes relate to efficacy, including fracture frequency, growth, bone mineral density over 20 months. Rapid exome sequencing using a panel targeted for skeletal disorders for definitive molecular diagnosis of OI will be developed. Experience, impact and perception of the therapy will be evaluated in both treatment groups.

RESULTS: We have established a European network centred around four clinical hubs in Stockholm, Cologne, London and Utrecht/Leiden. Early studies have shown that rapid diagnosis of skeletal dysplasias using whole exome sequencing is possible. Production of MSC is underway and the clinical trial protocol for transplantation and follow-up is being finalised. Recruitment to the main treatment study will soon commence.
DISCUSSION & CONCLUSIONS: Prenatal stem cell transplantation shows promise for the treatment of inherited single gene disorders. Demonstration that MSC transplantation improves early outcome in patients with severe OI would represent a major step forward in the management of these patients. If successful, such treatment could be relevant for the management of a range of other inherited birth defects. The BOOSTB4 consortium welcomes clinical cases for diagnosis of OI using rapid exome sequencing and, for the first time, inclusion of European patients in the clinical trial on treatment of OI with fetal MSC pre- and/or postnatally. Contact Cecilia Götherström for more information: Cecilia.Gotherstrom@ki.se

ACKNOWLEDGEMENTS: This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 681045 and from the Swedish Research Council (E0720901).

in situ poly(carboxybetaine) hydrogel for bone tissue application

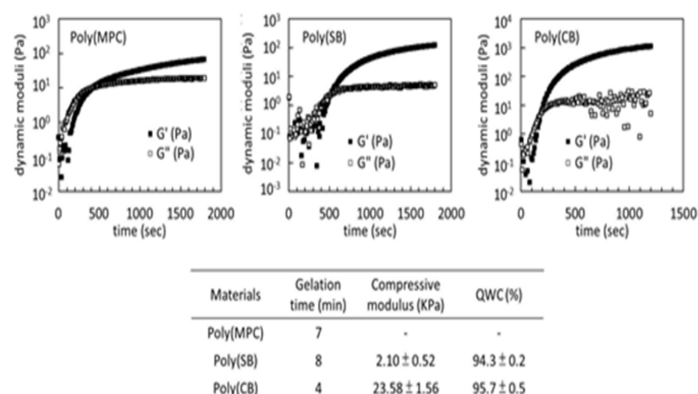
Hsiu-Wen Chien[1], Hsin-Yu Chen[1], Jiashing Yu*[1], Wei-Bor Tsai* [1]

[1] Biomedical and Tissue Engineering Lab, Department of Chemical Engineering, National Taiwan University, Taipei, Taiwan

INTRODUCTION: We re-investigated the cytotoxocity of CBMA, which was comparable to other commonly used zwitterionic monomers, such as 2-Methacryloyloxyethyl phosphorylcholine (MPC) and sulfobetaine methacrylate (SBMA). Biocompatibility and rheology of the polymerization process was also investigated. Next, the zwitterionic materials were directly injected into a mouse subcutaneously to study the tissue response. Finally, the hydrogels were prepared with bio-functional molecules, such as RGD peptides and hydroxyapatite (HAp) to examine the concentration effects of adhesive ligands for bone tissue engineering.

METHODS: Zwitterionic monomer solution was prepared in PBS at a concentration of 20% (w/v). Crosslinkers of NDMCC and PEGDMA and the initiator of APS/TEMED were added to the monomer solution at concentrations of 1 mol% of monomer and 10 mM, respectively. After polymerization, hydrogels were removed from the casts and soaked in PBS for complete swelling. The fully swollen hydrogel disks were compressed to failure at a rate of 35.4 mm/min with a mechanical tester (FGS-50V-H, NIDECSIMPO Corporation, Japan) and a digital force gauge (FGP-0.5, NIDECSIMPO Corporation, Japan). The Young's moduli of poly(zwitterion) hydrogels were calculated by applying 35 to 60% strain. Physical properties and the gelation times of each hydrogel were assessed with an AR2000 rheometer (TA In-struments) and a parallel plate geometry. Primary rat osteoblasts were isolated from calvariae bonds of neonatal rat. Cells were mixed and suspended in the solution to a final seeding den-sity of 5x106 cells /mL and allowed for polymerization at 37°C for 30 mins. For in vivo osteo-genic differentiation in the injectable poly(CB) hydrogels, the three experimental groups were designated as CB, CB/RGD, and CB/RGD/HAp hydrogels. The precursor solutions were in-jected into the subcutaneous of a nude mouse (4-5 weeks old), respectively.

RESULTS:



Materials	Gelation time (min)	Compressive modulus (KPa)	QWC(%)
Poly(MPC)	7	-	-
Poly(SB)	8	2.10±0.52	94.3±0.2
Poly(CB)	4	23.58±1.56	95.7±0.5

Fig. 1: Time dependence of storage modulus (G', solid) and loss modulus (G", hollow) of polymerized solutions gelled at pH 7.4 at 37°C.

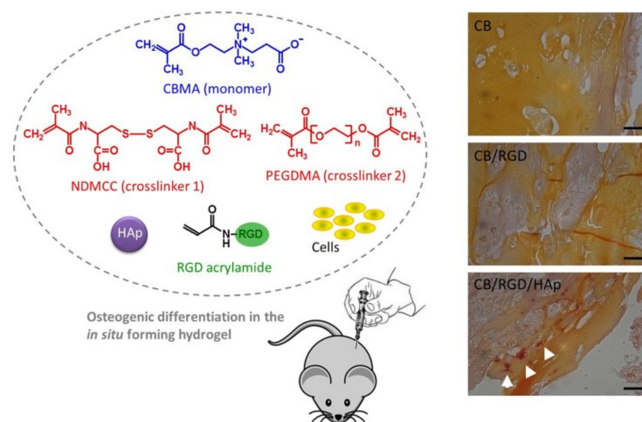


Fig. 2: The images of the dissection of MSC/scaffold constructs, stained by Alizarin red staining. The scale bar represents 100 µm.

DISCUSSION & CONCLUSIONS:

This study demonstrated that three zwitterionic monomers, CBMA, SBMA, and MPC, exhibited low cytoviabilityies. We subsequently prepared the CBMA precursor solution with Arg-Gly-Asp (RGD) and hydroxyapatite (HAp) nanoparticles for osteogenic tissue engineer-ing. Both in vitro and in vivo studies demonstrated that HAp containing poly(CB) hydrogels greatly enhanced the mineralization on the deposited substrates. These results indicated that poly(zwitterions) hydrogels may be useful in generation of biocompatible, implantable medical devices and tissue scaffolds.

Novel approach to control drug release and adherence of PLA based implant coatings

E Choinska[1], W Swieszkowski[1]

[1]*Faculty of Materials Science and Engineering, Warsaw University of Technology, Warsaw, Poland*

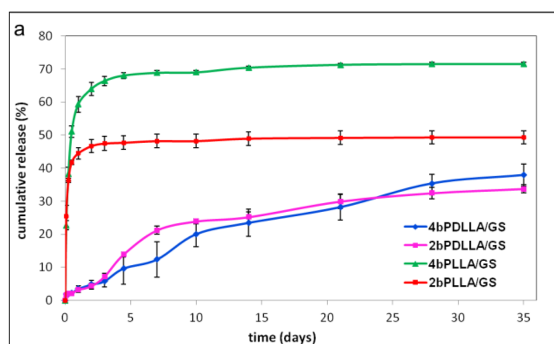
INTRODUCTION: The typical approach to control drug release from polymeric implant coatings is based on modifying composition and molecular weight of carrier or geometric parameters of the system [1]. In such DDS also the proper adhesion of polymer to substrate have to be provided. Usually it is done by increasing of contact area (eg. surface development), or chemical treatment of substrate (eg, silanization, or phosphating) [2]. In this work novel approach, based on changing of macromolecular structure of PLA, is presented.

METHODS: Two linear (2b) and two 4-arms (4b) polylactides were synthesized by ring opening method (Tab.1). Model drug, gentamicin sulphate (GS), and ion complex GS-AerosolOT (GS-AOT) were incorporated into polymer carrier during dissolving polymer in CHCl3, and then coatings were prepare by dip-coating of silanized [3] stainless steel substrate. The adherence of polymers was measured by scratch test (F=1-10N, 2N/min, l=3mm) and critical loads (L_c) were determined. Drug release test was perform in PBS, at 37°C for 5 weeks and fitting of obtained data to different mathematical models was done.

Table 1. Polymers used for implant coatings preparation.

Polymer	M_n (kgmol^{-1})	M_w (kgmol^{-1})	T_g (°C)	T_m (°C)
4bPdlLA	34.3	39.9	37.7	-
2bPdlLA	34.9	45.0	38.5	-
4bPlLA	40.7	46.0	60.6	171.0
2bPlLA	34.2	37.8	60.9	173.5

RESULTS: The kinetics of drug release is shown in figure 1.



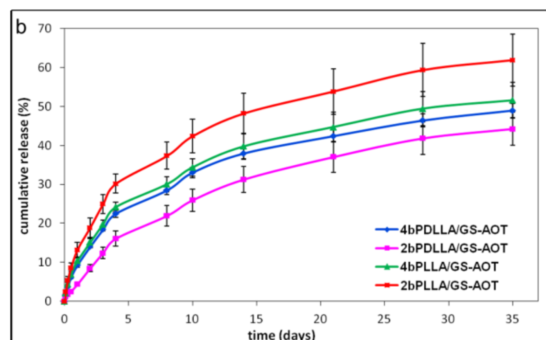


Fig. 1: Release of GS (a) and GS-AOT (b) from linear and 4-armes PLAs.

Table 2. Results of scratch test.

Polymer	4bPdlLA	2bPdlLA	4bPlLA	2bPlLA
Lc (N)	3.6	2.8	3.5	2.8

DISCUSSION & CONCLUSIONS: Drug release test showed that drug is faster release from 4bPLAs. GS diffuse according Higuchi model, but for GS-AOT the best fitting was obtained for Korsmeyer-Peppas model [4] . The analysis of determined values of critical load and comparison of area of damaged coating have shown that adherence of polymers is increasing in following order: 2bPlLA < 4bPlLA <2bPdlLA< 4bPdlLA.

It can be concluded, that changing of macromolecular structure allow to control drug release and adherence of PLA based implant coatings.

ACKNOWLEDGEMENTS: This study was financially supported by the National Centre for Research and Developments (MentorEye project -STRATEGMED1/2333624/4/NCBR/2014).

The efficacy of local bisphosphonate and BMP-2 delivery in improving bone mass and mechanical implant stability

L Freitag[1]*, C Günther[1]*, L Kyllönen[1]*, U Eberli[1], K Thompson[1], D Arens[1], S Zeiter[1], D Eglin[1], VA Stadelmann[1]

[1] *AO Research Institute, AO Foundation, Davos, CH.*
**Equivalent contributions*

INTRODUCTION: Worldwide, osteoporosis causes more than 8.9 million fractures, which means one fracture every three seconds[1]. For people older than 50 years, one out of three women and one out of five men will have an osteoporotic fracture [2 3 4].
Implant fixation in osteoporotic bones can be quite challenging due to low bone mass and reduced mechanical properties.

This study aims to improve implant stability in osteoporotic bone using a hyaluronan hydrogel for local delivery of bisphosphonates (BP) and bone morphogenetic protein 2(BMP-2).

We hypothetised, that BP would prevent early resorption in response to interventional trauma and BMP-2 support bone formation, which is impaired in osteoporotic bone.

METHODS: 41 female wistar rats were divided into 7 groups: Groups 1 and 2 were the healthy controls, groups 3 to 7 were ovariectomized at 13 weeks. All animals received a BaSO4-PEEK miniscrew in the proximal tibia at 25 weeks. In groups 2 and 4, pure hydrogel was pipetted into the drill hole before screw insertion. ZOL-BMP2 loaded hydrogel was given in group 5. Group 6 received zoledronate systemically, group 7 zoledronate systemically and BMP-2 locally.
Rats were euthanised 28 days post screw insertion.

Bone mineral density (at 12, 24 and 29 weeks) and implant osseointegration (0, 3, 6, 9, 14, 20 and 28 days post-op) were monitored using in-vivo microCT. Post mortem, samples underwent histological examinations to determine osseointegration on a cellular level.

RESULTS: Our preliminary data show, that the pure hydrogel is bioinactive in terms of implant fixation. ZOL-BMP2-hydrogel induces significant increase of bone-implant contact and peri-implant bone fraction, mostly through reduced resorption.

DISCUSSION & CONCLUSIONS: In conclusion, combination of zoledronate and BMP-2 might be able to improve implant stability in osteoporotic bones significantly and local delivery might be a potent alternative to systemic drug administration at significantly lower bisphosphonate doses.

ACKNOWLEDGEMENTS: This study was funded by AO Trauma.

Critical size bird bone defect healed with xenogeneic ostrich cancellous bone grafts seeded with aviary bone cells – experimental bird model

D Harvanova[1], T Spakova[1], J Plsikova[1], J Amrichova[1], S Hornak[2], J Rosocha[1]

[1] *Associated Tissue Bank, Faculty of Medicine of P. J. Safarik University and L. Pasteur University Hospital, Kosice, Slovakia,*

[2] *Small animal's clinic, University of veterinary medicine and pharmacy, Kosice, Slovakia*

INTRODUCTION: Large bone defects do not heal autonomously. Gold standard in treatment of critical bone defects is autologous bone grafting. Treatment of bone fractures in avian species has many critical limitations and one of these is lack of donor cancellous bone source for eventual bone autografting. Lack of autologous bone grafts in birds has prompted investigation of avian xenografts for bone augmentation. The purpose of this study was to evaluate model bone regeneration of critical segmental bone defects in pigeons by application of ostrich cancellous bone grafts (OCBG) seeded with allogeneic donor bird bone cells.

METHODS: The experiments were performed on model animals - pigeons with the approval of the University of Veterinary Medicine and Pharmacy ethical committee. Critical segmental bone defects (1 x 0,5 cm) were created on ulna and pigeons were divided into 5 groups according to the bone defects healing process. Bone defects were filled with demineralized and non-demineralized OCBG separately or in combination with aviary bone cells. Control group was healed spontaneously without treatment. Isolation and cultivation of aviary bone cells and methods of their seeding on OCBS was described previously [l] Bone regeneration was evaluated by radiograph and histology 1, 2, 3, 4, 5, 6, 9, 12 months after implantation.

RESULTS: Bone regeneration was significantly enhanced in the defects treated with demineralized OCBG in combination with aviary cells when compared with other experimental groups. Three months after implantation, OCBG's resorption was observed in the defect. At 6 and 9 months after implantation, complete bone regeneration without residual bone substitute in the defect was obtained (Fig.1). New bone tissue in the defect was confirmed by histology. In the groups, where the OCBG was implanted without aviary bone cells, incomplete bone regeneration was observed. Nine months after demineralized OCBG implantation, smooth radiolucent line still remained on the radiological images. Nevertheless, the new bone formation was histologically confirmed in the defect even in the groups without applied aviary bone cells. No bone regeneration in the defect was observed in the untreated group

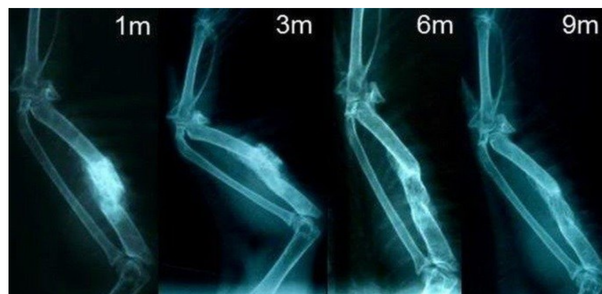


Fig. 1: RTG evaluation of bone regeneration after 1,3,6,9 month's implantation of demineralized OCBG with aviary bone cells into bone defects on pigeon's ulna

DISCUSSION & CONCLUSIONS:

Ostrich bone grafts used for bone regeneration were biocompatible but also have an appropriate time of degradation. When the rate of substitute degradation is too slow, the bone substitute itself can prevent bone regeneration. We demonstrate that OCBG was completely resorbed by 3 months after implantation in all cases. More important fact is that OCBG provided an effective biological support for aviary bone cells. We conclude that OCBG in combination with aviary bone cells had osteoconductive and osteoinductive properties which are necessary for the regeneration of bone defects in birds.

ACKNOWLEDGEMENTS:

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Effect of zoledronate on periodontitis in a compromised rat model

L Huo[1], LW Zheng[1]

[1] *Discipline of Oral diagnosis & Polyclinics, Faculty of Dentistry, The University of Hong Kong, Hong Kong SAR, China*

INTRODUCTION: Bisphosphonate is a powerful antiresorptive medication which has been widely used to manage osteoporosis and bone complications in cancer patients. Some studies found that bisphosphonate may benefit the treatment of periodontitis, however, other studies implied that periodontitis is a precipitating factor which may increase the risk of bisphosphonate related necrosis of the jaw. The aim of this study was to assess the risk and benefit of bisphosphonate on treating periodontitis using a compromised rat model.

METHODS: All 45 female SD rats received bilateral ovariectomy and then were assigned randomly to three groups, 15 in each: Group A received zoledronic acid (ZA) at a high dose (67µg/kg, thrice per week, *i.p.*), Group B received ZA at a low dose (88µg/kg, once per month, *i.p.*), and Group C was injected with saline[1]. Along with ZA treatment, dexamethasone (DEX) was administrated to all the animals to mimic a compromised condition[2, 3]. At the same day of the injection, ligature was placed around the left mandibular first molar (L-M1) of each rat. 12 weeks later, all the rats were sacrificed and the whole mandibles were collected for micro-CT scanning. The distance between the cemento-enamel junction and alveolar ridge crest (attachment loss) of L-M1 was measured, the bone mineral density (BMD) was also detected and statistically analysed.

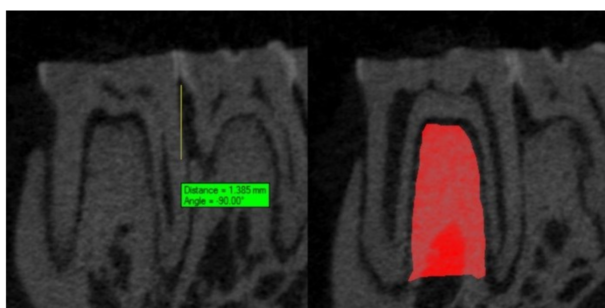

Fig. 1: The measurements of Bone loss (left) and BMD (right)

RESULTS: Compared with saline treated group, the attachment loss of the ligature site was inhibited significantly in both low dose and high dose ZA group (*$p<0.05$*), while no significant different between low and high dose ZA was noted. Compared with both saline treated and low dose ZA treated groups, the BMD in the high dose ZA treatment group was significantly improved.

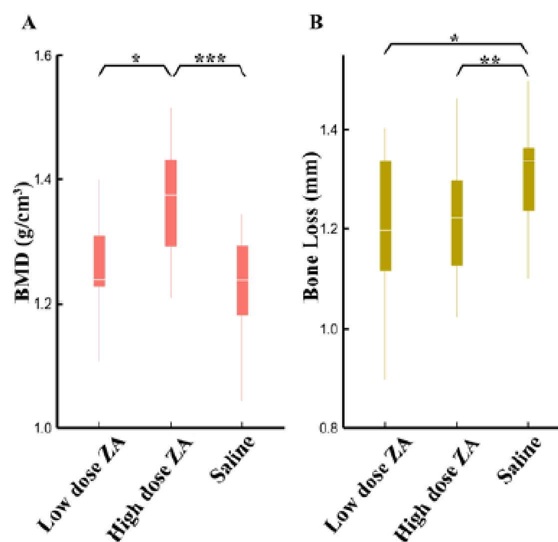

Fig.2: Comparison of BMD (A) and Bone loss (B) between different treatments

DISCUSSION & CONCLUSIONS: The systemically administrated zoledronate could benefit the treatment of periodontitis, either in high dose or in low dose, by inhibiting alveolar bone loss in the compromised rat model.

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

Impact of cell substrate curvature on matrix deposition and osteoblastic behavior

L. JUIGNET [1,2], B. CHARBONNIER[1,3], V. DUMAS[4], C. LAURENT[1,3], L. VICO[1,2], D. MARCHAT[1,3], N. DOUARD[1,3] & L. MALAVAL[1,2]

[1]*INSERM U1059, Saint-Etienne, France.* [2]*Université de Lyon, Saint-Etienne, France.* [3]*Ecole des Mines de Saint-Etienne, Saint-Etienne, France.* [4]*LTDS, UMR 5513 CNRS, Ecole Nationale d'Ingénieurs de Saint Etienne, Saint-Etienne, France.*

INTRODUCTION: One of bone tissue engineering applications is to develop *in vitro* models of living tissues to better understand their physiology. *In vivo*, cells reside in a complex and three-dimensional microenvironment. However, most of our knowledge on cell physiology has been obtained from cell cultures in Petri dishes, on plastic and in two dimensions. In those conditions, the spatial relationships between cells and their environment can only be deeply modified. Very few studies have shown its role at a tissue level, mostly focused on the matrix deposition rather than on the osteoblastic differentiation [1,2].

METHODS: Calcium phosphate biomaterials (hydroxyapatite: HA) with macroscopic grooves of different geometries were cast via an additive manufacturing process. These bioceramics display three different patterns of increasing curvature: circular grooves ("waves"), triangular grooves with a 90° angle ("90°") and triangular grooves with a 45° angle ("45°") [Fig.1]. Mouse calvaria primary cells were seeded on the biomaterials and cultured for 15 days in osteogenic medium. Different characterization methods ware used to investigate cell behavior (attachment, orientation, growth) and differentiation on these curved substrates, compare to flat HA.

RESULTS: Cell attachment and growth are accelerated in the early culture times on grooved materials. After 15 days, the cells formed a large and complex network of extracellular matrix (ECM) on the three types of architectures. However, the volume of ECM is significantly higher in the pattern "45°" (33.8±3.2 μm^3) compared to "90°" (8,8±1,8 μm^3). This suggests that ECM synthesis increases with substrate curvature. Quantitative immunodetection of osteoblastic differentiation markers (osteopontin, osteocalcin) at different depths in grooves [Fig.2] reveals distinct paterns of expression on the bioceramincs, suggesting distinct differentiation kinectics. It was demonstrate that the more curvature is important, the more osteocalcin quantity is important. These preliminary data suggest that osteoblastic differentiation is earlier in high curvature structures.

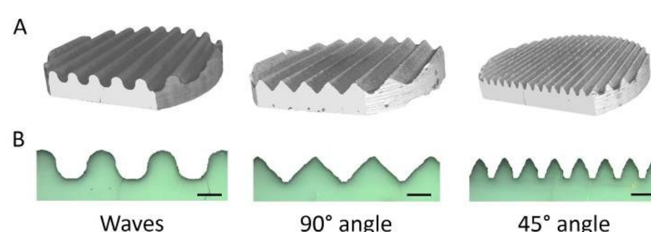

Fig. 1: Images in µCT (A) and sections (B) of HA scaffolds, showing the three different patterns tested ("waves", "90°", "45°"). Bar=500µm

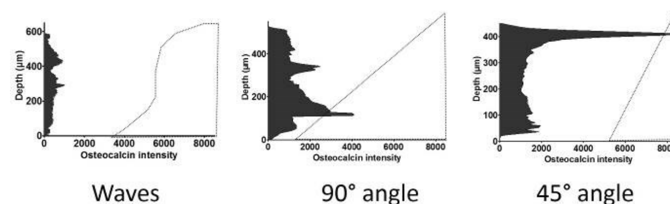

Fig. 2. Quantitative immunodetection of osteocalcin, a late osteoblastic differentiation marker, at different depth between the top of the ridges and the bottom of the grooves in the 3 bioceramic patterns.

DISCUSSION & CONCLUSIONS: Substrate curvature seems to affect both the deposition of extracellular matrix and osteoblast differentiation. These observations could provide insights on fundamental cellular mechanisms in bone biology but also the design of innovative biomaterials for bone tissue engineering.

ACKNOWLEDGEMENTS: We gratefully acknowledge the Rhône-Alpes Region (ARC program, PhD scholarship to LJ).

A new metaphyseal implant associated fracture healing model applied in osteoporotic knockout mice

V Kauschke [1], M Schneider[1], A Jauch[1], M Kampschulte[2], M Schumacher[3], M Gelinsky[3], C Heiss[1,4], KS Lips[1]

[1] *Institute of Experimental Trauma Surgery, Justus-Liebig-University Giessen, Giessen, Germany*
[2] *Department of Radiology, University Hospital of Giessen-Marburg, Giessen, Germany*
[3] *Centre for Translational Bone, Joint and Soft Tissue Research, Medical Faculty and University Hospital, Technische Universität Dresden, Dresden, Germany*
[4] *Department of Trauma Surgery, University Hospital of Giessen-Marburg, Giessen, Germany*

INTRODUCTION: Osteoporosis emerges when an imbalance between osteoblastic bone formation and osteoclastic bone resorption exists. It is a systemic disease, characterized by alterations in bone microarchitecture and reduced bone mineral density, often resulting in fractures. There is an urgent need for new bone substitution materials adapted to properties of osteoporotic bone.
Calcium phosphate cements are known to support mesenchymal stem cell differentiation into osteoblasts and therefore considered as suitable bone substitution materials [1]. However, in osteoporosis osteoblast differentiation requires additional stimulating agents. Analyses of fracture gap tissue revealed that Brain-derived neurotrophic factor (BDNF) was up-regulated during the bone formation process, indicating that BDNF is involved in fracture healing [2]. Recently it has been shown that knockout (KO) of the muscarinic acetylcholine receptor 3 (mAChR 3) resulted in an osteoporotic phenotype in mice [3]. We therefore conducted an osteoporotic murine metaphyseal fracture healing model using muscarinic acetylcholine receptor 3 knockout (M3 mAChR-KO) mice for implantation of a calcium phosphate cement paste containing BDNF-doped mesoporous bioactive glass.

METHODS: Prior to *in vivo* experiments, BDNF release from mesoporous bioactive glass incorporated in calcium phosphate cement was determined by Enzyme Linked Immunosorbent Assay (ELISA) *in vitro.*
Surgeries were conducted on anaesthetized 16-week-old female homozygous M3 mAChR-KO mice and their corresponding wildtypes. A metaphyseal titanium locking plate (AO Foundation, RISystem, AO Research Institute Davos, Switzerland) was placed on the anterolateral side of the right femur as described by T. Histing *et al*. [4]. Subsequently, a 1.2 mm osteotomy was performed in the distal metaphyseal region using a Piezosurgery osteotomy bone saw. The fracture gap was filled with paste-like calcium phosphate cement containing BDNF-doped mesoporous bioactive glass. Mice were sacrificed after 35 days and femurs obtained for micro-CT and histological analyses.

RESULTS: ELISA confirmed that BDNF was released from the mesoporous bioactive glass embedded in the calcium phosphate cement.
Micro-CT images showed direct contact of newly formed bone to the BDNF-doped mesoporous bioactive glass modified calcium phosphate cement.

DISCUSSION & CONCLUSIONS: Preliminary results showed good biocompatibility of mesoporous bioactive glass modified calcium phosphate cement as well as osseointegration. To our knowledge this is the first study introducing a metaphyseal implant associated fracture healing model in osteoporotic KO mice. The advantages of using mice instead of rat or large animal models are faster reproduction cycles and lower costs.

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2

Chondrogenically differentiated xenogeneic MSCs in endochondral bone regeneration

MKE Koolen[1,2], A Longoni[2,3], AJWP Rosenberg[3], HH Weinans[1,2], D Gawlitta[2,3]

[1]*Dept. of Orthopaedics, University Medical Center Utrecht, Utrecht, NL,* [2]*Regenerative Medicine Center Utrecht, Utrecht, NL,* [3]*Dept. of Oral and Maxillofacial Surgery & Special Dental Care, University Medical Center Utrecht, Utrecht, NL.*

INTRODUCTION: In search for alternatives to gold standard treatment of large bone defects or non-unions, the potential of multipotent mesenchymal stromal cells (MSCs) to initiate endochondral bone regeneration via a cartilage intermediate, has been recognized.

Previously, implantation of human MSC pellets in immunocompromised rats has led to efficient bone regeneration in an orthotopic defect model[1]. Besides, endochondral bone regeneration by autologous cells in immunocompetent animals was also confirmed. However, considering large variations in chondrogenic differentiation potential of MSCs from different donors and logistical challenges, using non-autologous cells would ease clinical application of this approach.

Limited data are available on the feasibility of bone regeneration using allogeneic or xenogeneic MSCs following chondrogenic differentiation. Nevertheless, HLA expression patterns of chondrogenically differentiated MSCs are similar to those of undifferentiated MSCs[2]. While implantation of undifferentiated xenogeneic MSCs led to impaired bone formation compared to autologous MSCs[3], implantation of allogeneic MSCs that were osteogenically stimulated resulted in bone formation comparable to implantation of autologous MSCs[4]. Whether chondrogenically prestimulated non-autologous MSCs hamper bone formation remains unknown.

We evaluated if immune reactions were evoked to implanted xenogeneic, chondrogenically prestimulated MSCs and, if so, endochondral bone regeneration was affected by this.

METHODS: Human MSCs were isolated on Ficoll-Paque from consenting donors prior to undergoing harvest of bone from the iliac wing. At passages 5, the MSCs were centrifuged to form pellets of 200,000 cells each. They were differentiated in medium containing TGFβ for 21 days. Then, pellets (~1 mm) embedded in fibrin glue were implanted into critical size femur defects of immunocompetent rats, and controls of fibrin without pellets were included.

Following implantation in male Wistar rats, blood was sampled at 4, 8, and 12 weeks to monitor the levels of immune markers by ELISAs. Baseline levels were collected from untreated rats. At 12 weeks, samples were explanted and decalcified for paraffin sectioning. Histological staining for bone formation (H&E) and immunohistochemistry for vascularization (CD34), macrophages and B and T lymphocytes are currently under analysis. Micro-CT scans were performed at 0, 4, 8, and 12 weeks.

RESULTS: Micro-CT analysis showed around 20% of defect filling with new bone in both groups after 8 weeks of implantation (Fig.1). Also, on histology new bone formation was observed.

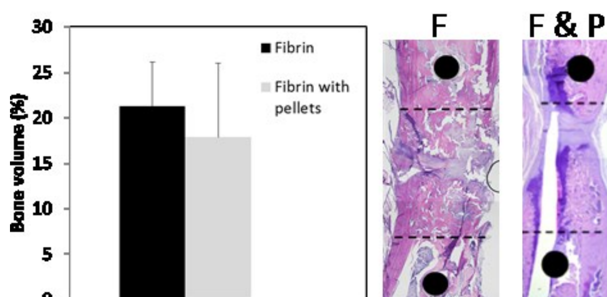

*Fig.1: percentage of defect filling (left, *p<0.05) and H&E of fibrin (F) +/- pellets (P)(right), both after 8 weeks.*

DISCUSSION & CONCLUSIONS:
No clear reactions of the animals to the xenogeneic implants were observed during the course of the experiment. Further analysis is ongoing.
The bone defects were filled to the same extent for fibrin with or without pellets at 8 weeks.

ACKNOWLEDGEMENTS: Contributions of E. Huethorst and A. Garcia Duran are appreciated.

human intra-articular stem cells isolated during arthroscopy surgery as a new cell source with high potential for cartilage regeneration

J Li[1, 2], YH Liang[1], ZY Zhou[1], WM Zhu[3], WJ Peng[1], and GQ Zhou[1]*

[1] The Centre for Anti-ageing and Regenerative Medicine, Shenzhen University, Shenzhen, China, [2] Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, Shenzhen University, Shenzhen, China, [3] The First Affiliated Hospital of Shenzhen University, Shenzhen, China

INTRODUCTION: Mesenchymal stem cells (MSCs) from various adult tissues have emerged as a potential therapy for osteoarthritic joint degeneration[1]. However, there remain significant challenges in obtaining optimal cell source for cartilage repair. Two primary criteria that are generally considered are the regenerative properties of the cells and their ease of access[2 _ENREF_2]. Hereby, we will introduce a new available and valuable way to get highly potential cell source, human intra-articular-derived mesenchymal stem cells (hIAMSCs) from arthroscopy surgery.

METHODS: Isolation of human intra-articular-derived cells (hIA-derived cells). With the approval of university and hospital institutional review board, 10 patients from 40 to 50 years old who had a diagnosis of knee osteoarthritis (based on OARSI) were perfomanced with arthroscopic lavage and debridement. We collected the lavage liquid of knee intra-articular in the early surgery. After centrifuging, the deposit with cells were resuspended in MEM with 20% FBS, and were plated and cultured to form colonies. Non-adherent cells were removed 2 days after initial plating. **Characterizations of hIAMSCs.** *Cell proliferation assay* -- Passage 5 hIA-derived cells were cultured in triplicate for designated time points and were measured via CCK-8 assay. *Induced differentiation assays* -- After incubating in adipogenic, osteogenic, and chondrogenic media, the induced hIA-derived cells were stained with 0.36% fresh oil red O, 0.1% alizarin red S, and 1% toluidine blue O solution, respectively. *Flow cytometry assay* -- One million passage 5 hIA-derived cells were incubated for 1 hour at 4℃ with conjugated antibodies to CD34-FITC, CD45-FITC, CD73-APC, CD90-PerCP-Cy5.5, or CD105-PE. Then these labelled cells were re-suspended and subjected to flow cytometry analysis.

RESULTS: During primary culturing, hIA-derived cells started to adhere after plating 24hrs and form obvious distinctive colonies at 7 days (**Fig. 1 A-D**). During subculturing the hIA-derived cells proliferated, with a typical population doubling time of 23.8 hrs (**Fig. 1E**). The multi-differentiation potential of hIA-derived cells was checked by culturing them in adipogenic, osteogenic, and chondrogenic media, respectively. After 14 days of culture in adipogenic medium, lipid droplets were formed within hIA-derived cells and observed with oil red O stainning (**Fig. 2A**). Meanwhile, osteogenic and chondrogenic differentiation of these cells was revealed by positive alizarin red-S and toluidine bule O staining at 21 days (**Fig. 2B-C**). Fluorescently activated cell sorting (FACS) analysis was utilised to label the hIA-derived cells for a series of mesenchymal stem cell markers. We found that these cells expressed in over 99% of the positive markers of MSCs, including CD73, CD90, and CD105, whereas they seldom expressed less than 1% in the negative markers such as CD34, and CD45 (**Fig. 3A-E**).

DISCUSSION & CONCLUSIONS: Within arthroscopic lavage liquid, we have successfully and efficiently isolated hIA-derived cells which possess clonogenicity, self-renewing capability, multidifferentiation potential, and stem marker expression, the common characteristics of MSC. Future research will explore the cartilage repair capacity of hIAMSCs in vivo animal and human, and compare the efficiency with other commonly used MSCs. It may open a new avenues to achieve highly potential cell source for cartilage repair and renewal using cell therapy or tissue engineering approaches.

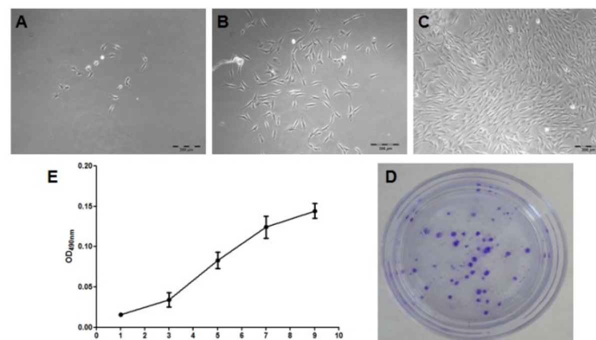

Fig. 1. *Colony formation and growth curve of hIA-derived cells. (A-C) Cell colony formation at 1, 3, and 7 days, respectively. (D) Total colonies stained with crystal violet at 7 days. (E) Cell growth curve. Scale bars, 200μm.*

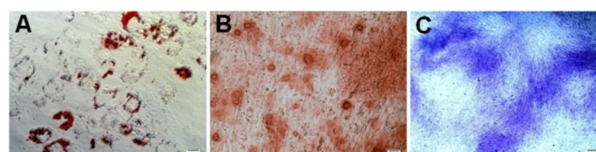

Fig. 2. *Induced differentiation of hIA-derived cells. (A-C) Oil red O, alizarin red S, and toluidine blue O staining for adipogenic, osteogenic, and chondrogenic differentiation, respectively. Scale bars, 200μm.*

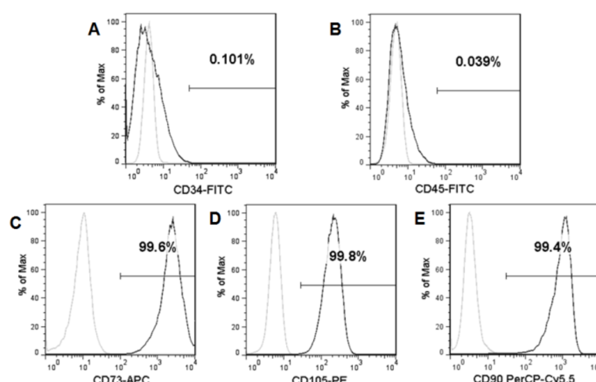

Fig. 3. *Flow cytometry assay of hIA-derived cells. These hIA-derived cells were identificated by labelling for of MSC surface markers CD34 (A), CD45 (B), CD73 (C), CD90 (D) and CD105 (E).*

ACKNOWLEDGEMENTS: This study was supported by NSFC (81472126) Shenzhen commission on innovation and technology grant. (#JCYJ20150626090344603).

Chondrogenic differentiation of bone marrow derived stem cells (MSCs) versus adipose derived stem cells (ASCs) at pro-inflammatory conditions

***C. Neidlinger-Wilke*[1], *G.Q. Teixeira*[1-3], *A. Rapp*[1], *D. Kletsas*[4], *A. Ignatius*[1] [1]**

[1]Institute of Orthopaedic Research and Biomechanics, Center for Musculoskeletal Research, University of Ulm, Germany, [2]Institute of Biomedical Engineering (INEB), Universidade do Porto, Portugal, [3]Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, Portugal, [4]Laboratory of Cell Proliferation & Ageing, Institute of Biology, NCSR Demokritos, Athens, Greece.

INTRODUCTION: Stem cells are considered as suitable cell source for regenerative therapies of cartilaginous tissues and bone due to their potential to differentiate towards osteogenic, adipogenic or chondrogenic phenotypes [1]. Human adipose-derived stem cells (ASCs) gained increasing interest as an alternative cell source to bone marrow-derived stem cells (MSCs) due to their easy accessibility and lower donor site pain. Regarding the treatment of degenerated cartilaginous tissues it has to be considered that degeneration is often associated with an increased occurrence of inflammation factors such as IL-1β and TNF-α [2]. These conditions are challenging for regenerative cell therapy approaches as they may influence cell physiology and differentiation. For ASCs it has been shown that inflammatory conditions influence their morphology and proliferation but their osteogenic and adipogenic differentiation capacity remains unaffected [3]. For human MSCs an induction of osteogenic differentiation by IL-1β has been reported [4]. Regarding chondrogenic differentiation at pro-inflammatory conditions, a direct comparison of both cell types is missing. Purpose of the present study was to characterize human MSCs and ASCs and to compare their chondrogenic differentiation capacity at pro-inflammatory conditions with regard to their suitability for cell therapy of degenerated intervertebral discs or osteoarthritis.

METHODS: Human MSCs and ASCs from each three donors (18-50 years old) that were pre-tested regarding the expression of stem cell markers (CD105, CD73, CD44, CD90 and SSEA4) were cultured for four weeks in micromass-pellets of each 200.000 cells in chondrogenic differentiation medium with TGFβ3 as described [5]. For simulation of pro-inflammatory conditions, the chondrogenic medium of parallel pellet cultures was supplemented by IL-1β during the whole culture period. Cultures with standard medium without TGFβ3- or IL-1β-supplementation served as controls. At the end of the differentiation period each three pellets/group were characterized with regard to morphology and matrix formation (Alcian blue staining) and expression of chondrogenic target genes (Sox-9, Coll-II, Aggrecan). Groups were compared by descriptive statistics.

RESULTS: In cultures supplemented by medium with TGFβ3, pellet formation occurred within the first culture week with an increasing size and optical density of the pellets during the four weeks of culture time. Cultures supplemented with IL-1β showed a 0.5-0.75-fold reduced pellet size. Cultures with standard medium without TGFβ3 failed to form pellets. Differentiation of both MSCs and ASCs could be confirmed by an up-regulation of Aggrecan (up to 30-fold), Coll-2 (up to 600-fold) and Sox-9 (up to 26-fold) expression at presence of TGFβ3. This effect was strongly decreased (0.6-0.2-fold) in IL-1β-supplemented pellet cultures of both MSCs and ASCs with high variability of the absolute gene expression levels with cells from different donors. The impaired differentiation capacity of ASCs and MSCs at presence of IL-1β could also be confirmed by reduced Alcian blue staining of the pellets. This effect was similar with both ASCs and MSCs with variability of staining intensity of pellets from different donors.

DISCUSSION & CONCLUSIONS: Our findings suggest that a pro-inflammatory stimulation with IL-1β impairs chondrogenic differentiation capacity of both MSCs and ASCs in pellet cultures. Therefore, inflammatory conditions may reduce matrix-formation if these cells are applied for cell therapy, e.g. in osteoarthritis or in a degenerated disc environment. As this effect could be shown for ASCs and MSCs, cells from both tissue sources appear to be similar sensitive towards pro-inflammatory conditions. These findings suggest that an anti-inflammatory treatment previous to cell therapy might improve the microenvironment for a successful cell therapy of disc degeneration or osteoarthritis.

THE EFFECTS OF HUMAN BONE GRAFTS ON OSTEOGENIC DIFFERENTIATION OF ADIPOSE TISSUE DERIVED MESENCHYMAL STROMAL CELLS *IN VITRO.*

J Plsikova[1], D Harvanova[1], T Spakova[1], S Gromosova[1], M Lacko[2], T Kluka[3], J Sevc[4], J Rosocha[1]
[1]Associated Tissue Bank, Faculty of Medicine, University of P.J. Safarik and L. Pasteur University Hospital, Kosice. [2]Department of Plastic, Reconstructive and Aesthetic Surgery, Faculty of Medicine, University of P.J. Safarik and L. Pasteur University Hospital, Kosice. [3]Department of Orthopaedics and Traumatology of Locomotory Apparatus, Faculty of Medicine, University of P.J. Safarik and L. Pasteur University Hospital, Kosice. [4]Institute of Biology and Ecology, Faculty of Science, University of P. J. Safarik, Kosice.

INTRODUCTION: Human donor cancellous bone, as originally allogeneic material is a well utilized bone tissue engineering scaffold. Cell free allograft bone has longer incorporation time than autograft and cannot elicit the same osteoinductive response as autograft bone. Processing of allogeneic bone minimizes the risk of an immunologic response of the recipient. Adipose tissue derived human MSCs share many of the characteristics of bone marrow derived MSCs including extensive self-renewal capacity and capacity to undergo differentiation into many mesenchymal types with low immunogenicity in recipient´s organism [1]. Many studies have reported bone regeneration using MSCs from adipose tissue [2]. It has been demonstrated, that combination of the cancellous bone scaffolds with MSCs accelerate and enhance bone formation within osseous defects when compared with the matrix alone [3]. Stem cell-seeded bone allografts therefore have great potential for bone regeneration.

METHODS: Adipose tissue derived human MSCs (hADCs) were isolated from human subcutaneous tissue with the enzyme Collagenase type I and cultured *in vitro* up to 3rd passage. Immunophenotype characterization (CD105, CD90, CD73, CD29, CD45) of hADCs was performed by flow cytometry and analyzed in a BD FACSCalibur using CellQuest software. Differentiation potential of hADCs was also performed. Human cancellous bone scaffolds (hCBS) were prepared according to standard operating procedures used in Tissue Bank. ADCs were labelled with PKH 26 dye and seeded on hCBS. After 4 weeks of static *in vitro* cultivation, cryosection of hCBS with cells were visualized on fluorescence microscope. Proliferation of ADCs alone and seeded on hCBS in standard and osteogenic medium was analysed by MTS colorimetric assay. Adherence, localization and differentiation of hADCs on hCBS was observed by HE staining, scanning electron microscopy (SEM) and actin/vinculin double staining on confocal microscopy.

RESULTS: Flow cytometric analysis showed that ADCs were positive for CD90 (98.9%), CD105 (82.93%), CD73 (99.62%) and CD29 (98.27%) antigens and negative for CD45 (0, 73%) antigen. Mesenchymal character of ADCs was also confirmed by in vitro differentiation test. Attachment of PKH26 labeled cells on the surface of hCBS was confirmed by fluorescence microscopy. Proliferation of hADCs alone and seeded on hCBS measured with MTS colorimetric assay was comparable in both standard (nonosteogenic) and osteogenic medium. The effect of hCBS on osteogenic differentiation of hADCs was observed with SEM. hADCs cultured in osteogenic medium formed on the hCBS surface sheets of cells with flat morphology like osteoblasts. In comparison with hADCs cultured in standard medium, hADCs did not formed layers, they were observed like colonies of cells. The adherence and localization of hADCs on hCBS was confirmed also by HE staining and confocal microscopy.

DISCUSSION & CONCLUSIONS: hADCs seeded on hCBS adhere mainly on the graft surface. hCBS had no significant effect on differentiation of hADCs in comparison with osteogenic medium.

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Characterization of a novel dynamizable external fixator for ovine tibial segmental defects

Nicholas P. Quirk[1], Andrew Thoreson[1], Rodolfo E. De la Vega, M.D.[1], Michael J. Coenen[1], Miguel Trujillo, Ph.D[1], Consuelo M. Lopez De Padilla, M.D.[1], Slobodon Tepic, D.Sc.[2], Christopher H. Evans, Ph.D.[1]

[1] Mayo Clinic, Rochester, MN, [2] Kyon AG, Zurich, Switzerland

INTRODUCTION: Large segmental defects in long bones present a clinical challenge to surgeons. There is much interest in the influence of the mechanical environment on the healing of these defects. Dynamization of the fracture gap has been shown to promote the subsequent stages of healing and maturation. This occurs through stiffness modulation of the fixation construct, stabilizing fracture during healing and has been successfully evaluated in rodent models. Prior to large animal study translation, a suitable, adjustable, well-characterized, external fixator is required.

The ultimate goal of using this fixator is to modify the defect mechanical environment in conjunction with recombinant human BMP-2 to improve healing in an ovine tibial segmental defect model.

METHODS: Fixators were characterized through mechanical testing by sawbone and ovine cadaver tibiae samples, and data was used to validate a finite element (FE) model. A 30mm fracture defect and 20 mm 'bone-to-fixator' offset was used on all samples. Extensometers were attached across the defect for inter-fragmentary movement (IFM) and at fixator ends to characterize flexural deformation of the fixator. Plastic and elastic axial compressive testing, torsional testing and cyclic axial testing were performed on the constructs. A FE model was developed using ANSYS and utilized quadratic tetrahedral elements (390052 elements and 624494 nodes), simplified geometry (no screw threads / fixator 'nuts & bolts') and fixed (bone-screw, fixator bodies) and frictional contact (screw-fixator) conditions.

RESULTS: Plastic axial testing showed yielding for low stiffness configuration at 520 N and 550 N for high stiffness. Elastic axial testing showed corroboration between sawbone and cadaveric samples. Elastic axial testing and torsional testing confirmed FE model predictions. IFM exhibited a mean value of 1.526 mm and 0.901 mm for low and high stiffness, respectively, for elastic axial testing. Cyclic fatigue testing showed plateaued deformation across 100,000 cycles for all groups.

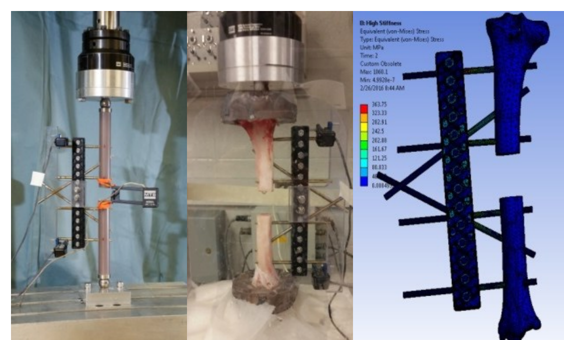


Fig. 1: Methods of characterization for external fixator (left to right); sawbone mechanical testing, cadaveric sample mechanical testing, FE analysis.

DISCUSSION & CONCLUSIONS: Fixator dynamization increased the construct stiffness by approximately 2-fold. Based on prior results from rat models, this is appropriate for enhanced bone healing. Moreover, negligible IFM differences of the fracture gap occurred during repeated load-cycling to mimic the projected lifecycle of the fixator while attached to the sheep. This shows stability of fixator across its life span and efficacy in a weight bearing animal. FE model results were generally in agreement with bench testing in key mechanical properties.

The successful design, manufacture and characterization of this external fixator provides the means to evaluate the efficacy of dynamization in ovine models of bone healing. This fixator may be useful in small animal veterinary practice and could form the basis for a device suitable for use in humans.

ACKNOWLEDGEMENTS: We would like to thank Lawrence Berglund for assistance with mechanical testing. This study was funded as part of a Department of Defense research grant (award number W81XWH-13-1-0324).

One Year Follow Up on Hybrid System in Spinal Tuberculosis Surgery

Rahyussalim AJ[1], D. Y. Pranatha[1], T Kurniawati[2]

[1]*Department of Orthopaedic and Traumatology Faculty of Medicein Universitas Indonesia –Ciptomangunkusumo Hospital*
[2]*Stem Cell Integrated Medical Service Faculty of Medicein Universitas Indonesia –Ciptomangunkusumo Hospital*

INTRODUCTION: Tuberculous spondylitis causes damage to the corpus and makes spinal instability. Operating procedures have been developed treating tuberculous spondilytis with some combination of conventional techniques and minimally invasive technique (Hybrid System) which allows the incision is required at the level of the vertebrae which will be stabilized.

METHODS: Patient who have tuberculous spondylitis which had been had corpus damages on vertebrae Th12-L1 and L4-L5, had been treated with posterior spine stabilization with Hybrid System and percutaneous abscess drainage. Blood loss, duration of surgery, length of stay, Visual Analogue Scale (VAS) and fusion status were evaluated for one year. The improvement of neurological was documented and its functional outcome was assessed using measurements Oswestry Disability Index (ODI)

RESULT: Intra operative blood loss was 150cc, 5 hours operating time and 5 days duration of hospitalized. After one year of follow-up, we found several data such as : a deflation in VAS score from 7-8 into 0-1; an x-ray photograph of anteroposterior thoracolumbal and lateral showed appearance of bridging trabeculae at the anterior and posterior column, there was no significant changes on motoric function at lower limb and improvement of neurological on L1 dermatome. The functional scoring based ODI before surgery was on 27 points (54% severe disability category) and after one year of follow-up, it changed into 2 points (4% minimum disability category)

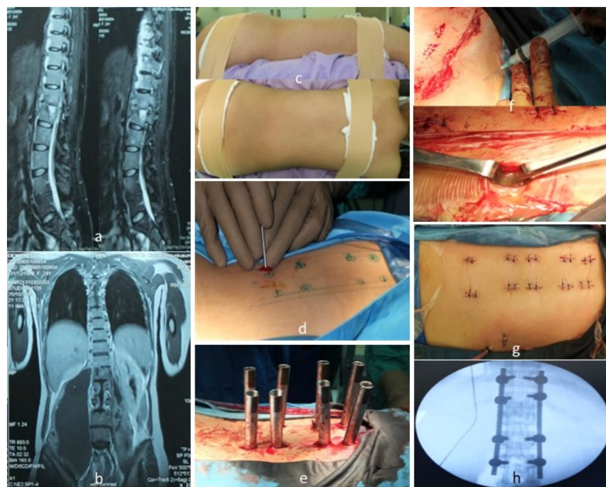


Fig. 1: Overview of Hybrid System procedure of spinal tuberculosis surgery a, b: MRI showed tuberculous spondylitis Th12-S1 with destruction of intervertebral disc on T12-L1 and L4-L5. Bilateral paravertebral abscess on Th7 into S2-S3 with involvement on bilateral m. Psoas, c: prone position, d: pre-operative localization, e: placement Illico MIS System, f: percutaneous abscess drainage, g: result , e: result on C-arm.

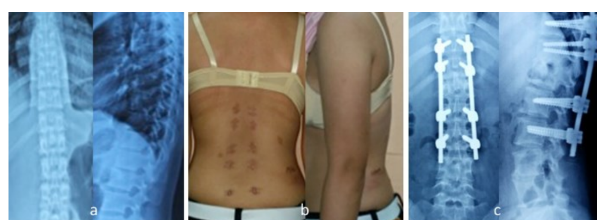


Fig. 2: Patient of one year follow up. a:Pre operative x-ray shows abscess and vertebral damage, b:Multiple incission after one year, c:Post operative x-ray shows implant on thoracolumbar area.

DISCUSSION AND CONCLUSION: Compared with conventional techniques, Hybrid System procedures have similar effectiveness in achieving spinal stability and abscess drainage. This prosedur proved to be more effective in reducing the amount of bleeding, tissue damage and length of stay, although this procedure still requires a longer operating time.

Single Concave Correction Technique for Main Thoracic Curve Scoliosis Lenke Classification Type 1

Rahyussalim AJ[1], Ifran Saleh[1,] T Kurniawati[2], M. Triadi Wijaya, Ahmad Yanuar Safri[3]

[1]Department of Orthopaedic and Traumatology Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital.
[2]Stem Cell Integrated Medical Service Unit Cipto Mangunkusumo Hospital-Faculty of Medicine Universitas Indonesia.
[3]Neurophysiology Division, Department of Neurology Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital.

INTRODUCTION: Idiopathic scoliosis Lenke 1 are found in 30-40% of all patients with idiopathic scoliosis. Usually, type 1 Lenke curvature is not as extreme and commonly found in adolescents and adults. In term of anatomical structure, the main thoracic curve in this scoliosis is supported/ surrounded by ribs which strengthen the structure of the spine in order to gain stability. Scoliosis surgery for this type can be done by using the anterior approach technique, thoracoplasty, or posterior approach using a spinal derotation technique which typically done by fixing a pedicle screw and rod on both sides. We have developed another approach to operations idiopathic scoliosis Lenke-1 called Single Concave Correction Technique (SCCT) based on anatomical structure and the biomechanic of main thoracic curve which works mainly on the concave side to provide adequate correction. This paper reports the results of operations using SCCT approach to evaluate the advantages of SCCT compared to other techniques currently established.

METHODS: SCCT is a scoliosis surgery using posterior approach on one side of the concave area. The correction is done by installing 4 to 5 screws to adjust the curvature shape of the main thoracic on the upper spine curve and 4 to 5 screws on the lower vertebrae curve, straightening is done by unbending the curvature, and derotating of the spinal rotation. Meanwhile, kyphosis can be managed by manipulating the anteropesterior side.

RESULT: We reported 3 cases of adolescent scoliosis and 1 case of adult scoliosis surgery with SCCT with follow-up period of 6 months, as follows:

Table 1. Evaluation of three cases with SCCT approach. It shows less screws and bloodless

Evaluation	Patient		
	KN	YN	AP
Screws amount (pcs)	7	9	11
Rod amount (pcs)	2	2	2
Rod connector amount (pcs)	1	1	1
Duration of surgery (minutes)	130	110	150
Bleeding (cc)	200	300	260
Post operation hospitalized (days)	5	4	5
Pre operation curve (degree)	70	72	79
Post operation curve (degree)	26	21	36
Correction achievement (%)	63%	71%	54%
Height increasement	6	8	5

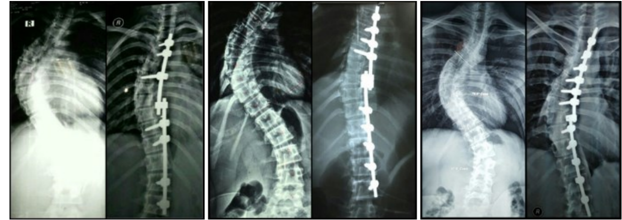


Fig. 1: Overview of curve after single concave correction technique manipulations. It shows optimal result with more than 50% correction.

DISCUSSION AND CONCLUSION: SCCT improved surgery duration, minimal bleeding and shortening of length-of-stay. This was due to the correction that was done on one side so there would be less damage on the tissue compared to the conventional techniques which were done on two sides. SCCT also provided satisfactory corrections and significant addition of height because only a small number of screw and rod are used hence financially more efficient. In long term follow-up, we were optimistic that we will not find any major problems regarding the application of SCCT in scoliosis. Management of Idiopathic scoliosis type 1 (lenke classification) using SCCT approach provided good result after 6 months follow up.

Toxicity and Biocompatibility Profile of Scaffold developed by Universitas Indonesia on Mesenchymal Stem Cells: A Preliminary Study

Rahyussalim AJ[1], T Kurniawati[2], Aprilya D[1], R Anggraini[2], Yudan Whulanza[3], G Ramahdita[3]

[1]*Department of Orthopaedic and Traumatology Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital.*
[2]*Stem Cell Integrated Medical Service Unit Cipto Mangunkusumo Hospital-Faculty of Medicine Universitas Indonesia.*
[3]*Department of Metallurgy and Material Faculty of Engineering Universitas Indonesia*

INTRODUCTION: Scaffold as a biomaterial must fulfill some requirements to be safely implanted to the human body. Toxicity and biocompatibility test are needed to evaluate scaffold material in mediating cell proliferation and differentiation, secreting extracellular matrix and carrying bio molecular signals for cell communication. This study aims to evaluate the toxicity and biocompatibility profile of various scaffolds with different materials developed by Universitas Indonesia by an in vitro study with mesenchymal stem cells.

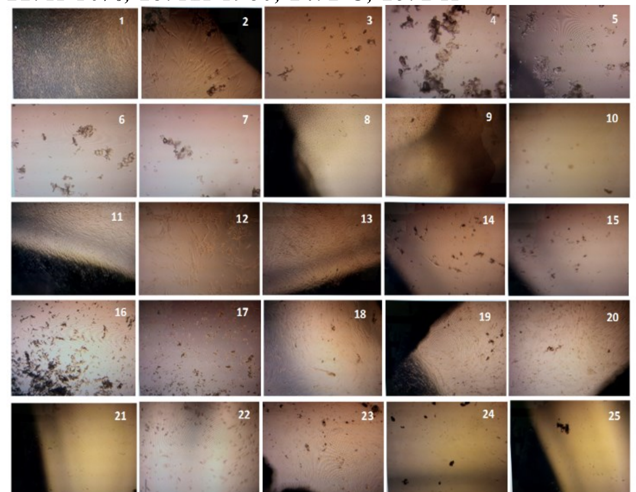
METHODS: Toxicity and biocompatibility test were conducted on 24 scaffolds made of poly-L-lactic acid (PLA), hydroxyapatite (HA), chitosan, Alginates, dicalcium phosphate dihydrate (DCPC), and polyvinyl alcohol (PVA) with direct contact test and indirect contact test using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay. To investigate cell toxicity by direct contact test, scaffolds were placed in the wells of mesenchymal stem cell (MSC) culture plates and then the cell proliferation inhibitions were determined. In MTT assay, scaffolds and cultured cells are tested with Vybrant® and the absorbance value was determined by ELISA reader at 570 nm wave length. Cell morphology, proliferation and cell adhesion to plastic material were evaluated at day-2 and day-6.

RESULT:

Table 1. Toxicity and biocompatibility of 24 scaffolds and control

Scaffolds	Direct Inhibition (%)	Indirect (MTT assay) Inhibition day-1 (%)	Indirect (MTT assay) Inhibition day-7 (%)	Material
Primo	54.90	40.25	27.75	PLA
ALBAB 20	100.00	-43.50	-37.00	HA-Chitosan
ALBAB 30	100.00	-45.25	-41.25	HA-Chitosan
ALBAB 40	100.00	-48.5	-48.5	HA-Chitosan
ALBAB 80	100.00	-45.25	-48.75	HA-Chitosan
C2	100.00	-47.00	-48.50	HA-Chitosan
C3	100.00	-44.75	-53.00	HA-Chitosan
C4	100.00	-48.25	-46.75	HA-Chitosan
DM 0%	94.12	34.00	-45.25	HA-PVA
DM 25%	94.12	16.00	-24.25	HA-PVA
DM 40%	94.12	30.50	6.50	HA-PVA
DM 50%	96.08	33.50	-7.25	HA-PVA
N-20	74.51	-46.75	-10.00	HA-Chitosan
N-30	100.00	-45.00	-44.00	HA-Chitosan
N-40	100.00	52.50	-43.75	HA-Chitosan
N-80	98.04	-48.25	-45.75	HA-Chitosan
R-Alg 50	80.39	-43.75	-37.75	HA-Alginate
R-Alg 60	62.75	-48.25	-51.75	HA-Alginate
R-Alg 70	92.16	-44.25	-48.75	HA-Alginate
H-0%	100.00	-51.25	-51.75	DCPD-HA
H-10%	98.04	-47.00	-45.75	DCPD-HA
RA-1980	68.63	5.75	11.25	DCPD-HA
HC	100.00	-46.75	-46.25	HA-Chitosan
DH	100.00	-41.25	-44.5	DCPD-HA
Control	0.00	100.00	100.00	cells & medium

Fig. 1: Cell morphology and proliferation of various scaffolds were observed at day-6 1. Control; 2. Primo; 3. ALBAB 20; 4. ALBAB 30; 5. ALBAB 40; 6. ALBAB 80; 7. C2; 8. C3; 9. C4; 10.DM 0%; 11. DM 25%; 12. DM 40%; 13. DM 50%; 14. N-20; 15. N-30; 16. N-40; 17. N-80; 18. R-Alg 50; 19. R-Alg 60; 20. R-Alg 70; 21. H-0%; 22. H-10%; 23. RA-1980; 24. DC; 25. DH



DISCUSSION AND CONCLUSION: There were cells-substrate adhesion impairment, morphological changes, cell death and reduction in cell proliferation seen at day-2 and day-6 in most tested scaffold except Primo, DM 0%, DM 25%, DM 40%, N-20, R-Alg 50, R-Alg 50, R-Alg 60, and RA 1980 scaffold. Cell count result at day-6 showed proliferation inhibition of more than 50% cell death (inhibition value > 50) in all tested scaffold. In MTT assay, Primo, RA-1980, and DM 40% were proven non-cytotoxic. Various scaffold materials showed different cytotoxicity effect. Primo as a polymer based scaffold showed the least cytotoxic effect, followed by DM 40% and RA-1980.

Implant Failure after Posterior Instrumentation on Extreme and Progressive Congenital Scoliosis with Some Comorbidities: 4 Years Follow Up

Rahyussalim AJ[1], Ifran Saleh[1], T Kurniawati[2], M. Triadi Wijaya, Ahmad Yanuar Safri[3]

[1]*Department of Orthopaedic and Traumatology Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital.*
[2]*Stem Cell Integrated Medical Service Unit Cipto Mangunkusumo Hospital-Faculty of Medicine Universitas Indonesia.*
[3]*Neurophysiology Division, Department of Neurology Faculty of Medicine Universitas Indonesia-Cipto Mangunkusumo Hospital.*

INTRODUCTION:
There are various complexities in congenital scoliosis ranging from the characteristic of rapid progressive, associated with fleeting cardiac and pulmonary function deterioration, worsening postural deformity predisposing limited range of motion and presence of other congenital disorders complicating the treatment. Until recently, the management of complex congenital scoliosis has not been satisfying as a consequence of poor accurate diagnosis, associative congenital comorbidities, and the possibility of crank shaft phenomenon and short stature development. Surgical techniques using posterior instrumentation has been the treatment of choice due to unsatisfactory conservative results. This paper showed implant failure after posterior instrumentation.

METHODS: It was a case report with 4 year follow up and done observations of surgical intervention, implant failure, progression and achievement of correction.

Case illustration: Nine year old boy with corrected anal and esophageal atresia which was diagnosed with congenital scoliosis at 2 year age with 40 degree of Cobb angle. He had undergone conservative and operative treatments since 5 year old but no optimal outcome, delay or progressivity and neither postural correction (figure 1).

RESULT: This case was performed two kinds of surgical interventions. The first surgery was single concave correction approach by using cervico-thoracic junction rod. The rod was failure 7 month after the first surgery. The second surgery was done to change screw and rod with bigger one. The second implants were also failure 35 month after the second surgery. (figure 1).

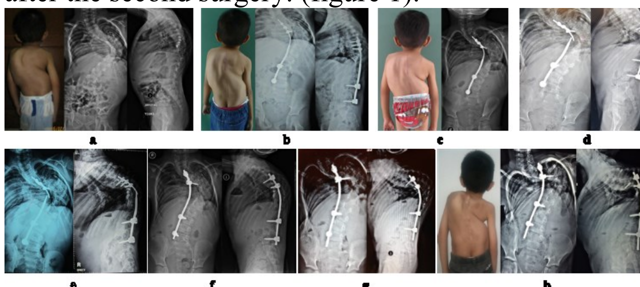


Figure 1. Four years follow up: (a) immediately pre operatif I; Cobb's angle 120° (b) post operatif I; Cobb's angle 89° (c) 3 month follow up post operatif I; Cobb's angle 100° (d) 7 month follow up post operatif I; Cobb's angle 102°; implant failure (e) 9 month follow up post operatif I; Cobb's angle 113°; implant failure (f) post operatif II; Cobb's angle 76° (g) 21 month follow up post operatif II; Cobb's angle 78° (h) 35 month follow up post operatif II; Cobb's angle 95°; implant failure.

Table 1. Evaluation of four years follow up on extreme and progressive congenital scoliosis with some comorbidities. It shows implant failure after posterior instrumentation.

Scoring Factor	Pre Operatif (2009)	Pre Operatif I (Feb 2012)	Post Operatif I (Apr 2012)	Follow Up I (Jul 2012)	Follow Up II (Nov 2012)	Follow Up III (Jan 2013)	Post Operatif II (Feb 2013)	Follow Up IV (Nov 2014)	Follow Up V (Jan 2016)
Cobbs Angle	40°	120°	89°	100°	102°	113°	76°	78°	95°
Erect Hide	NA	94cm	105cm	100cm	98cm	94cm	108cm	105cm	100cm
Rod Failure	NA	NA	NO	NO	YES	YES	NO	NO	NO
Screw Failure	NA	NA	NO	NO	NO	NO	NO	NO	YES

DISCUSSION AND CONCLUSION:
Evaluation to the measures taken have been evaluated, following steps ahead and prediction of future outcome has been a continuous homework for our multidisciplinary team to improve his quality of life.

Failure of implants in this case may be caused by inappropriate harmony of biomechanical force between growing bone and strength of implant loading.

Effect of in vitro differentiation of human mesenchymal stem cells on cartilage repair in osteoarthritis of knee joint

T Spakova[1], J Plsikova[1], D Harvanova, M Lacko[2], J Rosocha[1]

[1] Associated tissue bank and [2]Department of orthopaedics and traumatology of locomotory apparatus of Faculty of Medicine of P. J. Safarik University and University Hospital of L. Pasteur, Trieda SNP 1, Kosice, Slovakia

INTRODUCTION: Osteoarthritis (OA) - degenerative joint disease is characterised by progressive degeneration of cartilage, subchondral bone changes such as sclerosis, subchondral bone cysts, osteophytes, and synovitis [1]. Enhanced chondrogenesis can be achieved by kartogenin (KGN) and mesenchymal stem cells (MSCs) delivered on the cartilage surface. MSCs tend to undergo terminal differentiation, which means that cartilaginous tissue formed by MSCs is not stable and cells become hyperthrophic. This process is characterised by the production of hypertrophy-related factors and mimics the embryonic process of endochondral bone formation [2]. In our study an osteoarthritis (OA) model was used to evaluate the effect of KGN *in vitro*. We hypothesized that KGN would promote chondrogenic differentiation of MSCs without negative side effect on terminal differentiation. Additionally we hypothesized that local delivery of KGN with bone marrow derived MSCs (BMSCs) to a cartilage defect would improve the quality of the tissue formed and induce immunomodulatory responses after differentiation.

METHODS: Bone marrow and osteochondral cylinders were obtained from OA patients undergoing total knee joint replacement with full ethical approval. The effects of prolonged exposure of KGN on BMSCs in scaffold-free 2D culture were monitored by xCelligence (RTCA) system. BMSCs were expanded and seeded onto osteochondral cylinders as previously described [3]. Cylinders were embedded in agarose gel and cultured in DMEM/F12 supplemented with 2% ITS-A and 1% ATB. Culture media was changed and collected two times a week. Secretome analysis was done to control changes in response of BMSCs to the cylinders before and after induction with KGN. Analysis was performed using the RayBio® Quantibody Human Array on media collected during 21 days of co-culture. SEM images were captured from both loaded (with BMSCs and w/wo KGN) and non-loaded OA cartilage surface to evaluate cell distribution and collagen fibre orientation. Final tissue products of 3D cultures of OA cylinders with cells were checked by histological and biochemical assays to demonstrate chondrogenic differentiation and evaluate terminal differentiation of human BMSCs. All experiments were performed in scaffold-free 2D cultures, too.

RESULTS: BMSCs in monolayer were exposed to medium containing 1, 10, 100 µM KGN and viability and proliferation were monitored by RTCA after 1, 3 and 7 days. An increase in proliferation during 7 days was observed in BMSCs cultured in 10 µM KGN. After three weeks of co-culture anti-inflammatory factors were produced at a higher level in loaded cylinders than in non-loaded control. The retention and differentiation of BMSCs at the fibrillated surface of osteoarthritic articular cartilage was demonstrated by SEM and histological assays. KGN caused an upregulation in expression of Col II, aggrecan and downregulation of osteocalcin, MMP-13 and ALP activity. FACS analysis of BMSCs phenotype after KGN exposure showed higher expression of chondrogenic markers (CD49e, CD26, CD54) compared to control.

DISCUSSION & CONCLUSIONS: In the present study, we described an approach to the assessment of effect of KGN in an *in vitro* OA model. Preliminary results suggest that KGN may be an effective accelerant for cartilage tissue engineering by promoting chondrogenic differentiation of MSCs but with no significant effect on hypertrophic differentiation.

2

ACKNOWLEDGEMENTS: This work was supported by the Slovak Research and Development Agency under the contract No. APVV-0684-12 and by VEGA grant 1/0217/16.

Osteointegration in a CapFlex-PIP© finger implant: a histological case report.

CM Sprecher[1], SF Schindele[2], S Hensler[3], S Milz[4]

[1] AO Research Institute, AO Foundation, Davos, CH. [2] Department of Hand Surgery, Schulthess Klinik, Zurich, CH. [3] Department of Teaching, Research and Development, Schulthess Klinik, Zurich, CH. [4] Department of Anatomy II, Ludwig-Maximilians-University of Munich, Munich, DE

INTRODUCTION: Primary press fit and secondary osteointegration is a precondition for component anchoring in articular surface replacements of proximal interphalangeal (PIP) joints. However, this outcome fails in many existing prosthesis designs. In order to improve osteointegration of the implant, the modular prosthesis CapFlex-PIP© (KLS Martin Group, Germany), a modern polyethylene-metal surface replacement consisting of a proximal and distal component, was developed[1]. We suspected that long-term cementless fixation of the CapFlex-PIP© could be achieved with initial press-fit technique allowing secondary osteointegration at the bone with the pure titanium pore backside of the components. However, it is difficult to obtain evidence of such osteointegration using standard radiographs. The most detailed and accurate statement can be made by histological analysis, but this requires retrieval of the implant-bone interface. We report such a rare case of an explantation of a CapFlex-PIP© implant due to a soft tissue complication.

CASE REPORT: We present the case of an 84-year-old woman who had a traumatic rupture of the radial collateral ligament 11 months after CapFlex-PIP© replacement at the index finger. Fixed ulnar deviation with functional limitations provided the indication for revision surgery with removal of the CapFlex-PIP© prosthesis and joint arthrodesis.

For histological analysis of osteointegration, the removed implants and attached tissues were immediately fixed in 70% methanol. After dehydration, the blocks were cut in the transversal plane and selected sections were stained with Giemsa-eosin. The Bone-Implant-Contact (BIC) rate, as a quantitative indicator for osteo-integration, was measured on all stained sections using a Zeiss Axioplan microscope (Fig. 1). The average BIC value was 40.7% for the proximal and 46.5% for the distal implant component. The observed values were within the BIC range published for particular dental implants and higher than those reported for the humeral parts of resurfacing shoulder prostheses[2,3].

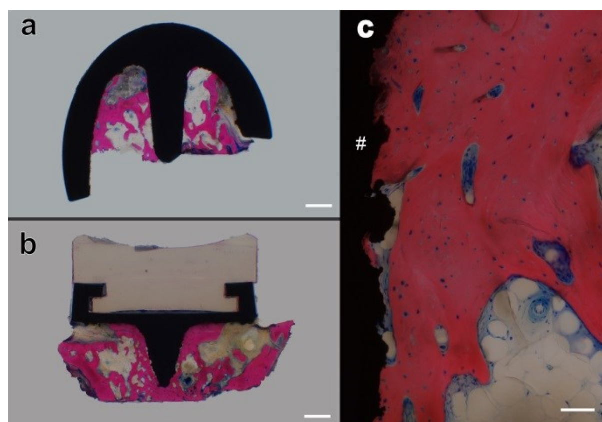


Fig. 1: Undecalcified sections of implants and adherent tissues stained with Giemsa-eosin. The red-stained bone is in direct contact with the surface of the implant. a) Proximal component, b) Distal component, c) Higher magnification of the bone-implant interface showing intimate contact.

DISCUSSION & CONCLUSIONS: The present case for the first time demonstrates a successful osteointegration of an implant used for human proximal interphalangeal joint replacement. The histological result is in line with the radiographic evaluation. The investigated CapFlex-PIP© implant shows osteointegration of both components, a result which is comparable to that of other load-bearing and articulating implants at different locations in the human body.

ACKNOWLEDGEMENTS: We would like to thank Mrs Andrea Altwegg for organizing the implant exchange and primary fixation of the components after explantation.

2

Regeneration of critical size bone defects via the implantation of novel ß-tricalcium phosphate scaffolds in transgenic mice

M Tohidnezhad[1], T Heigl[1], N Barahmand Pour[1], C Bergmann[2], M Bienert[3], P Lichte[4], H.C Pape[4], S Neuß Stein[3], H Fischer[2], T Pufe[1]

[1]Department of Anatomy and Cell Biology; [2]Dental Materials and Biomaterials Research; [3]Department of Pathology; [4] Department of Trauma Surgery, all RWTH, Aachen, Germany

INTRODUCTION: Large bone defects still challenge the orthopaedic surgeon. The quality of the bone graft and soft tissue envelope are crucial and presence of adequate blood supply is required to allow for a high standard of care. Vascular endothelial growth factors (VEGF) and their receptors (VEGFRs) are important in generating a microenvironment that facilitates bone growth[1,2].
Aim of the present study was the investigation of the fracture healing process in VEGFR2-luc and NFκB-luc mice using a novel 3D-printed ß-tricalcium phosphate (ß-TCP) scaffolds (with and without strontium (Sr)).
In vivo longitudinal measurements on VEGFR2-luc and NFκB-luc mice allow real-time monitoring of ongoing angiogenesis and inflammation at the fracture area, respectively.

METHODS: A critical size fracture was performed and stabilized using external fixation derived from AO (Arbeitsgemeinschaft Osteosynthese, Davos). The fracture was bridged using a synthetic 3D-printed scaffold with a defined porosity (Fig. 1) to promote regeneration. The ß-TCP and ß-TCP+Sr scaffolds were investigated for their regenerative potential and were implanted in the bone defect of VEGFR-2luc and NFκB-luc mice.

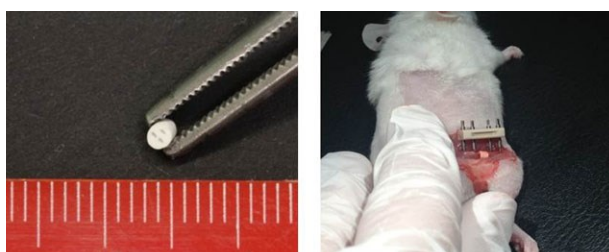


Fig. 1: Strontium doped ß-TCP scaffold with integrated capilar (left). Implanted scaffold in critical size femur fracture and stabilization of bone using external fixation.

These transgenic mouse strains express a VEGFR2- or NFκB-driven luciferase. In consequence, the expression levels of VEGFR2 and NFκB and thus represents either the revascularization or inflammation could be monitored non-invasively in a longitudinal fashion using the Xenogen imaging system. After two month, animals were euthanized and the fracture sites were histologically examined.

RESULTS: We observed the first peaks of luciferase activity in the VEGFR2-luc mice at the early angiogenesis periods (10th day) in all groups. While the level of VEGFR2-activity increased in the ß-TCP+Sr group at the 15th day, luciferase activity began to decrease in the other groups. Additionally, Sr reduced inflammation by means of NFκB activity in the early phase of healing (15th days), but it was increased again in the late healing stage.
The histological analysis showed that much more osseous tissue has been formed in ß-TCP+Sr when compared to ß-TCP. In both, ß-TCP and ß-TCP+Sr, the connection of newly formed tissue within the scaffold area to the fracture ends was clearly visible.

DISCUSSION & CONCLUSIONS: This study for the first time gives an overview of VEGFR2 and NFκB expression profiles during fracture healing. A New tissue was observed along the inside of the scaffolds. These tissue bridges filled the fracture gaps, which may accelerate the fracture healing. Addition of Strontium in scaffolds influence the inflammation in different stage of the healing and leads to increase of ossification. This effect might influence the healing process. Further histochemical and immunohistochemical analysis of femora should reveal the quality of callus

. 1

ACKNOWLEDGEMENTS: We thank our technical assistants supporting this work.

Human dental pulp stem cells stabilize blood vessels by secreting vascular basement membrane proteins collagen type IV and laminin *in ovo*

A Woloszyk[1], TA Mitsiadis[1]

[1]*Institute of Oral Biology, Department of Orofacial Development & Regeneration, Center of Dental Medicine, University of Zurich, Switzerland*

INTRODUCTION: The requirement for tissue and organ replacement is high and is expected to increase in the future due to population aging, especially in highly developed countries. Current shortage of suitable donors might be overcome by generating cell- and material-based biological substitutes with the potential to regenerate and replace lost tissues and organs. Teeth present an easily accessible source of autologous multipotent mesenchymal stem cells [1], which have been previously shown to possess pro-angiogenic properties [2]. As rapid vascularization and tissue integration into the surrounding host tissues is a prerequisite for the long-term survival of tissue engineered implants, we investigated the effect of human dental pulp stem cells (DPSCs) to improve neovascularization of a 3D silk fibroin scaffold using the chorioallantoic membrane (CAM) assay.

METHODS: Biocompatible silk fibroin scaffolds (height: 3 mm; diameter: 5 mm; pore size: 200-300 nm) were seeded with DPSCs and incubated for 24 h *in vitro* before performing the CAM assay for 7 days. Qualitative and quantitative blood vessel analysis was performed on H&E-stained paraffin sections. Cells of human origin were identified immunohistochemically using an anti-NuMA antibody, while anti-Collagen type IV and anti-Laminin antibodies were applied to visualize two of the main components of the vascular basement membrane. Empty scaffolds served as controls.

RESULTS: Cell-seeded scaffolds were found to attract more vessels than empty scaffolds, while creating their own extracellular matrix between the scaffold fibers. Cells of human origin were localized next to functional blood-perfused vessels, where they contributed to vessel stabilization by producing Laminin (Lam) (Fig. 1A) and Collagen type IV (Coll-IV) (Fig. 1B) within as short as 7 days. Therefore, the presence of DPSCs improved and accelerated vascular and tissue integration of the implants.

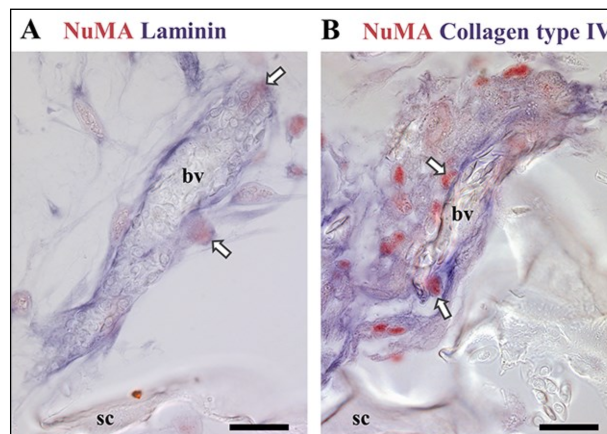


Fig. 1: Expression of Lam and Coll-IV by hDPSCs. (A, B) Human cell nuclei are stained in red (white arrows), while Lam (A) and Coll-IV (B) appear in purple. bv, blood vessel; sc, scaffold fiber. Scale bar = 25 µm.

DISCUSSION & CONCLUSIONS: The study has shown that a vascularized 3D tissue constructs can be created by combining DPSCs with 3D silk scaffolds. The cells have the potential to attract vascularization through their natural pro-angiogenic properties [2] without previous induction. The production of Lam and Coll-IV helps to stabilize ingrowing vessels, thereby accelerating tissue integration, which is important for clinical applications of cell-based tissue regeneration strategies. Following the '3Rs' principles of replacement, refinement, and reduction of animal use in research, the CAM assay provides a valuable intermediate platform for initial assessments prior to pre-clinical studies in mammals.

ACKNOWLEDGEMENTS: This work was supported by the Swiss National Foundation (SNSF) grant 31003A_135633 and by institutional funds from the University of Zurich. The authors thank Dr. Jolanda Baumgartner (Institute of Biomechanics, ETH) for manufacturing the silk scaffolds and Trudel Silk Inc. for providing silk cocoons.

Osteoblast-like cells seeded on 3D bone graft scaffold with hierarchical pore structure are responsive to mechanical loading

F Yang[1], SCF Rawlinson[2], KA Hing[1]

[1] School of Engineering and Materials Science, Queen Mary, University of London, UK. [2] School of Medicine & Dentistry, Queen Mary, University of London, UK

INTRODUCTION: Hierarchical structure silicon substituted hydroxyapatite (Si-HA) as a bone graft scaffold (BGS) has shown excellent bone ingrowth and repair in vivo [1] with increased osteogenic behaviour of BGS with higher levels of strut porosity [2]. One of the hypotheses of this enhancement is the inductive behaviour of BGS regulating a cellular mechanobiological pathway because bone is subjected to a complete combination of cyclical stresses and dynamic nutrient exchange in physiological environment. This study is to develop a system in which real clinical use 3D BGS can be screened under condition of "physiological" fluid flow and cyclic loading to test whether cyclic loading affects cell response when seeded on 3D BGS.

METHODS: The materials were characterized by XRD, FT-IR and SEM and seeded with MG-63 (osteoblastic like) cells. In the perfusion system they received culture medium at a rate of 0.07ml/min. Simultaneously, they were loaded intermittently (cyclic compressive strain of 0.5%, 1Hz.) using Bose ElectroForce load Frame System. Samples only receiving medium flow and cultured under non-loaded conditions were controls. After 3 days of culturing, sample granules with cells were fixed and stained appropriately for SEM and confocal microscopy. Cell lysates were extracted and total DNA and alkaline phosphatase (ALP) specific activity quantified.

RESULTS: DNA and ALP data is consistent with perfusion supporting osteoblast-like cell proliferation and differentiation. Cyclic loading further enhanced these responses. The promotion of proliferation was also corroborated with SEM examination (Fig. 1a, c and e). Immunofluorescence staining results validated the enhancement of differentiation by demonstrating that mechanical loading facilitated nuclear localization of Runx2, a major regulator in osteoblastic differentiation (Fig. 1b), compared with perfused BGS static cultures (Fig. 1d and f) .

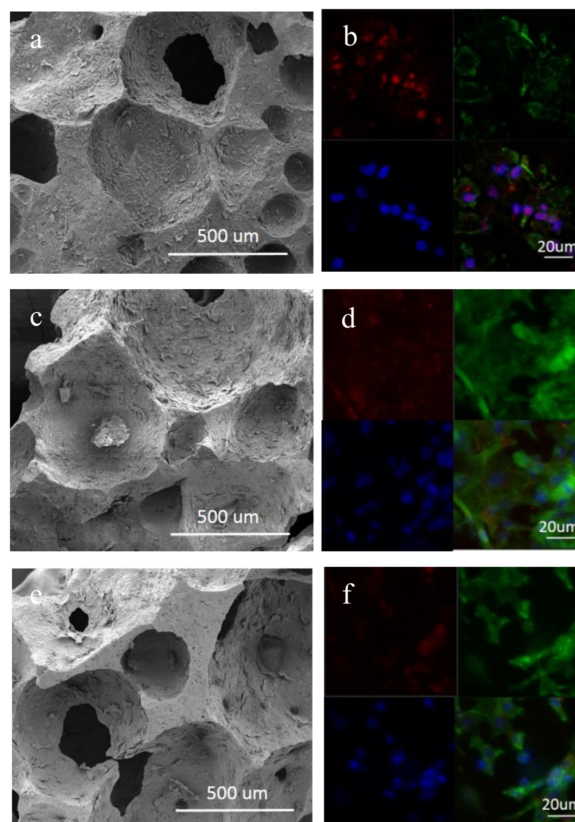


Fig. 1: Cell populations were visualized under scanning electron microscopy (SEM), and Runx2 nuclear localization was examined by immunofluorescence and confocal microscopy after culture in the perfusion system with intermittently mechanical loading (a)(b), culturing in the system with perfusion only (c)(d), and static culture (e)(f).

DISCUSSION & CONCLUSIONS: This study demonstrated that osteoblastic-like cells seeded on 3D BGS respond to changes in the mechanical environment. Proliferation and differentiation are significantly promoted. Follow up studies will investigate whether there are any differences when the same mechanical regimen is applied to BGS with different levels of strut porosity.

1

Influence of scaffold geometry on chondrocyte fate to tissue engineered cartilage

Kai-Chiang Yang [1, 2], Hsin-Hui Hu [1], Ing-Ho Chen [3], Chen-Chie Wang [2, 3, *]

[1] *School of Dental Technology, College of Oral Medicine, Taipei Medical University, Taipei, Taiwan* [2] *Department of Orthopedic Surgery, Taipei Tzu Chi Hospital, The Buddhist Tzu Chi Medical Foundation, New Taipei City, Taiwan* [3] *Department of Orthopedics, School of Medicine, Tzu Chi University, Hualien, Taiwan*

INTRODUCTION: Scaffold, the microenvironment to cells, plays an important role in tissue engineering. Several studies reveal that the environmental factors, such as the composition, mechanical properties, geometry/topography, and other biophysical cues modulate the phenotype to cells [1]. Cells cultured in a three-dimensional (3D) scaffold show a different biological performance when compared to the conventional 2D monolayer model. Therefore, the influence of scaffold geometry on chondrocyte fate shall be critical to tissue engineered cartilage.

METHODS: Microfluidic technology was used to prepare a honeycomb-like gelatin scaffold in this study [2]. A random foam gelatin scaffold was also prepared by the freeze-dried method for comparative purposes. The physical properties of these two scaffolds were compared. Primary chondrocytes were harvested from rabbit articular cartilages, expanded, seeded into these two scaffolds, and cultured for 4 weeks. The mRNA expressions of the seeded chondrocytes were evaluated by using qPCR, and the secreted components of extracellular matrix (ECM) were identified by histological examinations at predetermined intervals.

RESULTS: The honeycomb-like gelatin scaffolds showed a higher swelling ratio, porosity, and compressive strength when compared to the freeze-dried scaffolds. Rabbit chondrocytes also had a good viability, survival rate, glycosaminoglycans production within the honeycomb-like scaffolds. In addition, cells maintained a functional phenotype when analysed by qPCR. The mRNA levels of type I collagen were down-regulated, while the aggrecan and type II collagen were up-regulated when compared with chondrocytes cultured in the freeze-dried scaffolds. Histological examinations revealed that chondrocytes produced proteoglycan (Fig. 1) and other ECM proteins normally. Interestingly, chondrocytes expressed proliferating cell nuclear antigen (PCNA) pronounced in the honeycomb-like gelatin scaffolds (Fig. 2).

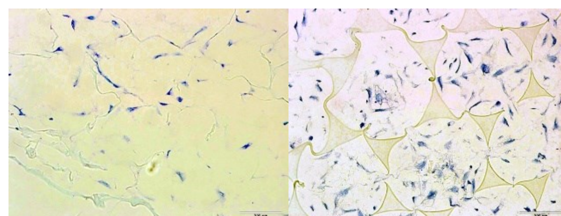


Fig. 1: Rabbit chondrocytes cultured in the honeycomb-like gelatin scaffolds (right). Cells produced proteoglycan normally.

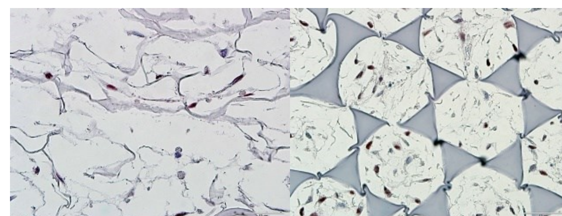


Fig. 2: Chondrocytes expressed PCNA pronounced in the honeycomb-like gelatin scaffolds (right) rather than in the freeze-dried scaffolds (left).

DISCUSSION & CONCLUSIONS: Previously, Schlegel et al. reported that the raw materials of scaffolds determined the fate of seeded chondrocytes [3]. The nature of matrix influenced the differentiation of dedifferentiated cells dramatically. We further found that the scaffold geometry mediated the mRNA expressions of ECM proteins to cells. Chondrocytes possessed normal phenotype with ECM productions within the honeycomb-like scaffold. Furthermore, the cell proliferation was also modulated in terms of PCNA expressions. In conclusion, the scaffold geometry influences chondrocyte fate in tissue engineered cartilage.

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Promotion of the osteogenic differentiation potential of human nasal inferior turbinate-derived mesenchymal stem cells by mechanical memory

Byeong Gon Yun[1], Sun Hwa Park[2], Se Hwan Hwang[1], Mi Hyun Lim[1], Sang A Back[1], Jung Ho Jeon[1], Sung Won Kim[1,2]

[1]*Department of Biomedical Science, the Catholic University of Korea, College of Medicine, Seoul, Korea*

[2]*Department of Otolaryngology Head and Neck Surgery, the Catholic University of Korea, College of Medicine, Seoul, Korea*

INTRODUCTION: Mesenchymal stem cells (MSCs) are used for cell-based therapy to prevent degenerative bone defects. Therefore, since bone regeneration is required, autologous tissues can be used to generate implants in the appropriate anatomical shape without risk of immunological rejection. Human nasal inferior turbinate-derived mesenchymal stem cells (hTMSCs) can be potentially used as a source of adult stem cells for therapeutic application due to their easy availability, cultivation, and high proliferative ability. We evaluated the capacity of hTMSCs for osteogenic differentiation. We hypothesized that different surfaces of the nano-pore surface plate affect the morphology, proliferation, and osteogenic differentiation of hTMSCs.

METHODS: We used human turbinate-derived MSCs after the second passage. hTMSCs (4 x 10^4 cells) were seeded in flat surface plates (20 mm × 20 mm) and nano-pore surface plates (20 mm × 20 mm; diameter, 200 nm; depth, 500 nm, pore-to-pore distance, 500 nm). The seeded hTMSCs were assessed using a scanning electron microscope (SEM) and focal actin staining to determine the morphology. The proliferation of hTMSCs was evaluated by measuring the cell metabolic activity using a Cell CountingKit-8 (CCK-8). The media used for osteo-differentiation was changed every 2–3 days. Osteogenic differentiation of hTMSCs was identified using alkaline phosphatase (ALP), alizarin red S staining, and vonkossa staining, Real-time quantitation of mRNA of COL1A1, osteocalcin, BMP-2, osterix, and bone sialoprotein were also performed.

RESULTS: There was no significant difference in the proliferation of seeded hTMSCs on a flat surface plate and those on a nano-pore surface plate. However, high osteogenic differentiation was observed using alkaline phosphatase (ALP), alizarin red S staining, von Kossa staining, and real-time PCR of seeded hTMSCs on the surface of a nano-pore plate.

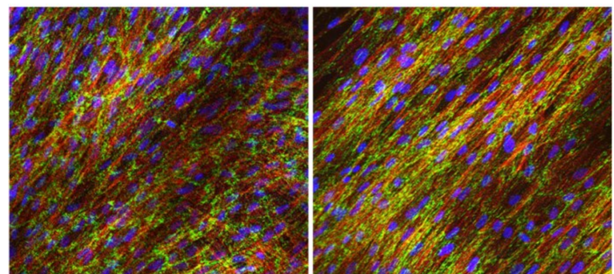

Fig. 1: Human nasal inferior turbinate-derived mesenchymal stem cells(hTMSCs) grown in the flat surface plates and nano-pore surface plates of through focal adhesion staining(FAK100). 21day culture in osteogenic differentiation media.

DISCUSSION & CONCLUSIONS: These findings suggest that hTMSCs are apparently redirected toward the osteogenic phenotype in an in vitro culture under specific conditions using bone-formation stimulating factors. Further, the osteogenic potentials observed were superior to other tissues originating from MSCs.

ACKNOWLEDGEMENTS: The authors wish to acknowledge the financial support of the National Research Foundation of Korea (NRF), funded by the Ministry of Science, ICT, and Future Planning (2014R1A2A2A01004325), and the Korea Health Industry Development Institute, funded by the Ministry of Health and Welfare (HI14C3228).

A tuneable, adenoviral BMP-2 gene delivery system to bone

JJ Bara[1], I Dresing[1], S Zeiter[1], M Anton[2], G Daculsi[3], D Eglin[1], D Nehrbass[1], VA Stadelmann[1], DC Betts[4], R Müller[4], M Alini[1], MJ Stoddart[1]

[1] AO Research Institute, AO Foundation, Davos, CH. [2]Klinikum rechts der Isar der Technischen Universität München, Institute of Experimental Oncology and Therapy Research, Munich, Germany. [3]INSERM U791, Laboratory for Osteoarticular and Dental Tissue Engineering, Dental Faculty, Nantes University, France .[4]Institute for Biomechanics, ETH Zurich, Switzerland.

INTRODUCTION: We report a novel non-integrating viral gene delivery approach that can be used to enhance bone healing achieved by the use of clinically approved biomaterials in an 'off-the shelf' manner. Specifically, a doxycycline inducible Tet-on adenoviral vector (AdTetBMP-2) in combination with mesenchymal stromal cells (MSCs), fibrin and a biphasic calcium phosphate ceramic (MBCP®) was used to repair large bone defects in nude rats.

METHODS: Human MSCs encapsulated in fibrin containing MBCP® granules (60% hydroxyapatite and 40% calcium phosphate, Biomatlante SA) were transduced with AdTetBMP-2 [1]. 4mm, internally fixated, femoral defects were created in nude rats with full ethical approval. MSCs were transduced, by direct application of AdTetBMP-2 or by pre-coating MBCP® with the virus. Doxycycline was administered in the animals feed. Control groups comprised un-transduced MSCs, transduced MSCs (-)doxycycline and doxycycline alone. Animals were euthanised at 12 weeks. Radiographs were performed post-operatively, at 6 and 12 weeks. Ex-vivo CT and histological analysis were performed post-mortem.

RESULTS: In vitro, BMP-2 transgene expression could be effectively tuned by alteration of doxycycline dose. Combined results from the *in vivo* study showed significantly improved defect healing in animals that had received direct delivery of the vector or when MBCP® were pre-coated with the virus. Radiograph scores were significantly improved in AdTetBMP-2+ doxycycline groups ($p=0.0033$). Micro-CT data showed a trend towards increased mineralised tissue volume within the defect in AdTetBMP-2+ doxycycline groups.

Bending stiffness EI, determined by micro finite element analysis of micro-CT images, was greater following delivery of AdTetBMP-2 compared to (-)doxycycline vector controls (224±136.9 vs. 148.5±48.2 Nmm2, respectively).

No adverse tissue reaction or ectopic ossification was seen histologically post-mortem. MBCP® granules integrated with both nascent and newly formed bone. Semi-quantitative analysis revealed that the percentage of bone within the defect site was greater, in animals where BMP-2 was overexpressed compared to controls (29.09±8.32% vs. 22.04±4.69%, $p=0.0267$). The presence of a cartilaginous tissue suggested that bone healing had occurred via endochondral ossification.

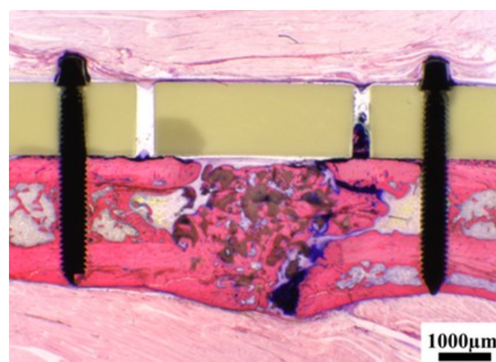

Fig. 1: Defect healing at 12 weeks following application of AdTetBMP-2 pre-coated MCBP® as shown by Giemsa-Eosin staining.

DISCUSSION & CONCLUSIONS: Adenoviral delivery of BMP-2 enhanced bone regeneration achieved by the transplantation of MSCs, fibrin and MBCP® *in vivo*. Our data show that this can be achieved with relatively low (ng/ml), levels of the growth factor. This approach may provide a powerful standardised model for the optimisation of growth factor delivery and release for the healing of large bone defects.

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Perfusion culture regulates the differentiation of mesenchymal stromal cells on polylactic-glycolic acid *in vitro*

C Moser[1,2], K Bardsley[3], C Wolfrum[2], AJ El Haj[3], M Alini[1], MJ Stoddart[1], JJ Bara[1]

[1] AO Research Institute, AO Foundation, Davos, Switzerland. [2]Institute for Translational Nutrition, ETH Zürich, Switzerland. [3]Institute for Science and Technology in Medicine, Keele University, United Kingdom

INTRODUCTION: I*n vitro* testing of biomaterial suitability for bone repair is typically performed in static culture. Yet mesenchymal stromal cells (MSCs), are known to be responsive to shear forces, which are present as a result of lacunar-canalicular interstitial fluid flow. Here, we investigate the behaviour of bone marrow-derived MSCs on porous polylactic glycolic acid (PLGA) scaffolds under dynamic culture conditions.

METHODS: PLGA (50:50 lactic: glycolic acid) scaffolds were fabricated by solvent casting/salt leaching as previously described.[1] Primary human MSCs from two donors were cultured on PLGA scaffolds in a bidirectional perfusion culture system in control vs. osteogenic induction media (UCUP, CELLEC BIOTEK AG). MSCs cultured on PLGA statically and on Thermanox™ served as controls. DNA content, secreted nitric oxide (NO), prostaglandin E2 (PGE2) and gene expression were assessed throughout the experiment. Histological analysis was performed at 3 weeks.

RESULTS: PLGA scaffolds degraded steadily which corresponded with a gradual reduction in DNA content. Gene expression analysis performed at day 7 revealed up-regulation of Runx2 in MSCs cultured in both control and osteogenic media on PLGA. Following culture in osteogenic media, Sox9 was down-regulated in Donor A and remained unchanged in Donor B at day 7. Collagen I and ALP were upregulated by MSCs cultured on PLGA in osteogenic media at day 7. Osteocalcin gene expression was higher in PLGA groups compared to Thermanox™ controls and greatest when MSCs had been cultured in osteogenic media under perfusion (Fig 1.A). Neither NO nor PGE2 were detectable in culture supernatant. Donor A, which exhibited a high Runx2/Sox9 ratio at day 7 following osteogenic induction in monolayer, demonstrated significant mineralisation on PLGA day 21 under perfusion (Fig 1.B) compared to static culture (Fig 1.C). Conversely, donor B, presented a comparatively lower Runx2/Sox9 ratio at day 7 after osteogenic induction in monolayer – and in this donor, collagen X was strongly upregulated following culture on PLGA scaffolds in osteogenic media – indicating hypertrophy. The lack of mineral deposition at day 21 with Donor B is in accordance with an immature hypertrophic phenotype.

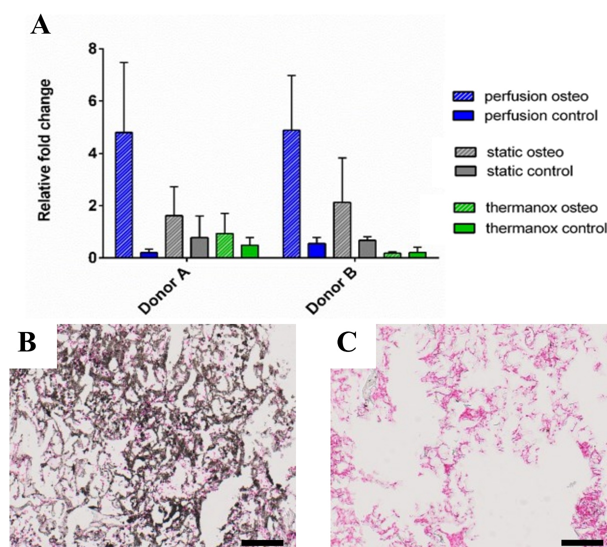

Fig. 1: (A) Osteocalcin gene expression at day 21.Mineral deposition as indicated by Von Kossa staining at day 21 following (B) perfusion and (C) static culture in osteogenic culture media.

DISCUSSION & CONCLUSIONS: Perfusion culture enhanced the expression of osteocalcin in osteogenically differentiated MSCs. The Runx2/Sox9 ratio during early osteogenic induction in monolayer may provide a reliable predictor of hypertrophic vs. direct osteogenic differentiation and mineralising potential of MSCs cultured on PLGA *in vitro*.

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